Original Article

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GHB Dependent Changes in PEA-15 Gene Expression in Different Human Cell Lines

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Abstract

Background. Gamma-hydroxybutyric acid (GHB) is found to be present endogenously in mammalian brains. It has been abused increasingly in recent years, particularly in date rape sexual assaults. Metabolism of GHB happens very rapidly; it disappears within 12 hours, making its detection in criminal cases very complicated.

Aim. This study intended to determine whether PEA-15 gene expression in human cell lines can be used as a marker for expanding the window of GHB detection beyond 12 hours.

Methods. The effects of GHB on cell viability was determined by use of the MTT assay. Quantitative real-time PCR and western blots were used to assess the effect of GHB exposure on PEA-15 mRNA and protein levels respectively in human brain and blood cells.

Results. The viability of human brain cells was decreased as GHB concentrations increased. PEA-15 mRNA levels increased in 1321N1 and SH-SY5Y cells by 4.2- and 3.7-fold respectively, after a 100 µM GHB treatment ($p<0.01$ both), but with no change at 1µM GHB. However, PEA-15 mRNA levels were increased significantly in THP-1 cells by 40.8-fold after a 10 µM GHB treatment ($p<0.01$), but with no change at 900 µM GHB. PEA-15 protein expression levels were increased in THP-1 cells by 1.9- and 1.6-fold ($p<0.001$) after 10µM and 900µM GHB treatments respectively, and increased in SH-SY5Y cells by 1.6-fold ($p<0.05$) after a 100 µM GHB treatment. However, there were no changes in PEA-15 protein levels in SH-SY5Y cells after 24 hours of GHB exposure.

Conclusion. The results of this study on human cell lines support the previous study in mice, which suggests PEA-15 levels as a possible surrogate marker for GHB administration beyond 12 hours. Additional studies are required.

Keywords. GHB, PEA-15, SH-SY5Y cells, SH-SY5Y cells, and THP-1 cells.

INTRODUCTION

GHB is a fatty acid with four carbons; it was first synthesized as an anaesthetic in 1960 and as a precursor of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) in the brain.[(1)] Furthermore, GHB was found to be present endogenously, mostly in the hypothalamus and the basal ganglia of the mammalian brain.[(2,3)] Although it has been more than 40 years since GHB was synthesised,[(1,4)] relatively little is known about its mechanisms of action. There has been a huge discussion about which receptors are involved in mediating GHB activity, but scant information is available concerning the

changes in gene expression that GHB may induce. GHB is known to have broad pharmacological activity, through binding to either the specific high-affinity GHB receptors or GABA receptors, interacting with the dopaminergic system, affecting serotonin levels, interacting with neurosteroids, interacting with the cholinergic system, decreasing acetylcholine levels, and increasing the secretion of growth hormone.[5] GHB's effects on the expression of a range of genes in various cell types has been studied previously.[6–9] One of these studies discovered that 9 mRNA transcripts were up-regulated after 24 hours of GHB exposure in mouse blood samples. This led researchers to conclude that GHB had induced changes in gene expression, with the specific changes depending on the time interval post exposure.[7] PEA-15 was involved with the up-regulated genes.

PEA-15

PEA-15 encodes a death effecter domain (DED). The N-terminal region contains the canonical death effecter domain that constitutes amino acids 1–80 and a nuclear export signal, and the C-terminal region contains two serine phosphorylation sites (Fig. 1). PEA-15 is a main phosphoprotein found in the astrocyte cells and is a substrate for the enzyme protein kinase C. Unphosphorylated PEA-15 binds extracellular-signal-regulated kinase (ERK) and prevents the accumulation of active ERK in the nucleus, thus blocking transcription and slowing proliferation.[10] The previous study showed that PEA-15 is important in protecting against cytokine-induced apoptosis.[11]

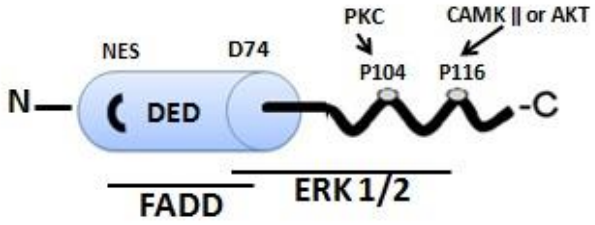


Figure 1. A schematic illustration of PEA-15 protein. It is 130 amino acids in length and comprised of an N-terminal region containing a canonical death effecter domain (DED) that constitutes 1–80 amino acids, along with a C-terminal region containing two serine phosphorylation sites, Ser-104 phosphorylated by protein kinase C (PKC) and Ser-116, phosphorylated by either CamKII or serine/threonine protein kinase (AKT). These phosphorylations are drawn by arrows. PEA-15 binding partners ERK and FADD are shown by lines.

