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Pretreatment of liver grafts *in vivo* by γ -aminobutyric acid receptor regulation reduces cold ischemia/warm reperfusion injury in rat

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Summary

Background: Gamma-aminobutyric acid (GABA) is found throughout the body. The regulation of GABA receptor (GABAR) reduces oxidative stress (OS). Ischemia/reperfusion injury after orthotopic liver transplantation (OLT) causes OS-induced graft damage. The effects of GABAR regulation in donors *in vivo* were investigated.

Material/Methods: Donor rats received saline, a GABAR agonist or GABAR antagonist 4 h before surgery. Recipient rats were divided into four groups according to the donor treatments: laparotomy, OLT with saline, OLT with GABAR agonist and OLT with GABAR antagonist. Histopathological, biochemical and immunohistological examinations were performed at 6, 12 and 24 h after OLT. Protein assays were performed at 6 h after OLT. The 4-hydroxynonenal (4-HNE), ataxia-telangiectasia mutated kinase (ATM), phosphorylated histone H2AX (γ H2AX), phosphatidylinositol-3 kinase (PI3K), Akt and superoxide dismutase (SOD) were assessed by western blot analysis.

Results: In the univariate analysis, histopathological and biochemical profiles verified that the GABAR agonist reduced graft damage. Immunohistology revealed that the GABAR agonist prevented the induction of apoptosis. Measurement of 4-4-HNE levels confirmed OS-induced damage after OLT, and the GABAR agonist improved this damage. In the γ H2AX, PI3K, Akt and antioxidant enzymes (SODs), ATM and H2AX were greatly increased after OLT, and were reduced by the GABAR agonist. In the multivariate analyses between multiple groups, histopathological assessment, aspartate aminotransferase level, immunohistological examinations for apoptotic induction and γ H2AX showed statistical differences.

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^AStudy Design

^BData Collection

^CStatistical Analysis

^DData Interpretation

^EManuscript Preparation

^FLiterature Search

^GFunds Collection

Conflict of interest

All authors have no financial conflict of interest.

Conclusions: A specific agonist demonstrated regulation of GABAR *in vivo* in the liver. This activation *in vivo* reduced OS after OLT via the ATM/H2AX pathway.

Keywords

reperfusion injury; oxidative stress; free radicals; cold ischemia; warm reperfusion

BACKGROUND

Oxygen is required for cell survival. However, it also poses a potential hazard via reactive oxygen species (ROS) and reactive nitrogen species (RNS), with modification of macromolecules (lipids, proteins and nucleic acids) and alteration of their biological functions [1-3]. Therefore, ROS/RNS were initially considered as harmful products of normal aerobic metabolism. Currently, it is evident that the control of ROS/RNS production plays a physiological role, especially in regulating cell signaling to involve cell proliferation, differentiation and apoptosis [1-3]. Oxidative stress mediated by free radicals is defined as an imbalance between the production of ROS/RNS and the antioxidant capacity of the cell [1-3]. These antioxidants are a defense against ROS/RNS-induced oxidative stress to lipids, proteins and deoxyribonucleic acid (DNA) [2].

Gamma-aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the mammalian brain. Most research that has focused on GABA or the regulation of GABA receptor (GABAR) has only been on the brain [4-8]. Currently, GABA is considered to be a multifunctional molecule with various physiological effects throughout the body [9,10]. Some studies have focused on the effects of regulation of GABAR in other fields, such as stem cell proliferation and metabolism [5,11]. In the brain, many studies have verified that GABA and/or the regulation of GABAR prevent oxidative stress-induced damage [5,7,8]. The results of these studies were mainly explained via specific pathways against oxidative stress [5,7]. It is known that the liver contains GABA and its transporter [10]. Hepatic GABAR has also been detected [12]. However, in the liver, the effects of GABAR regulation have not been well studied.

Orthotopic liver transplantation (OLT) is an accepted therapy for children and adults with end-stage liver disease, and currently provides long-term survival and a quality lifestyle. However, cold ischemia during organ storage and subsequent reperfusion with graft recirculation severely damages the liver graft [13]. During cold ischemic preservation, parenchymal cells swell and bleb [13]. Endothelial and Kupffer cells show some rounding and retraction of the cytoplasmic processes [13]. After warm reperfusion, hepatocytes resorb or shed their blebs, and the endothelial cells lose viability [13]. Moreover, Kupffer cells ruffle and release inflammatory mediators, and Kupffer and other cells trigger ROS/RNS production [13]. Ischemia/reperfusion injury is still a major cause of morbidity and mortality after OLT because of primary graft dysfunction or a non-functioning graft [13]. Reperfusion triggers a liver regeneration cascade, but also causes fatal damage in liver grafts because of oxidative stress [13,14]. Currently, several procedures have been proposed to improve liver tolerance against ischemia/reperfusion injury [15,16]. Proactive strategies through pharmacological pretreatment to limit graft damage from ischemia/reperfusion injury would be advantageous for excellent graft function after OLT.

