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A novel role for GAPDH-MAO B cascade in ethanol-induced cellular damage

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Abstract

Background—Alcoholism is a major psychiatric condition at least partly associated with ethanol-induced cell damage. Although brain cell loss has been reported in subjects with alcoholism, the molecular mechanism is unclear. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and monoamine oxidase B (MAO B) reportedly play a role in cellular dysfunction under stressful conditions and may contribute to ethanol-induced cell damage.

Methods—Expression of GAPDH and MAO B protein was studied in human glioblastoma and neuroblastoma cell lines exposed to physiological concentrations of ethanol. Expression of these proteins was also examined in the prefrontal cortex from human subjects with alcohol dependence

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and in rats fed with an ethanol diet. Co-immunoprecipitation, subcellular fractionation, and luciferase assay were used to address nuclear GAPDH-mediated MAO B activation. To test the effects of inactivation, RNAi and pharmacological intervention were used, and cell damage was assessed by TUNEL and H₂O₂ measurements.

Results—Ethanol significantly increases levels of GAPDH, especially nuclear GAPDH, and MAO B in neuronal cells as well as in human and rat brains. Nuclear GAPDH interacts with the transcriptional activator, transforming growth factor-beta-inducible early gene 2 (TIEG2), and augments TIEG2-mediated MAO B transactivation, which results in cell damage in neuronal cells exposed to ethanol. Knockdown expression of GAPDH or treatment with MAO B inhibitors selegiline (Deprenyl) and rasagiline (Azilect) can block this cascade.

Conclusions—Ethanol-elicited nuclear GAPDH augments TIEG2-mediated MAO B, which may play a role in brain damage in subjects with alcoholism. Compounds that block this cascade are potential candidates for therapeutic strategies.

Keywords

alcoholism; human brain tissues; rats-fed with an ethanol diet; ethanol-induced brain cell dysfunction; monoamine oxidase B; glyceraldehyde-3-phosphate dehydrogenase

Introduction

Alcoholism is a major psychiatric condition which causes about half of alcoholics in the United States to suffer from neuropsychological difficulties (1,2). Reduced volume of brain tissue, increased brain damage accompanied by cognitive deficits, and low density of neuronal and glial cells have been reported in alcoholism (3–8). Ethanol also induces neuronal cell death and cell cycle delay in cell model systems *in vitro* (9,10). Therefore, effective treatment against ethanol-induced cellular dysfunction and damage is eagerly awaited.

Monoamine oxidase B (MAO B) has been implicated in alcoholism (11). This enzyme degrades a number of biogenic amines and generates inert hydrogen peroxide (H₂O₂), which can interact with iron to produce reactive hydroxyl radicals that cause cellular dysfunction and death (12, 13). An MAO B transcriptional activator, transforming growth factor-beta-inducible early gene 2 (TIEG2), induces MAO B expression (14). TIEG2 reportedly inhibits cell growth (15) and mediates caspase-3-dependent apoptosis (16). Thus, the TIEG2-MAO B cascade has a role in cell dysfunction and damage.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a multifunctional protein. Recent studies from our group and others indicate that a small pool of GAPDH translocates to the nucleus and mediates stress signaling, resulting in cellular dysfunction and death (17–20). Although GAPDH protein levels were reportedly increased in brains from subjects with alcoholism (21), its significance in this disorder remains elusive.

Selegiline and rasagiline, inhibitors of MAO B, have been used mainly for treatment of Parkinson's disease because these compound prevent degradation of dopamine (22). In addition to inhibiting MAO B, selegiline and rasagiline selectively bind to GAPDH and block its nuclear translocation (23,24).

In this study, we report that ethanol elicited nuclear translocation of GAPDH, which activates the induction of MAO B via TIEG2-GAPDH protein interaction. The GAPDH-TIEG2-MAO B cascade is blocked by knockdown of GAPDH as well as selegiline, which leads to a decrease in ethanol-induced cell damage.

Materials and Methods

Cell lines and reagents

Human glioblastoma U-118 MG and neuroblastoma SH-SY5Y were purchased from ATCC. The antibodies used in this study were: mouse monoclonal antibodies for GAPDH (Santa Cruz. sc-32233), caspase-3 (sc-7272) and TIEG2 (BD Transduction Laboratory; 611402); rabbit polyclonal antibody for GAPDH (for immunoprecipitation assay, Santa Cruz. sc-25778) and goat polyclonal antibodies for MAO B (sc-18401). An MAO B inhibitor, selegiline (deprenyl), was purchased from Sigma-Aldrich USA. GAPDH small interfering RNA (siRNA) and the transfection kit were purchased from Ambion (Austin, TX). 2',7'-dichlorofluorescein-diacetate (for measurement of the generation of H₂O₂) was purchased from Sigma (D6883). In Situ Cell Death Detection Kit (for TUNEL staining) was purchased from Roche (Indianapolis, IN). EnzyChrom Ethanol Assay Kit (ECET-100) for the measurement of blood ethanol concentration was purchased from BioAssay Systems (Hayward, CA).