Aim of this Study

This study was intended to determine whether PEA-15 gene expression in human cell lines can be used as a marker for expanding the window of GHB detection beyond 12 hours, and to understand the conditions under which this marker is expressed. The study was also intended to investigate whether previous results from animal studies are consistent with findings in human cells. To achieve these aims, the effects of GHB on the level of PEA-15 mRNA and protein were studied using quantitative PCR and Western blots. Three human cell lines were selected: SH-SY5Y cells and 1321N1 cells, which are neuronal and non-neuronal cells respectively, and THP-1 cells, which are human blood cells.

Materials and Methods.

Chemicals and reagents

All chemicals used were of analytical grade and were purchased from Sigma-Aldrich (Poole, Dorset). Antibodies were obtained from Santa Cruz Biotech, Inc. (Santa Cruz, CA, USA); Acrylamide from Severn Biotech, Ltd (UK); and nitrocellulose membranes from Amersham Biosciences (UK).

Cell culture

Human THP-1 monocytic leukaemia cells[12] were obtained from a European collection of cell cultures in the UK. THP-1 cells were maintained in RPMI 1640 media, supplemented with 10% FBS, 1% L-glutamine, and 1% of 100 u/ml of penicillin-streptomycin. THP-1 cells were cultured and treated with 10µM and 900µM of the GHB drug for 24 hours.

SH-SY5Y cells (human neuroblastoma),[13,14] and 1321N1 cells (human astrocytoma),[15] were taken from American Type Culture Collection (ATCC). SH-SY5Y cells were grown in a 1:1 mixture consisting of Ham's F-12 and Eagle's minimal essential medium (EMEM) (Cat: M2279), supplemented with 1% nonessential amino acids, 1% sodium pyruvate, 1% L-glutamine, 100 units/ml each of penicillin and streptomycin, and 10% fetal bovine serum. 1321N1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Cat: D5796), supplemented with 10% FBS and 1% penicillin-streptomycin (10,000 units penicillin; 10 mg streptomycin/ml, 0.9% NaCl) solution. All cell culture chemicals were obtained from Sigma-Aldrich, UK.

Cell viability study (MTT assay)

Cells were cultured in a 96 well plate at a concentration of 3 X 105 cells/ml and allowed to attach for 24 hours. The cells were treated by the addition of 100 µl of media containing different concentrations of GHB dissolved in dimethylsulfoxide (DMSO). No more than 0.1% DMSO was added, and a negative control (DMSO and cells only) and a positive control (Triton x100) were used, as well as a media-only control (no cells). Cells were incubated for 24 hours. After that, 20 µl of MTT (1.2 mg of MTT dissolved in 1 ml of media) were added in each well, and incubated for a further 2 hours. Then the media were aspirated and 100 µl of DMSO was added to all wells, followed with gentle shaking for 10 minutes in order to obtain complete dissolution. Absorbance was read at 560 nm using the Labsystems iEMS microplate spectrophotometer, which was supplied by Labsystems and Life Sciences International Limited (UK). The background values (no cells control) were subtracted, and the results were the mean value as a percentage of the DMSO and cells-only control.

Sample preparation

SH-SY5Y and 1321N1 cells were cultured in triplicate in 6 well plates, at

a concentration of 106 cells in each well, and allowed to attach. They were then treated with 1µM and 100µM of the GHB drug for 24 hours. The cells were then washed with ice-cold, sterile 1x PBS and scraped into a microcentrifuge tube using a rubber policeman. THP-1 cells were cultured and treated with 10µM and 900µM of the GHB drug for 24 hours and then centrifuged, washed with ice-cold, sterile 1xPBS, and placed in a sterile microcentrifuge tube. The resultant pellets were then stored at -80°C to be used.