This study aimed to investigate the effect of GABAR regulation on postoperative liver graft function in the rat OLT model, and to examine possible pathways.

MATERIAL AND METHODS

Animals

Male Lewis rats (RT-1^l) were purchased from Harlan Laboratories, Inc. (Indianapolis, IN). Graft donors and recipients were 8–12 week-old rats (approximately 250 g). The experimental protocols were approved by the Ethical Committee of our institution (Mayo Clinic, Institutional Animal Care and Use Committee, No. A19609). Rats were cared for in accordance with the institutional guidelines for Animal Welfare based on The National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Intravenous injection of a GABAR agonist and or antagonist

The dose of 43.56 nmol/g body weight of a GABAR agonist (GABA_A receptor agonist, Mmscimol, 25 mg, 114.10 g/mol; 70015, Fluka, A Sigma-Aldrich Co., St. Louis, MO) and antagonist (GABA_A receptor antagonist, [+-]bicuculline, 25 mg, 367.35 g/mol; 14340, Fluka, Sigma-Aldrich Co.) was used. Four hours before donor surgery, donor rats intravenously received a total volume of 1.0 ml of agonist or antagonist from the penile vein. In our murine hepatectomy model, the GABAR regulation *in vivo* at 4 hours before surgery well worked. Therefore, we here employed the GABAR regulation *in vivo* 4 h before graft harvest, as a pretreatment to the donor rat.

OLT and postoperative care

Comprehensive details of the surgical procedures for rat OLT and postoperative care in our institution have been previously described [17,18]. In brief, the whole-liver graft was used in this study. Syngeneic grafts had a cold ischemic time of 2 h at 4°C in normal Ringer's solution, though cold storage time of 2 h without organ storage solution did not reflect clinical situation in OLT with whole liver graft. Liver grafts were washed out by 10 ml of normal Ringer's solution, immediately after the graft harvest and before graft implantation. To avoid any irrelevant signaling, the hepatic artery was reconstructed by ultra-microsurgery in this study [17,18]. Each rat was housed separately after OLT, and body temperature was maintained by a heating pad. Postoperative observations were performed every 30 min after OLT, and 1.0 ml of warm lactate Ringer's solution was routinely administered every 1 h until 6 h after OLT. For rat OLT, we previously demonstrated the importance of a short anhepatic phase and the exclusion of unreliable samples based on autopsy findings [17,18]. In the current study, the anhepatic phase was maintained within 20 min in each OLT, and no surgical complications were observed in each case at autopsy.

Biochemical assay and coagulation profile

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (T-Bil) levels, and the international normalized ratio of prothrombin time (PT-INR) were measured. Serum AST, ALT and T-Bil levels were assessed by commercial kits (SGOT, SGPT and total bilirubin reagent, respectively, Biotron, Hemet, CA). The PT-INR in plasma was measured by the i-STAT System (Abbott, Princeton, NJ).

Histopathological and immunohistological evaluation

Liver tissue was fixed in 10% neutral-buffered formalin, embedded in paraffin, and sliced into 4-μm sections. The morphological characteristics and graft injury scores were assessed after hematoxylin-eosin (H-E) staining. The graft damage score (point) has previously been described elsewhere [18]. Scores were counted in ten fields (×100) in each slide, and then these scores were averaged for each slide.

The induction of apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) (ApopTag Peroxidase *In Situ* Apoptosis Detection Kit, S7100, Chemicon International, Inc., Billerica, MA) and cysteine aspartic acid protease (caspase)-3 immunostaining (cleaved caspase-3 [Asp175] antibody, 9661S, Cell Signaling Technology, Inc., Danvers, MA). TUNEL-positive nuclei were stained brown and negative nuclei were counterstained light blue. Caspase-3-positive nuclei were stained brown and negative nuclei were counterstained blue. Caspase-3 staining in the rat liver seemed to be not convincing, and therefore highly accurate scanning system were used. Slides were scanned with an automated high-throughput scanning system (Scanscope XT, Aperio Technologies, Inc., Vista, CA). To quantify the immunohistological findings, positive-stained nuclei were counted by Aperio Imagescope software (Aperio Technologies, Inc.). All nuclei were classified into four color intensity levels, and the higher two levels were considered as positive. We calculated the ratio of positive-stained nuclei to all nuclei, and the mean ratio per mm² was determined.

