Neuroprotective effect of small heat shock protein, Hsp27, after acute and chronic alcohol administration

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Abstract Alcohol induces degeneration of neurons and inhibits neurogenesis in the brain. Small heat shock proteins are able to protect neurons in cerebral ischemia and oxidative stress. In this study, we investigated the neuroprotective effect of small heat shock protein, Hsp27, after acute and chronic ethanol administrations using transgenic mice overexpressing the human Hsp27 protein. Transgenic mice and wild-type littermates were injected with 2 g/kg ethanol intraperitoneally, and then motor coordination and muscle strength were analyzed using different behavioral tests, such as footprint analysis, balance beam, and inverted screen tests. Ethanol-injected transgenic mice showed similar footprints to control saline-injected mice, did not fall of the beam, and were able to climb to the top of the inverted screen, while wild-type mice showed ataxia and incoordination after ethanol injection. The effect of Hsp27 on chronic ethanol consumption was also investigated. Drinking water of mice was replaced by a 20% ethanol solution for 5 weeks, and then brain sections were stained with Fluoro Jade C staining. We found significantly lesser amount of degenerating neurons in the brain of ethanol-drinking transgenic mice compared to wild-type mice. We conclude that Hsp27 can protect neurons against the acute and chronic toxic effects of ethanol.

Keywords Hsp27 · Neuroprotection · Ethanol · Transgenic mice

Introduction

Stress proteins or heat shock proteins that have chaperone activity (that is, they are capable to protect newly made proteins from misfolding) belong to five conserved families: Hsp100, Hsp90, Hsp70, Hsp60, and the so-called small heat shock proteins. The HSPB (small Hsp) family consists presently of 11 members that are characterized by a signature conserved crystallin domain flanked by variable N- and C-termini. The best studied members of sHsps are HSPB1 (Hsp27), HSPB4 (α A crystallin), and HSPB5 (α B crystallin; Kampinga et al. 2009).

Small heat shock proteins are ubiquitous proteins found in all living systems. One of the most notable features is their large oligomeric structures with conserved structural organization. It is well documented that sHsps can capture unfolding proteins to form stable complexes and prevent their irreversible aggregation. In addition, sHsps coaggregate with aggregation-prone proteins for subsequent, efficient disaggregation of the protein aggregates. The release of substrate proteins from the transient reservoirs, i.e., complexes and aggregates with sHsps, and their refolding require cooperation with ATP-dependent chaperone systems (Nakamoto and Vigh 2007).

The amphitropic sHsps were shown to associate with membranes, sometimes by anchoring to them via rapidly formed stress-lipids (Balogi et al. 2005), although they do not contain transmembrane domains or signal sequences. These studies also indicate that membrane-associated sHsps, by altering the fluidity and non-bilayer propensity of the lipid matrix (Török et al. 2001; Tsvetkova et al. 2002), play an important role in the stability and quality control of membranes. Thereby, sHsps potentially contribute to the maintenance of membrane integrity especially under most various stress conditions (Horváth et al. 2008).

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Since the membrane physical state controls the signaling mechanism of certain Hsps, including sHsps (Balogh et al. 2005; Nagy et al. 2007), it is tempting to speculate that membrane association of sHsps may result in the inactivation of membrane-perturbing signals.

Small Hsps can form oligomers up to 1,000 kDa. This oligomerization is a dynamic process that depends on the phosphorylation status of the protein. The phosphorylation is catalyzed by the MAPKAP kinases 2 and 3 in response to a variety of stresses (Stokoe et al. 1992). The oligomerization of Hsp27 seems to play a central role in regulating the chaperone activity of the protein (Arrigo et al. 1988). Hsp27 is expressed in many cell types and tissues at specific stages of development and differentiation.