Generating GAPDH-expression vector and MAO B 2 kb promoter-luciferase reporter gene vector

The human GAPDH coding sequence was obtained by PCR. The primer sequences employed were 5'-TCGACAGTCAGCCGCATCTTCTTT-3' (forward) and 5'-TGTGCTCTTGCTGGGGCTGGTG-3' (reverse). The PCR product was subcloned into the pCR4-TOPO vector using TOPO TA cloning Kit (Invitrogen). Subsequently, the coding region of GAPDH within the PCR product was subcloned into pcDNA3.1 vector (EcoR I/EcoR I).

The human MAO B 2 kb promoter was obtained by PCR using a forward primer (with Sac I enzyme site) 5'-GAGCTCATTGCCAGTTGGACATAGAGAA-3' and a reverse primer (with Xho I enzyme site) 5'-AGTCCCCTCCCTGGTGCCCGCTGCTC-3'. The PCR product was subcloned into the pCR4-TOPO vector and then subcloned into pGL3 basic luciferase reporter gene vector (Sac I/Xho I).

Treatment of cells with ethanol and selegiline

Seventy-five millimolar (75 mM) ethanol or both ethanol and selegiline (0.25 nM) were added directly into the medium of each dish. As ethanol is volatile, a closed chamber system was utilized to stabilize the ethanol concentration in the culture medium as described previously (25,26).

The ethanol concentrations used in this study (75 mM) are within the level that results in physiological effects observed in alcoholics because ethanol at 50–100 mM reflects blood levels of ethanol in chronic alcoholics (27,28).

Quantitative real-time RT-PCR

Total RNA was isolated from each group by using RNA isolation reagent (Invitrogen). The extracted mRNAs were reverse-transcribed into cDNAs using Invitrogen random primer [Anchored Oligo(dT)20 Primer] and SuperScript III following the instruction of the manufacturer. Specific primers for the human MAO B and GAPDH were designed as follows: MAO B sense, 5'-GACCATGTGGGAGGCAGGACTTAC-3'; antisense, 5'-CGCCCACAAATTTCCTCTCCTG-3'; and GAPDH sense, 5'-GCAAATTCCATGGCACCGTCAAG-3'; antisense, 5'-GATGCTGGCGCTGAGTACGTCGT-3'. The mRNA content for each group was analyzed by real-time RT-PCR using a Bio-Rad iCycler system. The real-time PCR was performed with a SYBR supermix kit (Bio-Rad), and 18S Ribosomal RNA primer was used as the internal control in each plate to avoid sample variations (14,29).

Nuclear protein extraction

Cells (10-cm dish) were treated with ethanol or both ethanol plus selegiline for three days and harvested by scraping. The cell pellets were resuspended in 20 μ l of buffer A [10 mM KCl, 10 mM HEPES, 1.5 mM MgCl₂ (0.5 mM DTT and 0.1% NP-40 were freshly added just before using)] and centrifuged at 4 °C for 10 min (6,000 rpm). The pellets (containing nuclei) were resuspended in 15 μ l of buffer C [20 mM HEPES (pH 7.9), 25 % glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂ (0.5 mM DTT and 0.5 mM PMSF were added freshly)] and then centrifuged for 10 min (3,000 rpm) at 4 °C. The supernatant containing nuclear proteins was diluted with 75 μ l of buffer D [20 mM HEPES (pH 7.9), 20% glycerol, 50 mM KCl, 0.2 mM EDTA (0.5 mM DTT and 0.5 mM PMSF were added freshly)] and stored at –80 °C (30).

Immunofluorescence

Cells were plated on a four-well chamber slide (Nalge) 1 day before treatment with or without ethanol or with both ethanol and selegiline. Two days after the treatment, cells were fixed in 4% paraformaldehyde for 20 min and immunostained by a mouse anti-GAPDH antibody (Santa Cruz. sc-32233, 1:1000 dilution) and visualized with a Cy3-conjugated anti-mouse (red) secondary antibody (Amersham Biosciences, PA 43002; 1:1000 dilution). Nuclei were stained by DAPI (blue; VECTASHIELD Mounting Medium with DAPI, H-1200; Vector Labs) (29).