Western blot analysis

Primary antibodies for 4-hydroxynonenal (4-HNE) (4 hydroxynonenal antibody, ab46545; Abcam Inc., Cambridge, MA), ataxia-telangiectasia mutated kinase (ATM) (phospho-[Ser/Thr] ATM/ATR substrate [4F7] rabbit mAb; Cell Signaling Technology, Inc., 2909), phosphorylated histone H2AX (γ H2AX) (phospho-histone H2A.X [Ser139] antibody, 2577; Cell Signaling Technology, Inc.), phosphatidylinositol-3 kinase (PI3K) (phospho-PI3K p85 [Tyr458]/p55 [Tyr199] antibody, 4228; Cell Signaling Technology, Inc.), Akt (phospho-Akt [Ser473] [193H12] rabbit mAb, 4058; Cell signaling Technology, Inc.), superoxide dismutase (SOD) 1 (SOD1) (Cu/Zn superoxide dismutase, LS-B2907, LifeSpan BioSciences, Inc., Seattle, WA), SOD 2 (SOD2) (Mn superoxide dismutase [SOD2], LS-C62194; LifeSpan BioSciences, Inc.) and catalase (catalase, LS-B2554; LifeSpan BioSciences, Inc.) were used. Liver samples were collected, homogenized, and centrifuged at high speed for 10 min at 4°C. The supernatant was then collected and used for BCA protein determination (BCA Protein Assay Reagent, Thermo Fisher Scientific, Rockford, IL) and western blot analysis. A total of 40 μ g of protein was run on 4–20% tris-glycine gels (Invitrogen, Carlsbad, CA) and transferred onto a 0.45 μ m nitrocellulose membrane (Bio-Rad, Life Science Research, Hercules, CA). The membranes were then blocked with 5% nonfat milk in a Tris-buffered saline solution. After blocking the membranes, they were incubated at 4°C over night with the primary antibody. The following day, the membranes were washed three times for 10 min with a Tris-buffered saline solution and then incubated with the peroxidase-conjugated secondary for 1 h, by shaking at room temperature. After incubation, the membranes were once again washed three times for 10 min with the Tris-buffered saline solution and then developed using chemiluminescence (Amersham, Bioscience, Piscataway, NJ). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control. Signals were quantified by using ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

Study design

The scope of this work is the verification of the usefulness of GABAR regulation *in vivo* for a pretreatment of donor. Recipient rats were divided into four groups according to the donor treatments: (i) laparotomy only with saline (normal saline, 1.0 ml, i.v.), (ii) OLT with saline (normal saline, 1.0 ml, i.v.), (iii) OLT with GABAR agonist (muscimol, 43.56 nmol [4.98 μ g]/g body weight, 1.0 ml, i.v.) and (iv) OLT with GABAR agonist (bicuculline, 43.56 nmol [16.00 μ g]/g body weight, 1.0 ml, i.v.). In brief, the molecular doses of GABAR agonist and antagonist were the same, and this dose was similar to that in previous reports [5,19].

Cell signaling involved in cell proliferation, differentiation and apoptosis was confirmed from the early postoperative period after OLT [13,20-22], and subsequently, progressive necrosis was observed [13,20-22]. First, we performed a survival study ($n=10$, in each group). We then collected serum, plasma and histopathological/immunohistological samples at 6, 12 and 24 h after OLT in each group ($n=5$, at each time point). Finally, all samples for western blot analysis were obtained at 6 h after OLT ($n=5$ in each group).

Statistical analysis

The results are presented as mean $\pm$ standard deviation. At first, differences between 2 groups were evaluated by univariate analysis. Next, comparisons between multiple analyses were performed by analysis of variance (ANOVA) and post-hoc tests. The Student t test was used for the comparison of unpaired continuous variables between two groups. Survival curves were constructed by the Kaplan-Meier method. To compare multiple groups, analysis of variance (ANOVA) (one factor ANOVA) and post-hoc test (Dunnett's test) were used. Additionally, statistical results of Dunnett's test were verified by another post-hoc test (Tukey-Kramer's test). Statistical calculations were performed using SPSS Software Version 16.0 (SPSS Inc., Chicago, IL). A p value <0.05 was considered statistically significant.

RESULTS

Survival curves

Long term survival after OLT with whole-liver syngeneic grafts was confirmed in each group (Figure 1).

Parenchymal damage of grafts

We observed inflammatory cell infiltration, vacuolization, hepatocyte ballooning and necrosis after OLT. Histopathological findings of H-E staining are shown in Figure 2A–2D.

There were significant differences at each time point between laparotomy and OLT with saline and between OLT with saline and OLT with GABAR agonist. There were no significant differences between OLT with saline and OLT with GABAR antagonist (Figure 2E).

Biochemical and coagulation profiles

Changes in AST, ALT, and T-Bil levels and PT-INR are shown in Figure 3. There were significant differences in AST, ALT, and T-Bil levels and PT-INR at each time point between laparotomy and OLT with saline. There were significant differences in AST (6 and 12 h after OLT) and ALT levels (6 and 12 h after OLT), and PT-INR (24 h after OLT) between OLT with saline and OLT with GABAR agonist. There were no significant differences in AST, ALT, and T-Bil levels and PT-INR between OLT with saline and OLT with GABAR antagonist (Figure 3).

Induction of apoptosis

TUNEL immunostaining findings are shown in Figure 4A–4D. The ratio of TUNEL-positive nuclei was significantly different at each time point between laparotomy and OLT with saline, and it was also significantly different at each time point between OLT with saline and OLT with GABAR agonist (Figure 4E). Results of caspase-3 immunostaining are shown in Figure 4F–4I. The ratio of caspase-3-positive nuclei was significantly different at each time point between laparotomy and OLT with saline, and it was also significantly different at each time point between OLT with saline and OLT with GABAR agonist.