Quantitative Real Time-PCR

Oligonucleotide primers for the amplification of PEA-15 and GAPDH were synthesized by Eurofins MWG Operon (UK). For PEA-15 (NM_003768.3) the forward primer was 5´-GCTCCAGAGGCGTCATGGCTG-3´ and the reverse primer was 5´-TGAGTAGGTCAGGACGGCGGG-3´. For GAPDH (NC_000012.11) the forward primer was 5´-GGAGTCAACGGATTTGGT-3´ and the reverse primer was 5´-GTGATGGGATTTCCATTG-3´. The PCR Sprint Thermal Cycler was obtained from Thermo Life Sciences. 10µl RNA (about 0.5µg) and 1µl random primer (46ng final concentrations) were added together in a sterile microtube and incubated for 5 minutes at 70 °C. The mixture was quickly cooled on ice for 5 minutes after the incubation, and 5µl M-MLV reverse transcriptase buffer (5x), 1.25µl PCR nucleotide mix (0.5mM final concentration), 6.75µl nuclease-free water, and 1µl M-MLV reverse transcriptase (last) were added. The mixture was incubated for 10 minutes at 25°C and the PCR reaction was performed in two steps: incubation at 42°C for 50 minutes (step 1) and inactivation at 70°C for 15 minutes (step 2). The cDNA produced was stored at -20°C.

Q-RT-PCR was carried out using a set of oligonucleotide primers with the Light Cycler instrument (Roche Diagnostics), with the specific cycling conditions for the Light Cycler. Reactions were set up in 20µl volumes that consist of 5µl of first strand cDNA, 1µl of each primer (5pmol/µl, the final concentration of 0.25µM), 3µl of nuclease-free water, and 10µl of Perfecta SYBR Green Fast mix (obtained from Quanta Biosciences). DNA was amplified by an initial incubation at 95°C for 2 min followed by 24–27 cycles of 95 °C for 30 seconds, annealing temperature (primer-dependent) for 30 seconds, 72°C for 90 seconds, and a final extension at 72°C for 10 minutes. The number of cycles was determined following pre-testing of a range of cycles in which the product showed linear expression. The PCR products were separated by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining. Nuclease-

free water was used as a negative control. The relative amount of cDNA synthesized in each RT-PCR was compared with GAPDH mRNA levels. Relative expression levels were calculated for each sample after normalization to the GAPDH, using the ΔΔCT method for comparing relative fold-expression differences. The reproducibility of the quantitative measurements was evaluated by 3 independent cDNA syntheses.

Western Blot

Cells were treated with diverse concentrations of GHB (1μM and 100μM or 10μM and 900μM) in 6-well plates done in triplicate for 24 hours, and western blot analysis was performed on the whole-cell extracts. 40 μg of protein lysate were denatured with sodium dodecyl sulfate (SDS), reducing the buffer by boiling it for 5 minutes, and then separated on a 10% polyacrylamide SDS gel. The separated proteins were transferred to nitrocellulose membrane (Hybrid ECL) and probed with specific antibodies, followed by incubation with goat anti-rabbit IG-horseradish peroxidase-conjugated antibody (Bio-Rad). Reactive proteins were detected using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL, USA), according to the manufacturer's procedures. Western blots were carried out using PEA-15 (H-3) antibody (Santa Cruz Biotech, Inc., catalogue number sc-166678). GAPDH antibodies were used for normalization. Bands were quantified using ImageJ, and expression levels were calculated relative to the untreated control.

Statistical Analysis

Statistical analysis of results was done with one-way analysis of variance (ANOVA), unless otherwise stated. Comparison between groups was carried out using Dunnett's post-test. Statistical analysis was completed using Excel and Prism software.

Results.

Determination of appropriate concentration of GHB

To determine an appropriate concentration of GHB to use, brain cells (1321N1 and SH-SY5Y cells) were treated with diverse concentrations of GHB for 24 hours, and the effect on cell viability was measured with the MTT assay, which measures metabolic activity. The results show some decreased cell viability in the two cell lines at increasing concentrations of GHB (Fig. 2). However, the level of toxicity was not high and an IC50 could not be determined. Based on these results, a safe, nontoxic 1 μM dose and a slightly toxic dose 100 μM of GHB were selected for subsequent work.