MAO B catalytic activity

One hundred micrograms of total protein were incubated in a glass tube (31) with 10 μ M ¹⁴C-labeled phenylethylamine (Amersham) and assay buffer (50 mM sodium phosphate buffer, pH 7.4) at 37 °C for 20 min until the reaction was stopped by the addition of 100 μ l of 6 N HCl. The reaction products were then extracted with ethylacetate/toluene (1:1) and centrifuged for 10 min. The organic phase containing the reaction product was extracted, and its radioactivity was obtained by liquid scintillation spectroscopy (32).

Human subjects

Brain samples (Brodmann area 8/9) were collected at autopsy at the Cuyahoga County Coroner's Office in Cleveland, Ohio. Informed written consent was collected from the legal next-of-kin of all subjects. Next-of-kin from all subjects were interviewed, and retrospective psychiatric assessments were conducted in accordance with Institutional Review Board policies (8). A total of 20 subjects were diagnosed with alcohol dependence at the time of death according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) (American Psychiatric Association, 1995) as described previously (8,33), and 20 control subjects were assessed and diagnosed as psychiatrically normal (Table S1 in Supplement 1).

Animals, group size and feeding

Ethanol-preferring male Wistar rats [weighing 180–220 g, from the Indiana University Alcohol Research Center (34)] were treated with an ethanol diet (#710260; Dyets, Bethlehem, PA) or control glucose liquid diet (#710027; Dyets) for 4 weeks. All protocols for the animal experiments described in this study were carried out according to the Ethical Guidelines on Animal Experimentation and were approved by the Animal Usage Committee of the University of Mississippi Medical Center.

Ethanol-preferring Wistar rats were housed in individual cages in a temperature- and humidity-controlled room with a 12:12-h light-dark cycle. The group size in our studies was 10 rats per control or treatment group.

Rats were acclimatized for 3 days after arrival and provided with free access to Purina rat chow and water. Rats were then allowed free access to liquid diet without ethanol for 3 days and then

randomly assigned to the ethanol-fed or pair-control groups. The liquid ethanol diet contained increasing amounts of ethanol until a final diet containing 36% of calories from ethanol (6.4% EtOH) was achieved as follows: no EtOH for 3 days, followed by 2.5% for 3 days, then followed by 5.0% for 5 days and finally 6.4% for 17 days. For these diets, glucose was isocalorically substituted by ethanol according to the Lieber-DeCarli diet formula following the manufacturer's instructions. The final ethanol diet (6.4% EtOH) containing 36% of calories from ethanol (~14g EtOH/day/kg rat) achieved the blood ethanol concentrations of less than 50 mM (the average level was 40.8 ± 7.2 mM). Blood alcohol concentration was measured using an EnzyChrom™ Ethanol Assay Kit (BioAssay Systems) in all rats when they were sacrificed. The prefrontal cortex was immediately removed on dry ice and stored at -80°C until used.

Western blotting

Total proteins (50 μg /well for human MAO B or 40 μg /well for rat MAO B; 4 μg for human or rat GAPDH assay) were analyzed by 10.5 % SDS-polyacrylamide gel electrophoresis. Anti-MAO B antibody (1:500), anti-GAPDH antibody (1:2,500), and anti-TIEG2 antibody (1:500) were used. For the prefrontal cortex (PFC) tissue from both human and rat samples, pairs of subjects were immunoblotted on the same gel with duplicate samples immunoblotted on separate gels. The tissue standard was dissected from the anterior PFC of one control subject, and the same cortical tissue standard was used for all experimental gels. Band relative density (relative optical density $\times$ pixel area) from Western blotting was determined using gel analysis software and a computer-assisted image analysis system (35). Linear regression was used to plot a standard curve for each gel (Figure S1 in Supplement 1), from which relative optical density (ROD) values of samples were converted to cortical standard protein units for each experimental sample for each gel. The final data are expressed as a ratio of [protein of interest]/[actin].

Transfection and luciferase assay

Transfections in cells were performed using the Superfect Transfection reagent (Qiagen, Inc.). Cells were plated at a density of 5×10^4 cells/well in 6-well plates and grown until 50% confluency was obtained. 0.5 μg of MAO B 2 kb promoter-luciferase construct (for one well) was co-transfected with 0.5 μg of GAPDH-expression vector, GAPDH mutant (K160R, preventing activation of p300/CBP)-expression vector, or GAPDH mutant at the active cysteine site (Cys150) or 0.5 μg of TIEG2-expression vector or both GAPDH-expression vector (0.25 μg) and TIEG2-expression vector (0.25 μg) or 0.5 μg of empty expression vector (pCMV3.1) (14).