(Figure 4J). There was no significant difference in the ratio of caspase-3-positive nuclei between OLT with saline and OLT with GABAR antagonist (Figure 4).

Lipoperoxidation

The intensity of 4-HNE in each group is shown in Figure 5A. Normalized 4-HNE was significantly different between laparotomy and OLT with saline, and it was significantly different between OLT with saline and OLT with GABAR agonist (Figure 5B). There was no significant difference in normalized 4-HNE between OLT with saline and OLT with GABAR antagonist (Figure 5).

Response to and repair of DNA damage

The intensity of ATM and H2AX in each group is shown in Figure 6A. Normalized ATM was significantly different between laparotomy and OLT with saline, and it was significantly different between OLT with saline and OLT with GABAR agonist (Figure 6B). Normalized H2AX was significantly different between laparotomy and OLT with saline, and it was significantly different between OLT with saline and OLT with GABAR agonist (Figure 6C). There were no significant differences in normalized ATM and H2AX between OLT with saline and OLT with GABAR antagonist (Figure 6).

Promotion of cell survival

The intensity of PI3K and Akt in each group is shown in Figure 7A. Normalized PI3K was significantly different between laparotomy and OLT with saline, and it was significantly different between OLT with saline and OLT with GABAR agonist (Figure 7B). Normalized Akt was significantly different between laparotomy and OLT with saline. There was no significant difference in normalized Akt between OLT with saline and OLT with GABAR agonist (Figure 7C). There were no significant differences in normalized PI3K and Akt between OLT with saline and OLT with GABAR antagonist (Figure 7).

Antioxidant enzyme activity

The intensity of SOD1, SOD2 and catalase in each group is shown in Figure 8A. There were significant differences in SOD2, but not in SOD1 and catalase, between laparotomy and OLT with saline (Figure 8B–8D). There were no significant differences in SOD1, SOD2 and catalase between OLT with saline and OLT with GABAR agonist (Figure 8B–8D). There were also no significant differences in SOD1, SOD2 and catalase between OLT with GABAR antagonist and OLT with saline (Figure 8).

Differences between OLT with saline, OLT with GABAR agonist and GABAR antagonist

Comparisons between multiple groups in each factor were performed, and the results were summarized in Table 1.

At 6 h after OLT, ANOVA revealed that GABAR agonist showed statistical differences in graft damage score, AST level, TUNEL-positive rate, caspase 3-positive rate and γ H2AX, and post-hoc tests demonstrated that GABAR agonist showed statistical differences in AST level, TUNEL-positive rate, caspase 3-positive rate and γ H2AX.

DISCUSSION

GABA was initially thought to be confined to the central nervous system, but currently, GABA is considered to be a multifunctional molecule with various physiological effects throughout the body [9,10]. Although it is known that the liver contains GABA and GABAR [10,12], the functions of GABA and/or GABAR in the liver remain unknown. In the current

study, histopathological, biochemical and immunohistological results 24 h after OLT clearly showed that rat OLT accompanied by ischemia/reperfusion injury resulted in graft damage and apoptosis, and that GABAR activation by a specific agonist reduced this damage and apoptosis *in vivo*.

Oxidative stress, which causes DNA damage and subsequent apoptosis, is caused by an imbalance between free radical production and antioxidant defense [1-3]. With regard to production of free radicals, ROS/RNS can attack and damage a variety of critical biological molecules, including lipids, essential cellular proteins and DNA [1-3]. Products of lipid peroxidation can be easily detected in biological fluids and tissues, and can reliably and rapidly reflect sensitive and specific signals of lipid peroxidation that occur *in vivo* [23,24]. The aldehyde 4-HNE is an end product of lipoperoxidation with antiproliferative and proapoptotic properties [23,24]. In our study, measurement of 4-HNE levels showed that oxidative stress mediated by ischemia/reperfusion injury occurred after OLT, and activation of GABAR by a specific agonist reduced this oxidative stress *in vivo*.