GHB concentrations for the blood cells (THP-1 cells) were selected based on a study carried out to compare the window of GHB detection in blood and saliva after a GHB dose of 60 mg/kg

body weight (total of 4680 mg), administered orally on an empty stomach to a 54-year-old male (78 kg). The results of that study indicated that the maximum GHB level in the blood was 991.3 µM reached after 20 min of GHB administration, and the level after about 5 hours was 79.3 µM.[16] Therefore, in this study, THP-1 cells were treated with a low concentration (10 µM) and high concentration (900 µM) of GHB, to represent concentrations found following GHB exposure.

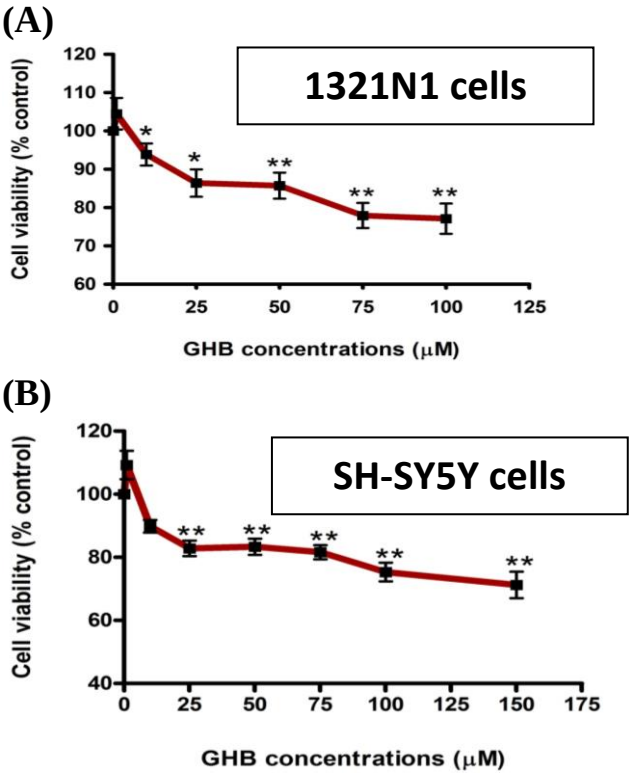


Figure 2. (n=6) and effects of GHB on the viability of (A) 1321N1 and (B) SH-SY5Y cells. Cells at 60% confluence were exposed to 1, 10, 25, 50, 75, 100, and 150 µM GHB for 24hr, and cell viability was assessed with the MTT assay as described in Materials and Methods. Values representing mean ± SEM are expressed as a percentage of control (untreated) cells. Asterisks indicate significance compared with untreated control (p<0.01 *p<0.05), using Excel and Prism software.**

Quantitation of PEA-15

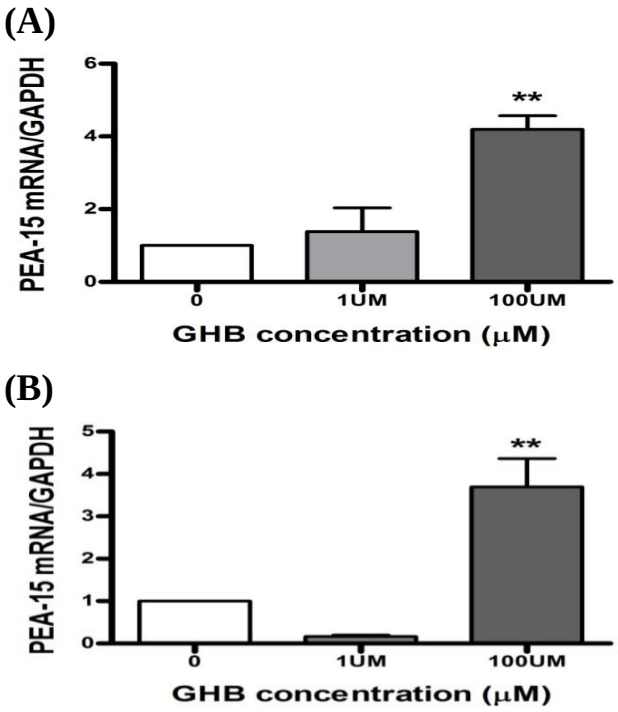
To assess the effect of GHB exposure on PEA-15 mRNA levels in brain cells (1321N1 and SH-SY5Y) and blood cells (THP-1), the brain cells were exposed to 1 and 100 µM, and the blood cells were exposed to 10 and 900 µM, GHB for 24 hours, and the level of PEA-15 mRNA expression was measured by Quantitative RT-PCR using the specific oligonucleotide primers. The amplification specificity of each primer set was checked by melting and amplification curve analysis, and the standard curves were prepared using plasmid DNA containing the cloned PEA-15 gene ranging from 10^5 to 10^9 copies/µl. PCR reactions were performed simultaneously with the samples.