Hsp27 can protect cells against diverse stressful stimuli. For example, Hsp27 modulates apoptosis by interacting with and inhibiting the key components of the apoptotic signaling pathway (Mehlen et al. 1996). Hsp27 can also increase the resistance of cells to oxidative injuries by reducing lipid peroxidation, protein oxidation, and f-actin architecture disruption. Hsp27 expression is also correlated with decreased level of reactive oxygen species (ROS) and nitric oxide. Hsp27 protects cells against ischemic insult in cell cultures (An et al. 2008). There are several evidences that Hsp27 can protect neurons against different harmful stimuli (ischemia, oxidative stress) or in neurodegenerative disorders (Muchowski and Wacker 2005). It can protect dorsal root ganglion neurons from apoptosis induced by nerve growth factor withdrawal, and Hsp27 also protects ND7 neuronal cells from retinoic acid-induced apoptosis (Wagstaff et al. 1999). Overexpression of Hsp27 can effectively attenuate the deleterious effect of oxidative stress induced by H₂O₂ (An et al. 2008).

The protective capacity of Hsp27 is not restricted to cultured cells; it was also detected in animal models. For example, overexpression of hsp27 in mice protects neurons against kainate-induced cell death (Akbar et al. 2003). Induction of Hsps by geldanamycin protects rat brain against focal cerebral ischemia (Lu et al. 2002). A number of studies have investigated the role of Hsps in neurodegenerative diseases and found that they can protect proteins against misfolding and aggregation and they are effective suppressors of neurodegeneration in animal models.

Ethanol has diverse effects on the central nervous system. Acute effect of alcohol administration involves euphoria, confusion, disinhibition, incoordination, and coma, depending on the dose. Chronic alcohol consumption generates neurodegenerative processes in different regions of the brain. There are several factors in the mechanism of ethanol-induced brain damage, and it is complicated by other nutritional deficiencies and coexistence of damage of other organs such as liver, pancreas, or heart. A substantial increase of oxidative stress may be also involved in

neurodegenerative action of ethanol (Fadda and Rossetti 1998).

The aim of the present study was to investigate the neuroprotective effect of Hsp27 protein after acute and chronic ethanol administration. For this purpose, we used an *in vivo* approach; a transgenic mouse line overexpressing the human Hsp27 protein was produced. The effect of acute ethanol administration was monitored using behavioral assays, such as footprint analysis, balance beam test, and inverted screen. The toxic effect of chronic alcohol consumption was investigated by detecting degenerating neurons specifically using FluoroJade staining.

Materials and methods

Generation of Hsp27 transgenic mice

The human Hsp27 cDNA clone was a kind gift of Prof. Wilbert Boelens (University of Nijmegen, Netherlands). Transgenic DNA construct contained the human Hsp27 cDNA driven from a cytomegalovirus (CMV) promoter. The DNA fragment containing the CMV-hsp27 transcription unit was separated from the vector by double digestion with *Apa*LI and *Ade*I restriction enzymes (Fermentas). The isolated linear transgene was microinjected at 2 ng/ul into the pronuclei of fertilized eggs collected from FVB mice. Microinjected and re-expanded oocytes were then transferred into the oviduct of pseudopregnant FVB mice. Tail biopsy was taken from 10-day-old offsprings, and transgenic mice were identified by polymerase chain reaction (PCR) analysis using the following primers: 5'-GTC CCT GGA TGT CAA CCA C-3' and 5'-GAC TGG GAT GGT GAT CTC GT-3'. The PCR amplification was done in an MJ Research PTC-200 Thermal Cycler using the following parameters: 94°C for 5 min followed by 37 cycles of 94°C for 40 s, 65°C for 40 s and 72°C for 40 s, finally 72°C for 5 min.

Western blot analysis

Transgene product was detected in the brain, heart, and liver tissues by western analysis. Total protein was isolated from frozen tissues homogenized in lysis buffer (containing Tris-HCl 50 mM, NaCl 0.1 mM, EDTA 10 mM, and protease inhibitor), centrifuged at 10,000 rpm for 30 min at 4°C, and the supernatant was used for western blot analysis. The protein concentration was measured at 260 nm using a NanoDrop spectrophotometer (NanoDrop Technologies). Protein samples (50 µg) were separated by SDS-PAGE (Cambrex) and then transferred onto HybondP PVDF membrane (Amersham). The membrane was blocked for 1 h in 5% milk powder. After a short wash in PBS, the membrane

was incubated with anti-hsp27 antibody (1:5,000, Stressgen Cat No: SPA-803), overnight at 4°C. After a brief wash, membrane was incubated with secondary antibody (Chemicon, Cat No: AP 132P, 1:10,000) for 2 h at room temperature; signals were developed using ECL Advance Western Blotting Detection Reagent (Amersham) according to the manufacturer's instructions.