As described above, ROS/RNS has been suggested as a major contributing factor of DNA damage in the progression of oxidative stress. The protein kinase ATM, which is a sensor of DNA damage responses, can be initiated through rapid intermolecular autophosphorylation induced by DNA damage [25,26], phosphorylate various proteins, and subsequently amplify the responses to DNA damage [26]. This DNA damage-inducible kinase activates histone H2AX [5]. H2AX is required for cell cycle arrest and DNA repair following double-stranded DNA breaks [5,27]. DNA damage results in the rapid phosphorylation of H2AX by ATM and ATR (ATM and Rad3-related kinase) [5,28,29]. Within minutes following DNA damage, H2AX is phosphorylated at sites of DNA damage [5,30]. This very early event in DNA-damage response is required for recruitment of a multitude of DNA-damage response proteins. Therefore, histone H2AX is activated by ATM after DNA damage [5]. In previous reports on the brain, many studies have suggested that GABAR regulation by specific agonists or antagonists can affect the response to and repair of DNA damage induced by oxidative stress via the ATM/H2AX pathway *in vivo* and *in vitro* [5,26,31]; however, H2AX may be related to an unknown pathway [5]. The current study results of ATM and H2AX clearly demonstrated that oxidative stress mediated by ischemia/reperfusion injury after OLT caused DNA-damage signaling and triggered a subsequent DNA-repair process, and that GABAR activation by a specific agonist appeared to reduce ischemia/reperfusion injury-induced oxidative stress after OLT via the ATM/H2AX-dependent pathway *in vivo*.

Akt plays an important role in controlling apoptosis [25,32,33], and promotes cell survival [33-36]. Activation of Akt inhibits the apoptotic machinery [32,37,38]. Akt is positioned a component of the anti-apoptotic process related to the activation of PI3K [39], and PI3K is upstream from Akt [33,40]. With regard to the effects of GABAR regulation against oxidative stress in the brain, it has been shown that PI3K/Akt is the cell survival pathway involved in DNA damage *in vivo* and *in vitro* [5,39,41,42]; however, Akt and PI3K may have an unknown pathway [32]. PI3K-mediated signals are associated with decreased liver content of phosphatase and tensin homologue [43,44], and the PI3K/Akt pathway is also considered as an important signaling for cell survival in the liver [44,45]. The present study results suggested that the PI3K/Akt signaling pathway, which is involved in the promotion of cell survival, was disrupted with oxidative stress mediated by ischemia/reperfusion injury after OLT, and that the preventive effect against the oxidative stress by a GABAR agonist did not depend on this pathway.

With regard to antioxidant defense, free radical scavenging enzymes, such as SOD and catalase, also play an important role in reducing DNA damage and subsequent apoptosis [2,3,46]. Normal cells are able to defend against oxidative stress through this scavenging

system [3,46]. Therefore, antioxidant enzymes reduce oxidative stress-induced damage. In the brain, these effects have also been reported in the regulation of GABAR [47,48]. Oxidative stress impairs mitochondrial import and processing of SOD2 (49), and prevention of DNA damage via SOD2 has been reported in the small-for-size liver graft [50]. Although our own results of a difference in SOD2 between laparotomy and OLT with saline appears to be consistent with a previous report [50], the scavenging system did not appear to be triggered by GABAR regulation *in vivo*. One possible explanation for these responses after OLT may be that this scavenging system failed some reactive molecules evaded the detoxification process and damaged potential targets because of the extensive damage after OLT, despite the fact these scavenging enzymes can cope with large amounts of ROS/RNS [51].

In the brain, the effect of GABAR regulation on the prevention of oxidative stress is still controversial. Some studies have reported that GABAR activation by a specific agonist was successful, and others have demonstrated that GABAR antagonists have a positive effect against oxidative stress [5-7]. Previous studies have focused on ATR/ATM/H2AX, ATM/H2AX, H2AX, PI3K, PI3K/Akt, Akt and antioxidant enzymes as a possible pathway and reasonable explanation in the brain [5,39,41]. We believe that these studies provide evidence that regulation of GABAR by either agonists or antagonists affects oxidative stress via these pathways in the brain [5,6,8,19]. In conclusion, a specific agonist of GABAR demonstrated regulation of GABAR *in vivo* in the liver, and this regulation appeared to reduce ischemia/reperfusion injury-mediated oxidative stress after OLT via an ATM/H2AX-dependent pathway *in vivo*.

In our preliminary study, a high-dose injection of GABAR agonist or antagonist temporarily mediated neurological convulsion during 15–20 seconds, and then we recognized that GABAR agonist or antagonist have a toxicity. Based on our preliminary study, we here employed harmless doses of GABAR agonist and antagonist. In OLT with living donor, a pretreatment of living donor is very limited, and there is an ethical dilemma. Therefore, we challenged to shift the GABAR regulation to the back-table procedure (i.e., a pretreatment *ex vivo*), in order to focus the possibility of the application to OLT. Because this pretreatment *ex vivo* is suitable for not only live donor but also deceased donor, we initially expected the beneficial effects. Although we challenged many pretreatments *ex vivo*, we completely failed to obtain even a subtle benefit in the OLT recipient rats [52]. Although possible explanations are that GABAR regulation in the liver required the optimal environment [2,4,5] and that the reduction of graft damages by GABAR regulation *in vivo* reflected the secondary effects mediated by extrahepatic or systemic effects of GABAR regulation [9,10,12], we can't explain our failure of GABAR regulation *ex vivo*, even by the literature review. We now investigate the mechanism of the effects of GABAR regulation and GABA itself on the hepatocytes by the experiments *in vitro*. In current status, we speculated that pretreatment by GABAR regulation may be suitable for OLT with not living donor but deceased donor. On the other hand, we speculated that the GABAR regulation to OLT recipient may have a therapeutic potential, because we currently demonstrated that the GABAR regulation has a beneficial effect on the murine hepatectomy model [53].