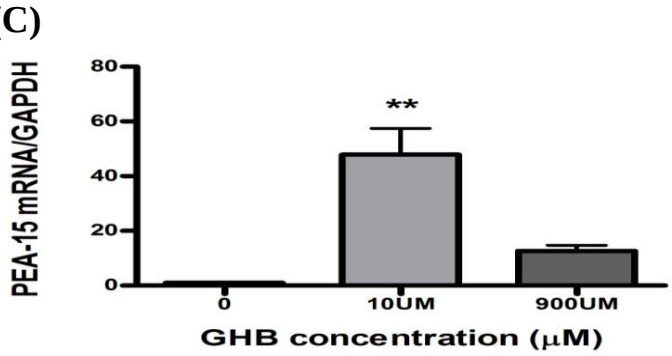
Copy numbers were calculated by extrapolation with the standard samples from the standard curves, and the copy number of the target gene (PEA-15 gene) was normalized with the reference gene (GAPDH gene) by using the formula.

$$\text{Normalized copy number} = \frac{\text{Copy number of target gene}}{\text{Copy number of reference gene}}$$

The normalized copy numbers of 3 independent readings were analyzed using Excel and Prism software by the use of one-way analysis of variance (ANOVA), and the comparison between groups was carried out using Dunnett's post-test.

The results showed that PEA-15 mRNA levels increased in 1321N1 and SH-SY5Y cells by 4.2- and 3.7-fold respectively, after 100 µM GHB treatment ($p<0.01$ both), but with no significant change at low concentrations of GHB (1 µM). However, the PEA-15 mRNA level was increased significantly in THP-1 cells by 40.8-fold after a 10 µM GHB treatment ($p<0.01$), but with no significant change at high concentrations of GHB (900 µM), as shown in (Fig. 3).





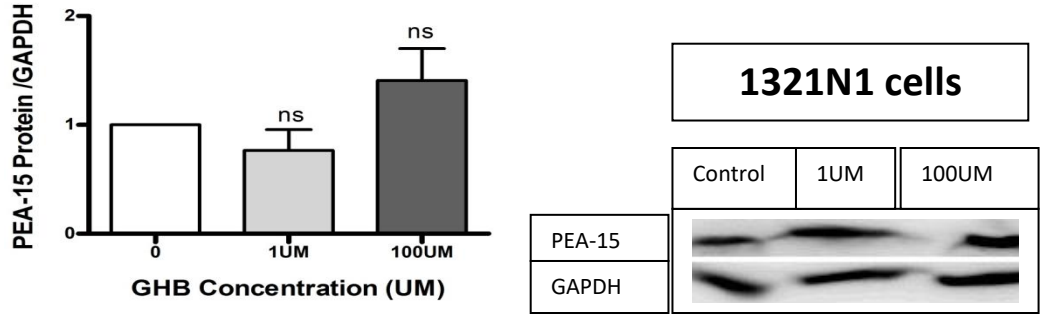
Secondary validation of PEA-15 protein expression after GHB exposure

In order to confirm whether GHB had an effect on PEA-15 protein expression in 3 cell lines, western blotting analysis for protein was carried out on these cells. Brain cells (1321N1 and SH-SY5Y cells) were treated with 1 µM and 100 µM concentrations of GHB, and blood cells (THP-1 cells) were treated with 10 µM and 900 µM concentrations of GHB, for 24 hours, and the results showed that PEA-15 protein expression levels were increased significantly in blood cells (THP-1) by 1.9- and 1.6-fold ($p<0.001$) after 10µM and 900µM GHB treatments respectively, and were also increased significantly in neuronal cells (SH-SY5Y) by 1.6-fold ($p<0.05$) after a 100 µM GHB treatment for 24 hours. However, there were no significant changes in the PEA-15 protein expression level in 1321N1 cells after 24 hours of GHB exposure, as shown in Fig. 4.