Immunohistochemistry

Frozen sections (10 µm) of the brain were processed for immunohistochemistry. Sections were fixed in acetone for 3 min. After a brief wash with PBS, sections were incubated with 1.5% H₂O₂ in methanol for 20 min and then with 3% H₂O₂ for 20 min to quench endogenous peroxidases. Washing in PBS was followed by treating with 0.1 mg/ml collagenase type II (Sigma) in a buffer containing 0.25 M NaCl 50 mM Tris-HCl pH 7.4 and 1 mM EDTA at 37°C for 5 min. After washing in PBS, sections were incubated in 5% goat serum for 1 h at room temperature to prevent nonspecific binding of the antibody. Sections were then covered with primary antibody (anti-Hsp27, Stressgen, Cat No: SPA-803, 1:100) overnight at 4°C. Sections were washed with PBS, and then secondary antibody was applied for 45 min (Chemicon Cat. No.: AP 132P, 1:2000). After washing, sections were incubated with developing reagent (10% dimethyl formamide, 10% imidazole, 0.02% aminoethyl carbazole, 0.04% H₂O₂) for at least 30 min at room temperature. Sections were then counterstained with hematoxylin solution for 4 min at room temperature followed by washing with PBS.

Animals

Four-month-old FVB transgenic mice and wild-type littermates were used throughout the experiments. Mice were housed in groups of four to six under standard conditions (24°C, 12-h light–dark cycle) with food and water available ad libitum. Experimental protocol was approved by the local animal care ethical committee (license no. XIX/05004/001/2005).

Behavioral tests

Transgenic and wild-type mice were randomly divided into two groups (ethanol-injected and saline-injected control), ten mice per group. Before each test, mice were trained three times on three consecutive days. On the test day, animals received an intraperitoneal injection of 20% Et–OH (2 g/kg) or an equal volume of physiological saline. Behavioral tests were performed 10 min after the injection to allow ethanol concentration to reach the maximum level in the brain (Crabbe et al. 2003).

Footprint analysis

Mice were made to walk along a 60-cm-long 7-cm-wide runner (with 10-cm walls) by shining a 60-W lamp at the start and placing a dark box at the end. The runner was lined with white paper, and the fore and hind paws of the animals were stained with different non-toxic colors to record the footprints. The middle steps of a series of steps were analyzed. The following parameters were measured: front- and back-leg stride length and width and front/hind paw overlap.

Balance beam walking

Mice were placed onto a 1-m-long 10-mm-round horizontal wooden beam. They were motivated to walk along by shining light at the beginning of the beam and placing a dark plastic box at the end of the beam. Time taken to run along the beam, slips, and falling-off were recorded. If a mouse fell off, it was picked up and put back on the beam to the same position. Between tests, beam was wiped with a paper towel moistened with 75% ethanol.

Inverted screen test

Mice were placed in the center of a 25×30-cm wire mesh elevated 50 cm above the ground and quickly inverted. Time climbing to the top of the wire was measured.

Swimming test

A glass tank (20×20×60 cm) was filled with 10-cm high of 30°C water. Mice were placed into the water at the end of the tank. On the other side of the tank, a platform was accommodated where the mice could get out of the tank. Time required to swim across the 60-cm-long tank was measured.

Chronic ethanol treatment

Four-month-old transgenic and wild-type mice were randomly divided into two groups. In the Et–OH-treated groups, the drinking water was replaced by 10% Et–OH solution for 4 days, then 15% for another 4 days (adaptation period), and finally 20% for 5 weeks. The control groups received drinking water during the same period. Both groups were allowed access to drink and food ad libitum.