Co-Immunoprecipitation

Nuclear proteins were extracted from cells (1×10^7) and adjusted to 200 $\mu\text{g}/\text{ml}$ with ice-cold PBS. The nuclear protein was immunoprecipitated by incubating with anti-TIEG2 antibody (4 μl of antibody in 1 ml of PBS) with BioMag beads (Anti-Mouse, QIAGEN) as described previously (14,30).

Measurement of intracellular H_2O_2 generation

H_2O_2 can oxidize 2',7'-dichlorofluorescein-diacetate into the highly fluorescent compound 2',7'-dichlorofluorescein which was measured by a fluorometer. In brief, 75 μl of cell suspension were transferred to a 96-well plate, and an equal volume of 2',7'-dichlorofluorescein-diacetate (final concentration, 10 $\mu\text{g}/\text{ml}$) was added to each well. Generation of H_2O_2 at 5 min after incubation was read using a fluorescence spectrophotometer (wavelength 485/535 nm) (36).

TUNEL Assay

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End Labeling (TUNEL) assay was used to assess the extent of apoptosis in treated cells. Cells were plated on a four-well chamber slide on the day preceding the experiment and treated with or without ethanol daily for two or three days. Cells were then washed with PBS and fixed using 4% paraformaldehyde. The slides were stained by adding fluorescein 12-dUTP to nicked ends of DNA and then visualized with a fluorescent light microscope. Green fluorescence was correlated with DNA fragmentation (37).

Statistical analysis

A cell means ANOVA model followed by Bonferroni correction for predefined contrasts was used to compare relevant means. The statistical package SPSS v 15.0 was used for all analysis. The F-values and group and experimental degrees of freedom are included in the main body of text. Within each experiment, the familywise Type I error rate was controlled by using $p < 0.05/k$. In experiments with two factors, we used contrasts to test for simple effects in the ANOVA. For experiments with two groups (e.g. the human subjects and rat brain tissues in Fig. 2 and 3), Student's t-test was used, and a value of $p < 0.05$ was considered statistically significant.

Results

Increased expression of MAO B and GAPDH as well as augmented nuclear GAPDH in cells treated with physiologically attainable levels of ethanol

To examine whether a stress sensor such as GAPDH may play a role in apoptosis under exposure to physiological levels of ethanol (75 mM), we treated two human brain cell lines, glioblastoma U-118 MG and neuroblastoma SH-SY5Y, with ethanol and examined the levels of GAPDH, especially the nuclear pool of GAPDH. Induction of GAPDH mRNA was observed after exposure to ethanol (Fig. 1A; $F_{1,6}=94.25$, $p < 0.0001$). The GAPDH mRNA levels were increased by ethanol significantly as shown in Fig. 1A (lanes 3 vs. 1 and 4 vs. 2; $p < 0.0001$). Both subcellular fractionation and immunofluorescent cell staining clearly indicated marked augmentation of nuclear GAPDH after exposure to ethanol (Fig. 1 B and C). Under this condition (Fig. 1D), we also observed significant increases in levels of MAO B mRNA ($F_{1,6}=91.22$, $p < 0.0001$) and enzymatic activity ($F_{3,12}=108.72$, $p < 0.0001$). MAO B mRNA levels significantly increased by 4-fold after ethanol treatment (Fig 1Da, lanes 3 vs. 1 and 4 vs. 2; $p < 0.0001$), and the catalytic activity also significantly increased (Fig 1Db, lanes 3 vs. 1 and 4 vs. 2; $p < 0.005$).

Furthermore, we found that ethanol not only increased the amount of GAPDH in the nucleus (~3.5-fold) but also significantly increased the overall GAPDH levels (in both the nucleus and cytosol, ~1.8-fold) as compared to those in untreated controls (data not shown). Thus, our results suggest that nuclear translocation and resultant enrichment of nuclear GAPDH certainly occurs.

Increased expression of MAO B and GAPDH in the prefrontal cortex from human subjects with chronic alcohol abuse and in rats fed with ethanol