Figure 3. PEA-15 mRNA levels in control, untreated, and treated cells. Total RNA was isolated from (A) 1321N1, (B) SH-SY5Y, and (C) THP-1 cells (treated and untreated cells). The PEA-15 mRNA level was

quantified by QRT- PCR using the Light Cycler SYBR green, as described in Materials and Methods. Asterisks indicate significance compared with the untreated control (p<0.01 and *p<0.05).**

(A)



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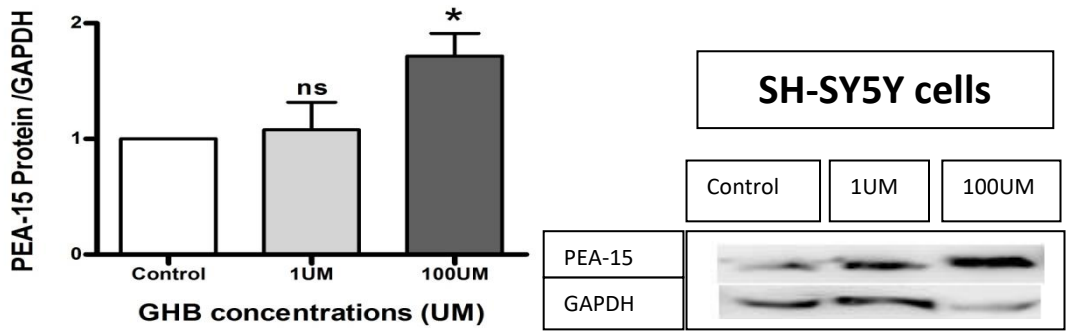

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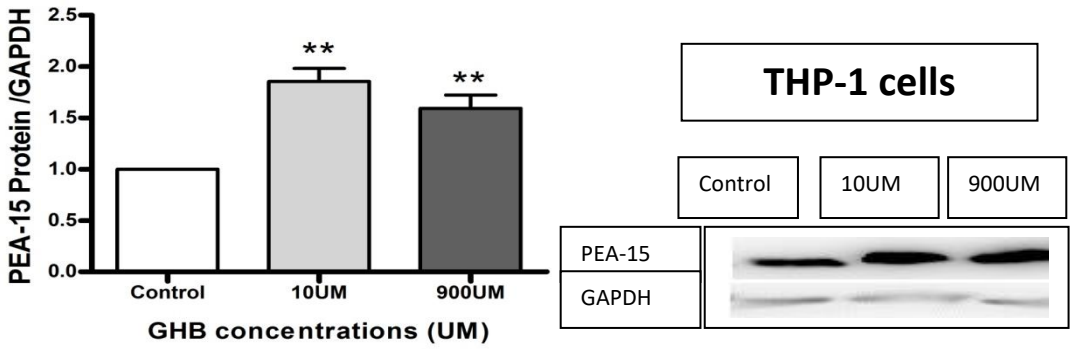

Figure 4. Change in PEA-15 protein expression in control, untreated, and treated cells after GHB exposure. (A) 1321N and (B) SH-SY5Y cells were treated with 1 or 100µM GHB, and (C) THP-1 cells were treated with 10 or 900µM GHB for 24 hours, and western blots were carried out on whole-cell extracts using the PEA-15 antibody (Santa Cruz Biotech, Inc.), with GAPDH used for the normalization and loading on 10% SDS-PAGE gel. Asterisks indicate significance compared with the control (p<0.01 and *p<0.05).**

Discussion

Despite a great deal of discussion about GHB pharmacodynamics and the receptors involved in mediating GHB action, little is known about the changes in gene expression that may be involved.

GHB toxicity

The results revealed that the toxicity of GHB was similar in both brain cell lines (SH-SY5Y and 1321N1 cells). GHB significantly decreases cell viability in a concentration-dependent manner in both cell lines, but is not significantly toxic at concentrations up to 150 µM. Our results are the first to show that GHB has the same effect on the viability of neuronal and non-neuronal brain cells. This is interesting because in the brain, astrocytes are known to provide metabolic and immune-function support to neurons, and therefore might be considered to be more robust in terms of metabolic processes and ability to withstand toxic insult. However, the results presented here indicate that both cell types are equally sensitive to GHB, which may indicate that metabolic processes are not involved in mediating any intrinsic protection.