CONCLUSIONS

Proactive strategies through pharmacological pretreatment to limit graft damage from ischemia/reperfusion injury has a large advantage for excellent graft function after OLT. Regulation of GABAR *in vivo* by specific agonist well worked in the liver, though the protective effect is moderate. We suggested that this regulation may have a strategic potential in the liver transplantation field, to reduce ischemia/reperfusion injury-mediated oxidative stress.

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Abbreviations

ROS	reactive oxygen species
RNS	reactive nitrogen species
DNA	deoxyribonucleic acid
GABA	γ -aminobutyric acid
GABAR	gamma-aminobutyric acid receptor
OLT	orthotopic liver transplantation
AST	aspartate aminotransferase
ALT	alanine aminotransferase
T-Bil	total bilirubin
PT-INR	international normalized ratio of prothrombin time
TUNEL	transferase-mediated deoxyuridine triphosphate nick-end labeling; caspase, cysteine aspartic acid protease
4-HNE	4-hydroxynonenal
ATM	ataxia-telangiectasia mutated kinase
H-E	hematoxylin-eosin
PI3K	phosphatidylinositol-3 kinase
SOD	superoxide dismutase
SOD1	superoxide dismutase 1
SOD2	superoxide dismutase 2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
ATR	ATM and Rad3-related kinase.

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Figure 1.

Survival curves after OLT with whole-liver syngeneic grafts. GABAR – γ -aminobutyric acid receptor; OLT – orthotopic liver transplantation.

Figure 2.

Histopathological findings of H-E staining and graft damage scores. (A) Laparotomy with saline, histopathological finding at 6 h after OLT (H-E, $\times 100$). (B) OLT with saline, histopathological finding at 6 h after OLT (H-E, $\times 100$). (C) OLT with GABAR agonist, histopathological finding at 6 h after OLT (H-E, $\times 100$). (D) OLT with GABAR antagonist, histopathological finding at 6 h after OLT (H-E, $\times 100$). (E) Changes in graft damage scores. There were significant differences in graft damage scores between laparotomy and OLT with saline (0.1 ± 0.1 vs. 4.0 ± 0.6 points, $p < 0.0001$ at 6 h; 0.1 ± 0.1 vs. 5.4 ± 0.2 points, $p < 0.0001$ at 12 h; 0.1 ± 0.1 vs. 7.8 ± 0.4 points, $p < 0.0001$ at 24 h) and between OLT with saline and OLT with GABAR agonist (4.0 ± 0.6 vs. 3.1 ± 0.2 points, $p = 0.0179$ at 6 h; 5.4 ± 0.2 vs. 4.8 ± 0.4 points, $p = 0.0127$ at 12 h; 7.8 ± 0.4 vs. 6.3 ± 0.4 points, $p = 0.0004$ at 24 h). There were no significant differences in graft damage scores between OLT with saline and OLT with GABAR antagonist (4.0 ± 0.6 vs. 4.1 ± 0.7 points, $p = 0.7718$ at 6 h; 5.4 ± 0.2 vs. 5.2 ± 0.6 points, $p = 0.5586$ at 12 h; 7.8 ± 0.4 vs. 7.5 ± 0.1 points, $p = 0.2699$ at 24 h). GABAR – γ -aminobutyric acid receptor; H-E – hematoxylin-eosin; NS – not significant ($p > 0.05$); OLT – orthotopic liver transplantation; * $p < 0.05$.

Figure 3.