On the basis of the observation that GAPDH and MAO B are augmented in cells exposed to the physiological level of ethanol, we hypothesized that these two proteins would be up-regulated in autopsied brains from subjects with chronic alcohol abuse. Thus, we used Western blotting to examine prefrontal cortex tissue from subjects with alcohol dependence as compared to normal control subjects. Indeed, protein levels of GAPDH and MAO B were significantly higher in the alcohol-dependent group ($p < 0.05$; Fig. 2) and there was no change in levels of

the housekeeper protein, actin, (Table S2 in Supplement 1) as analyzed by Student's t-test. There is a significant difference in the GAPDH/Actin ratio ($t=2.20$, $df=38$, $p\text{-value}=0.0338$) and in the MAO B/Actin ratio ($t=2.34$, $df=38$, $p\text{-value}=0.0269$) between the two groups. There was a significant negative correlation between GAPDH and PMI ($r = -0.48$, $p\text{-value}=0.002$) and a significant positive correlation between MAO B and age ($r = 0.49$, $p\text{-value}=0.002$) despite the lack of significant differences between cohorts regarding other factors (Tables S1 and S2 in Supplement 1). The difference between the groups not only remains significant but also yields a much smaller p-value for GAPDH ($t = 3.28$, $df=36$, $p\text{-value}=0.0017$) and for MAO B ($t = 3.13$, $df=36$, $p\text{-value}=0.0065$).

Furthermore, we used the rat model to determine the levels of GAPDH and MAO B in ethanol-fed rat brains. Our results showed significant induction of GAPDH (~2-fold; $p < 0.02$) and MAO B protein (~1.7-fold; $p < 0.05$) in the prefrontal cortex of rats exposed to ethanol for 4 weeks (Fig. 3), providing further evidence that the GAPDH-MAO B pathway plays an important role in ethanol-induced apoptotic cell death. As analyzed by Student's t-test, there is a significant difference in the GAPDH/Actin ratio ($t=2.8$, $df=18$, $p\text{-value}=0.0116$) and in the MAO B/Actin ratio ($t=2.2$, $df=18$, $p\text{-value}=0.0401$) between the two groups.

Nuclear GAPDH binds with MAO B transcription factor TIEG2 and induces MAO B gene expression upon ethanol treatment

Nuclear GAPDH is known to bind with transcription factors and their regulators. In the nucleus, GAPDH is acetylated at lysine-160 (K160) by p300/CBP (38). Augmentation of nuclear GAPDH translocation together with an increase in MAO B in cells after exposure to ethanol provided us with a working hypothesis that GAPDH may interact with Transforming Growth Factor-beta-Inducible Early Gene-2 (TIEG2), a key transcriptional enhancer of MAO B in the nucleus. TIEG2 interacts with Sp1-binding sites in the core promoter region of the MAO B gene (14). First, we examined the protein level of TIEG2 in the nucleus. In both U-118 MG and SH-SY5Y cells, nuclear TIEG2 was augmented after exposure to ethanol (Fig. 4A; $F_{3,12}=106.06$, $p < 0.0001$). Similar results were found for the GAPDH levels induced by ethanol.

Under these conditions, GAPDH-TIEG2 co-immunoprecipitation was increased in the nucleus (Fig. 4B; $F_{1,6}=93.18$, $p < 0.0001$). The levels of GAPDH interacting with TIEG2 were increased (Fig. 4B, lanes 2 vs. 1; $p < 0.0001$). To test whether GAPDH affected TIEG2-mediated transcriptional activation of MAO B, we performed a luciferase assay using a construct containing the MAO B promoter region upstream of luciferase in the presence of exogenous wild-type or a mutant GAPDH in which K160 is replaced by R (K160R GAPDH). As previously shown (14), over-expression of TIEG2 increased the MAO B promoter-mediated gene transcription. Consistent with nuclear enrichment of TIEG2 (Fig. 4A), ethanol treatment further increased the MAO B promoter-mediated gene transcription by TIEG2 (Fig. 4C, lanes 3 vs. 1; $p < 0.001$). TIEG2-elicited MAO B gene transcription is enhanced more by the co-expression of wild-type GAPDH (Fig. 4C, lanes 4 vs. 1, $p < 0.001$; 5 vs. 1; $p < 0.0001$) than of the K160R mutant (Fig. 4C, lanes 6 vs. 1, $p < 0.01$; 7 vs. 1, $p < 0.001$). The global comparison of the TIEG2-expression vector or both TIEG2- and GAPDH-expression vectors is significant ($F_{1,40}=148.5$, $p < 0.0001$). In addition to K160, the active site cysteine residue (Cys150) of GAPDH has been reported to play a crucial role in the nuclear translocation (39); thus, we tested its effects in our study. As shown in Fig. 4C, the mutation of GAPDH (Cys150) exhibited similar results to the K160R mutant (Fig. 4C, lanes 8 vs. 1 $p < 0.01$; 9 vs. 1, $p < 0.001$). Consistent with this notion, interaction of the GAPDH-TIEG2 protein complex with the core promoter of the MAO B gene was demonstrated by a chromatin immunoprecipitation (ChIP) procedure (Figure S2 in Supplement 1). GAPDH does not bind to the MAO B core promoter

(Sp1-binding sites) directly, as assessed by an electrophoretic mobility shift assay (data not shown).