The toxicity of GHB to THP-1 was not assessed, but previous results indicated that much higher concentrations of GHB could be tolerated by blood cells. The GHB concentrations for the blood cells (THP-1 cells) were selected based on a study carried out on a 54-year-old male (78 kg), administered a 4.68 g dose of GHB orally on an empty stomach. The results of that study indicated that the maximum GHB level in the blood was 991.3 µM, reached after 20 minutes of GHB administration; the level after about 5 hours was 79.3 µM.[16]

GHB dependent changes in PEA-15 mRNA and protein levels

The results revealed that human PEA-15 mRNA levels were increased in 1321N1 and SH-SY5Y cells, and were also increased significantly in THP-1 cells after GHB treatment. At the protein level, western blotting showed that PEA-15 protein expression was increased significantly in blood cells (THP-1) by GHB treatments, and also increased significantly in neuronal cells (SH-SY5Y). However, there were no significant changes in PEA-15 protein expression levels in 1321N1 cells.

PEA-15 is a main phosphoprotein in human astrocyte cells that is known to block the inhibition of integrin activation by Ras, and also to adjust the ERK MAP kinase cascade. PEA-15 is known to be regulated post-translationally by phosphorylation in astrocytes by protein kinase C in response to endothelin, and by CaMKII (or a related kinase).[11]

Despite its name (phosphoprotein enriched in astrocytes), PEA-15 is expressed in most tissues. In terms of

function, studies in knockout mice indicate that PEA-15 protects astrocytes from TNF-induced apoptosis. It is recognized to be broadly anti-apoptotic and controls caspase-3 function, thereby domineering cell survival. It is also expressed highly in type-2 diabetes mellitus, and it has been revealed that it can influence glucose uptake, causing insulin resistance. Another function appears to be in autophagy, as it can induce autophagy in myoblasts, along with differentiation.[17]

At the transcriptional and translational levels, PEA-15 has been observed to be regulated by a range of conditions and factors. It is overexpressed in some cancer cell lines and can be induced by exposure of cells to TGF-beta1.[17]

Morphine sensitization is known to lead to up-regulation of PEA-15.[18] PEA-15 is also up-regulated by 12-O-tetradecanoyl phorbol-13-acetate (TPA) in skin and keratinocytes, and this may explain its role in mediating TPA-induced effects on caspase-3 functions and apoptosis sensitivity.[19] In contrast, leuprolide acetate, which is a gonadotropin-releasing hormone (GnRH) agonist, decreases PEA-15 expression by 50%.[20] Leuprolide acetate has antiproliferative effects in uterine leiomyomas due to the suppression of the anti-apoptotic effects of PEA-15.

There is no apparent reason why PEA-15 would be regulated by GHB directly. However, it is known that GHB increases growth hormone secretion during sleep,[20] and it is also known that growth hormone leads to a multitude of changes within the cell, so it is likely that the effect observed could be indirect.

Conclusion.

The results of the present study demonstrated that GHB causes changes in PEA-15 mRNA and protein expression levels in the human cell lines used, and the results seem to support the previous study in mice which suggests the PEA-15 level as a possible surrogate marker for GHB administration after 12 hours.[7] Our results are the first to demonstrate that GHB induces changes in PEA-15 gene and protein expression levels in human cell lines.

Further experiments are needed to confirm that these results are a specific response to GHB exposure, by comparing PEA-15 levels induced in cell lines with levels induced by combinations of GHB, GABA, and the specific antagonists NCS-382 (GHB receptor antagonist) and CGP-35348 (GABAB receptor antagonist).

References.

1. Laborit H. Sodium 4-hydroxybutyrate. Int J Neuropharmacol. 1964 Sep 1;3(4):433-IN8.
2. Bessman SP, Fishbein WN. Gamma-hydroxybutyrate, a normal brain metabolite. Nature. 1963 Dec;200(4912):1207.