Biochemical and coagulation profiles. (A) Changes in AST levels. There were significant differences in AST levels between laparotomy and OLT with saline (42.5 ± 8.6 vs. 290.5 ± 31.9 U/L, $p < 0.0001$ at 6 h; 43.9 ± 12.0 vs. 264.1 ± 23.1 U/L, $p < 0.0001$ at 12 h; 46.6 ± 12.1 vs. 172.6 ± 26.3 U/L, $p < 0.0001$ at 24 h) and between OLT with saline and OLT with GABAR agonist (290.5 ± 31.9 vs. 189.6 ± 24.3 U/L, $p = 0.0005$ at 6 h; 264.1 ± 23.1 vs. 191.4 ± 37.3 U/L, $p = 0.0060$ at 12 h). There were no significant differences in AST levels between OLT with saline and OLT with GABAR antagonist (290.5 ± 31.9 vs. 288.6 ± 32.8 U/L, $p = 0.9299$ at 6 h; 264.1 ± 23.1 vs. 258.6 ± 45.1 points, $p = 0.8123$ at 12 h; 172.6 ± 26.3 vs. 175.2 ± 38.5 U/L, $p = 0.9038$ at 24 h). (B) Changes in ALT levels. There were significant differences in ALT levels between laparotomy and OLT with saline (59.8 ± 9.6 vs. 309.4 ± 38.3 U/L, $p < 0.0001$ at 6 h; 58.6 ± 12.0 vs. 254.7 ± 30.4 U/L, $p < 0.0001$ at 12 h; 56.9 ± 11.3 vs. 171.6 ± 29.5 U/L, $p < 0.0001$ at 24 h) and between OLT with saline and OLT with GABAR agonist (309.4 ± 38.3 vs. 257.8 ± 28.0 U/L, $p = 0.0413$ at 6 h; 254.7 ± 30.4 vs. 190.2 ± 52.8 U/L, $p = 0.0456$ at 12 h). There were no significant differences in ALT levels between OLT with saline and OLT with GABAR antagonist (309.4 ± 38.3 vs. 307.0 ± 44.9 U/L, $p = 0.9286$ at 6 h; 254.7 ± 30.4 vs. 255.3 ± 26.6 U/L, $p = 0.9743$ at 12 h; 171.6 ± 29.5 vs. 178.4 ± 34.5 U/L, $p = 0.7476$ at 24 h). (C) Changes in T-Bil levels. There was a significant difference in T-Bil levels between laparotomy and OLT with saline (0.30 ± 0.07 vs. 0.58 ± 0.15 mg/dL, $p = 0.0049$ at 6 h; 0.29 ± 0.12 vs. 0.93 ± 0.22 mg/dL, $p = 0.0050$ at 12 h; 0.28 ± 0.05 vs. 1.37 ± 0.32 mg/dL, $p < 0.0001$ at 24 h). There were no significant differences in T-Bil levels between OLT with saline and OLT with GABAR agonist (0.58 ± 0.15 vs. 0.59 ± 0.14 mg/dL, $p = 0.9329$ at 6 h; 0.93 ± 0.22 vs. 0.90 ± 0.22 mg/dL, $p = 0.7422$ at 12 h; 1.37 ± 0.32 vs. 1.18 ± 0.29 mg/dL, $p = 0.3371$ at 24 h) and between OLT with saline and OLT with GABAR antagonist (0.58 ± 0.15 vs. 0.58 ± 0.12 mg/dL, $p = 0.9287$ at 6 h; 0.93 ± 0.22 vs. 0.87 ± 0.29 mg/dL, $p = 0.8613$ at 12 h; 1.37 ± 0.32 vs. 1.37 ± 0.05 mg/dL, $p = 0.9920$ at 24 h). (D) Changes in PT-INR. There were significant differences in PT-INR between laparotomy and OLT with saline (0.99 ± 0.04 vs. 1.12 ± 0.04 , $p = 0.0008$ at 6 h; 0.99 ± 0.04 vs. 1.08 ± 0.04 , $p = 0.0053$ at 12 h; 0.98 ± 0.03 vs. 1.05 ± 0.06 , $p = 0.0442$ at 24 h) and between OLT with saline and OLT with GABAR agonist (1.05 ± 0.06 vs. 0.97 ± 0.05 ,

$p=0.0459$ at 24 h). There were no significant differences in PT-INR between OLT with saline and OLT with GABAR agonist (1.12 ± 0.04 vs. 1.12 ± 0.04 , $p=0.8864$ at 6 h; 1.08 ± 0.04 vs. 1.08 ± 0.05 , $p=0.9488$ at 12 h) and between OLT with saline and OLT with GABAR antagonist (1.12 ± 0.04 vs. 1.11 ± 0.05 , $p=0.8444$ at 6 h; 1.08 ± 0.04 vs. 1.09 ± 0.03 , $p=0.7717$ at 12 h; 1.05 ± 0.06 vs. 1.03 ± 0.14 , $p=0.7749$ at 24 h). AST, aspartate aminotransferase; ALT, alanine aminotransferase; GABAR – γ -aminobutyric acid receptor; NS – not significant ($p > 0.05$); OLT – orthotopic liver transplantation; PT-INR – international normalized ratio of prothrombin time; T-Bil – total bilirubin; * $p<0.05$.

Figure 4.