Fluoro Jade staining

Brains were removed from each animal, and 10 µm acetone fixed frozen sections were made. Slides were immersed into absolute ethanol for 5 min, then rinsed in 70% ethanol for

2 min, in 30% ethanol for an additional 2 min, and finally in distilled water for 2 min. Sections then were incubated in 0.06% potassium permanganate solution for 30 min. Following a 2-min water rinse, sections were transferred into a 0.0004% solution of Fluoro Jade C (Chemicon) dissolved in 0.1% acetic acid. Sections on slides were washed with three changes of distilled water for 1 min per change. After drying sections were cleared in xylene for 2 min and coverslipped with DPX nonfluorescent mounting media. Degenerating neurons emitting green fluorescence were visualized under Nikon Eclipse E600 fluorescent microscope using a 520-nm fluorescein filter set.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance using OriginPro8 software, followed by Fisher's post hoc test. The level of statistical confidence was set at $p < 0.05$.

Results

Generation of Hsp27 transgenic mice

Transgenic construct contained the human Hsp27 cDNA controlled by the viral CMV promoter. Ten-day-old offsprings were genotyped using PCR. Two independent transgenic lines were detected designated as lines 33 and 24. To analyze the expression of human Hsp27 gene, western blot analysis was performed on brain samples of the two transgenic lines. Antibody against the human Hsp27 was obtained from Stressgen (Cat No: SPA-803). Transgenic human Hsp27 was expressed at high level in the brain of transgenic line 33; however, the antibody did not cross-react with the endogenous mouse hsp25 protein (Fig. 1a).

Immunohistochemistry

To monitor the distribution of human Hsp27 protein in the transgenic brain, immunohistochemical analysis was performed. Strong expression of human Hsp27 was detected in the cerebellum, hippocampus, cerebral cortex, and olfactory and thalamic regions (Fig. 1b).

Behavioral tests

To investigate the acute effect of ethanol administration, a series of behavioral tests were performed. Motor coordination, imbalance, and ataxia were monitored using footprint analysis, balance beam walking, inverted screen tests, and swimming. In all test, 4-month-old transgenic mice and

their wild-type littermates were used, ten animal per group. Behavior tests were performed in a noise-attenuated room between 1 and 3 pm. Mice were adapted to the environment and tools in three-trial tests performed on three consecutive days.

Footprint analysis

In the footprint analysis, we compared the stepping patterns of the transgenic and wild-type mice injected with Et-OH or saline. Ten mice per group were used, and two trials were performed on every mouse with one trial per day. Saline-injected control mice had a normal gait; they used only the front part of the paws to walk. Contrarily, ethanol-injected wild-type mice walked on their whole paw and produced toe dragging (Fig. 2a). Footprint patterns of transgenic Et-OH-injected mice were much similar to the patterns of saline-injected control mice. For quantitative comparison of footprint patterns, front- and back-leg stride length and width and front/hind paw overlap were measured. Six steps were analyzed on each footprint. Statistical analysis showed a significant difference in the stride length between the ethanol-treated transgenic and wild-type groups (Fig. 2b, c). These data suggest significantly impaired forelimb-hindlimb coordination in the Et-OH-injected wild-type group compared to the transgenic group (forelimbs and hindlimbs, $p < 0.001$).

Balance beam walking

Motor coordination and balance were measured by beam walking test. Ten mice were in each group, and three trials were performed by each mouse. The time taken to run along the beam was measured. It took about 5 s for the saline-injected mice; however, after ethanol treatment, this was increased by 15 s in the wild-type group, while merely by 11 s in the transgenic group ($p < 0.001$, Fig. 3a). There was also significant difference in the number of falls. Saline-injected mice did not fall off from the beam, but after ethanol treatment, on average a wild-type mouse fell off more than two times from the beam, whereas a transgenic mouse less than once ($p < 0.001$, Fig. 3b). None of the saline-injected mice fell off the beam, but as a result of ethanol treatment, 86% of the wild-type mice did it at least once, while only 53% of the transgenic mice ($p < 0.01$, Fig. 3c).

Inverted screen test

The inverted screen test is a functional test requiring proper motor coordination and muscle strength. Mice not able to climb the top of the screen were considered to have impaired motor coordination or reduced muscle strength.