GAPDH mediates augmentation of MAO B and cell damage upon ethanol treatment

The above results suggest that GAPDH can increase MAO B via TIEG2 in the presence of ethanol. To establish that GAPDH mediates augmentation of MAO B and cell damage upon exposure to ethanol, we used RNA interference (RNAi) of GAPDH in both U-118 MG and SH-SY5Y cell lines in which endogenous GAPDH was knocked down to 15% (Fig. 5A). When cells were pretreated with GAPDH RNAi, we observed a decrease in ethanol-induced augmentation of MAO B (Fig. 5B, $F_{1,6}=91.26$, $p < 0.0001$). The MAO B mRNA levels were increased by the treatment of ethanol (Fig. 5B, lanes 3 vs. 1) but reduced by GAPDH-knockdown (Fig. 5B, lanes 4 vs. 3, $p < 0.0005$).

Consequently, ethanol-elicited cell damage assayed by TUNEL staining (Fig. 5C, $F_{5,18}=139.817$, $p < 0.0001$) and the expression of apoptotic protein caspase-3 (Figure S3A in Supplement 1) were both significantly decreased by knockdown of GAPDH (Fig. 5Cb, lanes 4 vs. 3, $p < 0.001$ and lanes 6 vs. 5, $p < 0.0001$; Figure S3A in Supplement 1, lanes 4 vs. 2, $p < 0.005$).

Blockade of ethanol-elicited nuclear GAPDH-MAO B cascade by selegiline

Selegiline (deprenyl) inhibits enzymatic activity of MAO B. This compound also binds to GAPDH and inhibits its nuclear translocation in many stress conditions (23). Thus, we tested whether ethanol-elicited nuclear translocation of GAPDH was prevented by treatment with selegiline. Immunofluorescent cell staining (Fig. 6A) and biochemical fractionations consistently indicated successful blockade of GAPDH translocation in the presence of ethanol (Fig. 6B, $F_{1,6}=112.64$, $p < 0.0001$) by ~70% in U-118 MG cells and ~50% in SH-SY5Y cells ($P < 0.0001$). Because nuclear GAPDH induces MAO B expression, we examined whether selegiline treatment reduces MAO B expression as well. In two independent neuronal cell lines, we observed a decrease in ethanol-induced MAO B expression upon this treatment (Fig. 6C, $F_{1,6}=128.52$, $p < 0.0001$) by ~50% compared to that of control cells ($p < 0.0001$). Selegiline also reduced ethanol-induced cytotoxicity (Fig. 6D, $F_{1,6}=137.25$, $p < 0.001$) as determined by the generation of the toxic chemical H_2O_2 by ~35% compared to control cells ($p < 0.005$). In addition, the effect of selegiline on the expression of apoptotic protein caspase-3 was examined by Western Blot analysis (Figure S3B in Supplement 1) and compared to that of rasagiline, a new MAO B inhibitor (40). The caspase-3 expression was significantly decreased to ~41% by selegiline and to ~70% by rasagiline as compared to that without the treatment of drugs (Figure S3B in Supplement 1, lanes 2 and 3 vs. 1).

Analyzed together, our results suggest that selegiline and rasagiline can inhibit the expression of GAPDH and MAO B and reduce GAPDH-MAO B mediated apoptosis.

Discussion

There are two major findings in this present study. First, we demonstrate a possible novel mechanism of ethanol-elicited MAO B induction: GAPDH is up-regulated by ethanol and translocates to the nucleus, where it binds to TIEG2 and augments TIEG2-mediated gene transcription for MAO B (see scheme in Figure S4 in Supplement 1). This action depends on lysine-160 (K160) (38) and cysteine-150 (Cys150) of GAPDH; K160 is crucial for the binding of GAPDH to the transcriptional co-activator p300/CBP (38) and Cys150 is a possible trigger of nuclear translocation (18). Furthermore, knockdown of GAPDH by RNAi and subsequent blockade of nuclear translocation of GAPDH (or by selegiline or rasagiline) decreases the levels of MAO B and its resulting cytotoxicity (Figs. 5 and 6; Figure S3 in Supplement 1).

Second, we demonstrate that both GAPDH and MAO B proteins are increased in the prefrontal cortex of alcohol-dependent subjects or in rats-treated with ethanol in comparison to normal controls

With regard to the fact that GAPDH has been widely utilized as a loading control, our results and the results of others (17–20,38) suggest that GAPDH is not an appropriate control to elucidate cellular effects-induced by cell stressors, including ethanol. Even in 1990–95, there were many papers indicating that GAPDH expression is dramatically increased upon hypoxia, ischemia (41–43).