3. Snead III OC, Morley BJ. Ontogeny of γ-hydroxybutyric acid. I. Regional concentration in developing rat, monkey and human brain. Dev Brain Res. 1981 Jul 1;1(4):579-89.
4. Caputo F, Vignoli T, Maremmani I, Bernardi M, Zoli G. Gamma-hydroxybutyric acid (GHB) for the treatment of alcohol dependence: a review. Int J Environ Res Public Health. 2009 Jun;6(6):1917-29.
5. Drasbek KR, Christensen J, Jensen K. Gamma-hydroxybutyrate–a drug of abuse. Acta Neurol Scand. 2006 Sep;114(3):145-56.
6. Wisor JP, Morairty SR, Huynh NT, Steininger TL, Kilduff TS. Gene expression in the rat cerebral cortex: comparison of recovery sleep and hypnotic-induced sleep. Neuroscience. 2006 Jan 1;141(1):371-8.
7. Larson SJ, Putnam EA, Schwanke CM, Pershouse MA. Potential surrogate markers for gamma-hydroxybutyrate administration may extend the detection window from 12 to 48 hours. J Anal Toxicol. 2007 Jan 1;31(1):15-22.
8. Schnackenberg BJ, Saini UT, Robinson BL, Ali SF, Patterson TA. An acute dose of gamma-hydroxybutyric acid alters gene expression in multiple mouse brain regions. Neuroscience. 2010 Oct 13;170(2):523-41.
9. Kemmel V, Klein C, Dembélé D, Jost B, Taleb O, Aunis D, Mensah-Nyagan AG, Maitre M. A single acute pharmacological dose of γ-hydroxybutyrate modifies multiple gene expression patterns in rat hippocampus and frontal cortex. Physiol Genomics. 2010 Jan 26;41(2):146-60.
10. Renganathan H, Vaidyanathan H, Knapinska A, Ramos JW. Phosphorylation of PEA-15 switches its binding specificity from ERK/MAPK to FADD. Biochem J. 2005 Sep 15;390(3):729-35.
11. Kubes M, Cordier J, Glowinski J, Girault JA, Chneiweiss H. Endothelin induces calcium-dependent phosphorylation of PEA-15 in intact astrocytes: identification of Ser104 and Ser116 phosphorylated, respectively, by protein kinase C and calcium/calmodulin kinase II in vitro. J Neurochem. 1998 Sep;71(3):1307-14.
12. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukaemia cell line (THP-1). Int J Cancer. 1980 Aug 15;26(2):171-6.

13. Biedler JL, Helson L, Spengler BA. Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. Cancer Res. 1973 Nov 1;33(11):2643-52.
14. Biedler JL, Roffler-Tarlov S, Schachner M, Freedman LS. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. Cancer Res. 1978 Nov 1;38(11 Part 1):3751-7.
15. Clark RB, Su YF, Ortmann R, Johnson GL, Perkins JP. Factors influencing the effect of hormones on the accumulation of cyclic AMP in cultured human astrocytoma cells. Metabolism. 1975 Mar 1;24(3):343-58.
16. Kintz P, Goullé JP, Cirimele V, Ludes B. Window of detection of γ-hydroxybutyrate in blood and saliva. Clin Chem. 2001 Nov 1;47(11):2033-4.
17. Iovino S, Oriente F, Botta G, Cabaro S, Iovane V, Paciello O, Viggiano D, Perruolo G, Formisano P, Beguinot F. PED/PEA-15 induces autophagy and mediates TGF-beta1 effect on muscle cell differentiation. Cell Death Differ. 2012 Jul;19(7):1127.
18. Ramos-Miguel A, Esteban S, García-Sevilla JA. The time course of unconditioned morphine-induced psychomotor sensitization mirrors the phosphorylation of FADD and MEK/ERK in rat striatum: role of PEA-15 as a FADD-ERK binding partner in striatal plasticity. Eur Neuropsychopharmacol. 2010 Jan 1;20(1):49-64.
19. Formisano P, Perruolo G, Libertini S, Santopietro S, Troncone G, Raciti GA, Oriente F, Portella G, Miele C, Beguinot F. Raised expression of the antiapoptotic protein ped/pea-15 increases susceptibility to chemically induced skin tumour development. Oncogene. 2005 Oct;24(47):7012.
20. Bifulco G, Miele C, Pellicano M, Trencia A, Ferraioli M, Paturzo F, Tommaselli GA, Beguinot F, Nappi C. Molecular mechanisms involved in GnRH analogue-related apoptosis for uterine leiomyomas. Mol Hum Reprod. 2004 Jan;10(1):43-8.
21. Van Cauter E, Plat L, Scharf MB, Leproult R, Cespedes S, L'Hermite-Baleriaux M, Copinschi G. Simultaneous stimulation of slow-wave sleep and growth hormone secretion by gamma-hydroxybutyrate in normal young men. J Clin Invest. 1997 Aug 1;100(3):745-53.

Conflict of interest:

The authors declare that they have no conflicts of interest.