Fig. 1 **a** Detection of human Hsp27 protein in the brain of transgenic and wild-type mice using western blot. **b** Immunostaining of sagittal brain sections of transgenic and wild-type mice. *White arrows* indicate human Hsp27 immunoreactivity (brown staining) in the transgenic brain. Nuclei were visualized using hematoxylin staining. Higher magnification of the hippocampal (**c**) and cerebellar (**d**) regions of transgenic and wild-type sections showed above (**b**)

All saline-injected mice were able to climb to the top of the screen; however, after ethanol treatment, almost every wild-type mice fell off the screen, whereas 43% of the transgenic mice could climb over the edge of the screen ($p < 0.001$, Fig. 4.)

Swimming test

Motor coordination was also measured by swimming. Time taken to swim across a 60-cm-long tank was measured.

Statistical analysis revealed a strong trend between the performance of ethanol-injected transgenic and wild-type groups ($p = 0.06$; data not shown).

Chronic ethanol treatment

To study the deleterious effects of chronic ethanol consumption on Hsp27 transgenic and wild-type mice, drinking water was replaced by 10% ethanol solution for 4 days, then 15% solution of Et-OH for 4 days to avoid aversion of

Fig. 2 **a** Photograph of footprint patterns of saline-injected wild-type and transgenic mice and Et-OH-injected wild-type and transgenic mice. Mice traveled right to left. Abnormalities in the footprint of ethanol-treated wild-type mice such as toe dragging (*arrow with*

dashed tail) and walking on whole feet of the hind leg (*arrow with solid tail*) are indicated. Statistical analysis of stride length of the forelimb (**b**) and hindlimb (**c**). ** denotes a statistical difference of $p < 0.001$

mice to a high ethanol concentration. After the adaptation period, mice received 20% ethanol for 5 weeks. In the end of ethanol treatment, brains of transgenic and wild-type mice were removed, and sagittal frozen sections were prepared. Degenerated neurons were detected with Fluoro Jade C staining. Fluoro Jade C is an anionic fluorochrome which sensitively and specifically binds to degenerating neurons (Schmued et al. 2005).

For quantitative comparison, we counted the Fluoro Jade positive neurons in five fields of vision in the cerebellum and cortex and in three fields of vision in the hippocampal region. Significantly less degenerated neurons was detected in the hippocampus ($p < 0.001$), cerebellum ($p < 0.001$), and cortex ($p < 0.001$) of the ethanol-treated transgenic animals compared to the ethanol-treated wild-type mice (Fig. 5).

Fig. 3 Statistical analysis of beam-walk test. Time taken to walk across the beam (a), number of falls per mice (b), and the number of mice falling-off per group (c). ** indicates $p<0.001$

Discussion

In this study, we showed that Hsp27 was able to protect neuronal cells against the harmful effects of acute and chronic ethanol administration. Statistical analysis revealed a significant difference between alcohol-treated transgenic and wild-type mice in three of the four behavioral tests performed to compare motor coordination, ataxia, and muscle strength. Ethanol-treated mice had a longer stride, and they moved faster than untreated controls. This is in agreement with previous findings of others, who showed

that administration of relatively low doses of ethanol (1–2 g/kg) increases locomotor activity in mice (Matchett and Erickson 1977; Smoothy and Berry 1984). Ethanol has a complex effect on neuronal system. The hydroxyl group of ethanol can form hydrogen bonds with proteins or membrane phospholipids. The effects of ethanol are nonselective; it can modulate directly or indirectly the function of several membrane proteins including receptors and ion channels in the neuronal membranes (Fadda and Rossetti 1998).