We have recently reported that a new MAO B inhibitor [rasagiline (40)] and its metabolite could decrease the ethanol-induced cell death by preventing nuclear translocation of GAPDH using *in vitro* cell cultures (44). However, whether the MAO B inhibitor is through the blockage of this active cysteine residue (Cys150) and/or the lysine-160 of GAPDH needs to be investigated. Our pharmacological study suggested that GAPDH cascade might be involved in ethanol toxicity in human alcoholism and animal models *in vivo*. Consistent with our recent findings, the current study provides direct evidence that GAPDH is required for ethanol brain toxicity. Furthermore, the two important sites at GAPDH, K160 (38) and Cys150 (39), play the important roles in mediating GAPDH-involved MAO B expression induced by ethanol. Our current results also show that caspase-3 expression was significantly decreased by selegiline and rasagiline, providing further evidence that the neuroprotective effects of MAO B inhibitors are through blockage of GAPDH-MAO B-mediated apoptotic cell death.

In summary, MAO B inhibitors inhibit nuclear translocation of GAPDH and MAO B activity, both of which playing roles in ethanol-induced cell dysfunction and brain damage. Thus, although selegiline or rasagiline is currently used in the treatment of Parkinson's disease and related senile dementias (45), it may be worthwhile to pursue their application in ethanol-associated brain disorders including alcoholism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
MAO	monoamine oxidase
Co-IP	co-immunoprecipitation assay
TIEG2	transforming growth factor-beta-inducible early gene 2
PMSF	phenylmethylsulfonyl fluoride

PBS	phosphate-buffered saline
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid disodium salt dehydrate

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Fig. 1.

Effects of ethanol on expression of GAPDH and MAO B. The human glioblastoma U-118 MG and neuroblastoma SH-SY5Y were treated with 75 mM ethanol for 48 h for mRNA assay or for 72 h for MAO B catalytic activity assay. (A) GAPDH mRNA levels and (B) nuclear GAPDH protein levels were determined. Histone H4 was used as a loading control for nuclear proteins. (C) Immunofluorescence microscopy was performed with anti-GAPDH antibody. U-118 MG cells were plated on a four-well chamber slide and treated with or without ethanol for 48 h. Then the cells were immunostained by mouse anti-GAPDH antibody, followed by fluorescein-conjugated secondary antibody (red). Stained slides were mounted in the presence of DAPI for nuclear staining (blue). The GAPDH (red) and nucleus (blue) and the merge of both GAPDH and the nucleus are indicated at the top. (D) MAO B mRNA levels and MAO B catalytic activities were determined. Data represent the mean $\pm$ S.D. of four independent experiments. *, $p < 0.0001$ and #, $p < 0.005$ compared with respective controls (ethanol 0 mM).

Fig. 2.

Protein expression of GAPDH and MAO B in the human prefrontal cortex in alcohol-dependent subjects. **(A)** Western Blot analysis of brain GAPDH and MAO B. A representative blot of protein expression from 4 normal controls and 4 alcohol-dependent subjects is shown. **(B)** Quantitative analysis of Western blotting. Each protein was analyzed separately. Graphs of the average optical density of GAPDH and MAO B (normalized to the density of actin) are shown for the control group and alcohol-dependent subjects. The relative intensity (relative optical density $\times$ pixel area) of autoradiographic bands from two independent preparations was evaluated. Graphs of the average optical density of GAPDH/actin and MAO B/actin for the individual subjects and mean values (horizontal lines) are shown with 20 subjects ($n = 20$) in both the control group (circles) and alcohol dependent group (diamonds or squares). Expression of GAPDH ($p < 0.05$) and MAO B ($p < 0.05$) is significantly increased in alcohol dependent subjects as compared to the normal control subjects.

A **B** **Fig. 3.**

Protein expression of GAPDH and MAO B in the prefrontal cortex of rats fed with ethanol. Rats were fed with an ethanol diet or control diet for 28 days, and the protein levels of GAPDH and MAO B in the prefrontal cortex were examined by Western blotting. **(A)** Representative Western blots showing the immunolabelling of GAPDH or MAO B in the prefrontal cortex of 6 untreated controls and 6 ethanol-treated rats. The anti-actin antibody was used as the loading controls. **(B)** Quantitative analysis of Western blot results. Each GAPDH or MAO B protein's autoradiographic band was evaluated from two independent preparations by its relative intensity (relative optical density $\times$ pixel area) and normalized to the density of actin. Graphs of the average optical density of GAPDH/actin and MAO B/actin for the individual subjects and mean values (horizontal lines) are shown with 10 rats ($n = 10$) for both the control group (circles) and ethanol-feeding group (diamonds or triangles). Expression of GAPDH ($p < 0.02$) and MAO B ($p < 0.05$) is significantly increased in the ethanol-fed group as compared to that of the unfed control group.

Fig. 4. Effect of ethanol-induced nuclear accumulation of GAPDH or TIEG2 (an MAO B transcriptional activator) and the presence of the GAPDH/TIEG2 complex in the nucleus. **(A)** Western blotting of nuclear TIEG2 or GAPDH. Nuclear proteins were isolated from cells that were treated with or without ethanol for 72 h and analyzed with anti-TIEG2 or with anti-GAPDH. Quantitative analysis of optical density of TIEG2 or GAPDH (normalized to the density of histone H4) is shown at the bottom. **(B)** Nuclear TIEG2 co-immunoprecipitation with GAPDH. Nuclear proteins were isolated from cells that were treated with or without ethanol for 72 h, immunoprecipitated by incubation with anti-TIEG2 antibody, and analyzed by Western blotting with anti-GAPDH antibody. Quantitative analysis is shown at the bottom. Data represent the mean $\pm$ S.D. of four independent experiments. $*p < 0.001$ compared with respective control group (without ethanol) which was taken as 1. **(C)** Transient transfection and luciferase assay for the interaction among GAPDH, TIEG2, and MAO B promoter in U-118 MG cells. MAO B 2 kb promoter-luciferase reporter gene was co-transfected with pcDNA3.1 (control), GAPDH (wild type)-, GAPDH mutant (K160R)- or GAPDH mutant (Cys150)-expression vector, TIEG2-expression vector or both GAPDH- and TIEG2-expression vectors into cells. After 24 h, cells were treated with ethanol (75 mM) daily for another 2 days. All measurements were performed in triplicate in three independent experiments. $*$, $p < 0.01$, $**$, $p < 0.001$ and $\#$, $p < 0.0001$ compared with control (transfected with pcDNA3.1; lane 1).

Fig. 5.

Effects of GAPDH-knockdown on MAO B mRNA level and DNA damage in U-118 MG cells. (A) Western blot analysis of GAPDH-knockdown mediated by siRNA. Cells were transfected with control-siRNA or GAPDH-siRNA for 3 days. Equal amounts of total protein from each supernatant solution were resolved by SDS/PAGE and blotted by anti-GAPDH antibody. (B) Effects of GAPDH-knockdown on MAO B mRNA level. Control-siRNA-transfected cells or GAPDH-knockdown cells were treated with ethanol (75 mM) for 2 days, and then the MAO B mRNA level was determined. *, $p < 0.0005$ compared with control-siRNA transfected cells in ethanol-treated group. (C) Fluorescence showing TUNEL(+) cells and TUNEL(-) cells after treatment with ethanol in control-siRNA transfected cells or in GAPDH-knockdown cells. (a) Photomicrographs show representative cells from each treatment group (ethanol treatment

was 2 days); arrows indicate apoptotic cells. **(b)** Percentage of cells that contain damaged DNA (green fluorescence) induced by ethanol (0, 2 or 3 days) as revealed by the TUNEL assay. Experiments were done in duplicate in three independent evaluations. The average counted cell numbers are 775, 694, 758, 983, 716 and 695 from lane 1 to lane 6, respectively. *, $p < 0.001$ and ** $p < 0.0001$ compared with control-siRNA-transfected cells in the ethanol-treated groups.

Fig. 6.

Effects of MAO B inhibitor (selegiline) on ethanol-induced GAPDH nuclear translocation, MAO B mRNA level and the generation of toxic H_2O_2 . Cells were treated with 75 mM ethanol without or with selegiline (0.25 nM) for 48 h (for immunofluorescence and mRNA level) or 72 h (for Western blotting and measurement of H_2O_2 generation). GAPDH nuclear accumulation was determined by (A) immunofluorescence microscopy and (B) Western blotting. Nuclear proteins were isolated from cells that were treated with ethanol or with both ethanol and selegiline for 72 h and were analyzed with anti-GAPDH antibody as indicated. Quantitative analysis of optical density of GAPDH (normalized to the density of histone H4) is shown at the bottom. *, $p < 0.0001$ compared with ethanol-treated group (without selegiline). (C) MAO B mRNA levels and (D) generation of H_2O_2 were also determined. Controls were cells treated with ethanol alone which were taken as 100%. *, $p < 0.001$ and #, $p < 0.005$. Data represent the mean $\pm$ S.D. of three independent experiments.