There are several theories to explain the action of ethanol on neuronal cells. Alcohol can directly interact with membrane proteins at specific sites on each protein producing conformational changes thus influencing their function (Fadda and Rossetti 1998). According to the lipid hypothesis, ethanol affects primarily the neuronal membranes and secondarily the proteins. Acute administration of ethanol can increase membrane disorder by hyperfluidizing the membrane, which in turn has an effect on receptors and ion channel functions. In a completely opposite way, the chronic administration of ethanol might induce a compensatory response or even a net membrane rigidification within membranes exposed to permanent hyperfluidization (Balogh et al. 2005). In fact, increased membrane ordering could reduce the prior disordering effect of ethanol on membranes, hence, ultimately can result in a reduced activation of ion channels (Fadda and Rossetti 1998). Apparently, such an “overcompensation” of

Fig. 4 Statistical analysis of inverted screen test. Mice were placed in the middle of the screen, and then the screen was turned upside down, 180° around a horizontal axis. The number of mice could climb over the edge of the screen was compared. $n=10$, ** denotes $p<0.001$

membrane hyperfluidization, together with the ethanol-induced membrane (protein and lipid) damage, can also downregulate the expression of Hsps of chronically ethanol-stressed cells.

As discussed before, several studies have demonstrated that there is a potential link between heat shock proteins and membrane function, as sHsps can bind to membrane, increase its physical order, and reduce its non-bilayer propensity. In *Oenococcus oeni*, a lactic acid bacterium, an 18-kDa sHsp (Lo18) is induced by several stresses such as heat or ethanol, and it is associated with the membrane

after ethanol treating. This sHsp has a direct effect on the phospholipids and modulates the membrane properties by increasing their molecular order, which decreases the membrane fluidity (Coucheney et al. 2005). The Hsp60 GroEL was also shown to increase the molecular order of lipid bilayers and stabilize the membrane under stress conditions (Török et al. 1997). In vertebrates, alpha crystallin has been shown to bind ocular plasma membranes and synthetic phospholipid vesicles (Cobb and Petrash 2000). Taken together, these data suggest that small heat shock proteins might play a pivotal role in membrane

Fig. 5 Detection of degenerating neurons after chronic ethanol consumption in wild-type and Hsp27 transgenic brains in cerebellar (a), hippocampal (c) and cortical (e) regions using Fluoro Jade staining. Statistical analysis of neurodegeneration in the cerebellar (b), hippocampal (d), and cortical (f) regions. ** denotes $p < 0.001$

Fig. 5 (continued)

protection. Hsps can influence the function of ion channels, either indirectly by affecting the lipid phase harboring these proteins, but they can also interact with them directly or regulate their expression (Ficker et al. 2003; Krieger et al. 2006; Kashlan et al. 2007). Due to these properties, Hsp27 might protect neuronal cells against the membrane protein damaging effects of acute ethanol administration.

We have also documented that Hsp27 can prevent neurodegeneration during chronic ethanol administration. Chronic alcohol consumption results in neurodegenerative processes in different brain regions. Several factors might

be involved in the mechanism of ethanol-induced brain damage. A substantial increase in oxidative stress might play a major role in the initial damages of neurons, which is further complicated with malnutrition and vitamin deficiency and by the effect of damage of other organs (i.e., liver and kidney). There is increasing evidence that chronic ethanol administration enhance free radical generation and lipid peroxidation in the brain. Brain is extremely sensitive to oxidative stress as neuronal membranes are rich in polyunsaturated fatty acid chains, and they have poor catalase and SOD activities. It was shown earlier that grape

Fig. 5 (continued)

polyphenols, which are powerful antioxidants, can protect neuronal membrane proteins against the toxic effect of ethanol (Sun et al. 1999).

Heat shock proteins are induced in response to several stresses including oxidative stress. Hsp27 was shown to protect against oxidative stress by decreasing the level of ROS. Several studies showed that Hsp27 had a protective effect in ischemia/reperfusion animal models where oxidative stress was an important factor in neuronal cell death. Transduction with Hsp27 can also increase viability of neuronal cells treated with hydrogen peroxide (An et al.

2008). These studies indicate that Hsp27 can attenuate the oxidative stress induced cell death.

The protective effect of ginseng was documented against ethanol-induced gastric damages in rat. Ginseng administration resulted in a significant induction of Hsp27 suggesting its contribution and capability to prevent of ethanol-induced gastric injuries (Yeo et al. 2008).

In conclusion, our results presented here clearly show that Hsp27 can protect neuronal cells against degradation after long-term ethanol consumption, probably through its antiapoptotic and oxidative stress-attenuating properties.

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