Immunohistological findings of TUNEL and caspase-3 staining. (A) Laparotomy with saline, immunohistological finding at 6 h after OLT (TUNEL, $\times 100$). (B) OLT with saline, immunohistological finding at 6 h after OLT (TUNEL, $\times 100$). (C) OLT with GABAR agonist, immunohistological finding at 6 h after OLT (TUNEL, $\times 100$). (D) OLT with GABAR antagonist, immunohistological finding at 6 h after OLT (TUNEL, $\times 100$). (E) Changes in the ratio of TUNEL-positive nuclei. There were significant differences in the ratio of TUNEL-positive nuclei between laparotomy and OLT with saline (0.01 ± 0.01 vs. 0.11 ± 0.01 , $p < 0.0001$ at 6 h; 0.00 ± 0.01 vs. 0.13 ± 0.04 , $p = 0.0002$ at 12 h; 0.00 ± 0.00 vs. 0.17 ± 0.04 , $p = 0.0002$ at 24 h) and between OLT with saline and OLT with GABAR agonist (0.11 ± 0.01 vs. 0.06 ± 0.02 , $p = 0.0023$ at 6 h; 0.13 ± 0.04 vs. 0.08 ± 0.04 , $p = 0.0045$ at 12 h; 0.17 ± 0.04 vs. 0.11 ± 0.04 , $p = 0.0269$ at 24 h). There were no significant differences in the ratio of TUNEL-positive nuclei between OLT with saline and OLT with GABAR antagonist (0.11 ± 0.01 vs. 0.10 ± 0.01 , $p = 0.3319$ at 6 h; 0.13 ± 0.04 vs. 0.12 ± 0.03 , $p = 0.0813$ at 12 h; 0.17 ± 0.04 vs. 0.18 ± 0.03 , $p = 0.5473$ at 24 h). (F) Laparotomy with saline, immunohistological finding at 6 h after OLT (caspase-3, $\times 100$). (G) OLT with saline, immunohistological finding at 6 h after OLT (caspase-3, $\times 100$). (H) OLT with GABAR agonist, immunohistological finding at 6 h after OLT (caspase-3, $\times 100$). (I) OLT with GABAR antagonist, immunohistological finding at 6 h after OLT (caspase-3, $\times 100$). (J) Changes in the ratio of caspase-3-positive nuclei. There were significant differences in the ratio of caspase-3-positive nuclei between laparotomy and OLT with saline (0.001 ± 0.001 vs. 0.063 ± 0.014 , $p < 0.0001$ at 6 h; 0.002 ± 0.003 vs. 0.078 ± 0.010 , $p < 0.0001$ at 12 h; 0.001 ± 0.001 vs. 0.115 ± 0.020 , $p < 0.0001$ at 24 h) and between OLT with saline and OLT with GABAR agonist (0.063 ± 0.014 vs. 0.0039 ± 0.012 , $p = 0.0215$ at 6 h; 0.078 ± 0.010 vs. 0.063 ± 0.010 , $p = 0.0434$ at 12 h; 0.115 ± 0.020 vs. 0.077 ± 0.021 , $p = 0.0217$ at 24 h). There were no significant differences in the ratio of caspase-3-positive nuclei between OLT with saline and OLT with GABAR antagonist (0.063 ± 0.014 vs. 0.059 ± 0.016 , $p = 0.6492$ at 6 h; 0.078 ± 0.010 vs. 0.074 ± 0.015 , $p = 0.6646$ at 12 h; 0.115 ± 0.020 vs. 0.108 ± 0.028 , $p = 0.6768$ at 24 h). Caspase – cysteine aspartic acid protease; GABAR – γ -aminobutyric acid receptor; HV – hepatic vein; NS – not significant ($p > 0.05$); OLT – orthotopic liver transplantation; PV – portal vein; TUNEL – terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; * $p < 0.05$.

Figure 5.

Western blot analysis of 4-HNE. **(A)** Changes in the intensity of 4-HNE and GAPDH. **(B)** Changes in quantitative 4-HNE/GAPDH. There were significant differences in quantitative 4-HNE/GAPDH between laparotomy and OLT with saline (1.00 ± 0.09 vs. 1.41 ± 0.20 , $p=0.0032$) and between OLT with saline and OLT with GABAR agonist (1.41 ± 0.20 vs. 1.12 ± 0.10 , $p=0.0221$). There were no significant differences in quantitative 4-HNE/GAPDH between OLT with saline and OLT with GABAR antagonist (1.41 ± 0.20 vs. 1.22 ± 0.50 , $p=0.4516$). 4-HNE – 4-hydroxynonenal; GABAR – γ -aminobutyric acid receptor; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; NS – not significant ($p \geq 0.05$); OLT – orthotopic liver transplantation; * $p < 0.05$.

Figure 6.

Western blot analyses of ATM and H2AX. (A) Changes in the intensity of ATM, H2AX and GAPDH. (B) Changes in quantitative ATM/GAPDH. There were significant differences in quantitative ATM/GAPDH between laparotomy and OLT with saline (1.00 ± 0.10 vs. 1.31 ± 0.10 , $p=0.0011$) and between OLT with saline and OLT with GABAR agonist (1.31 ± 0.10 vs. 1.09 ± 0.18 , $p=0.0461$). There were no significant differences in quantitative ATM/GAPDH between OLT with saline and OLT with GABAR antagonist (1.31 ± 0.10 vs. 1.28 ± 0.17 , $p=0.7902$). (C) Changes in quantitative H2AX/GAPDH. There were significant differences in quantitative H2AX/GAPDH between laparotomy and OLT with saline (1.00 ± 0.17 vs. 1.67 ± 0.38 , $p=0.0071$) and between OLT with saline and OLT with GABAR agonist (1.67 ± 0.38 vs. 1.06 ± 0.26 , $p=0.0184$). There were no significant differences in quantitative H2AX/GAPDH between OLT with saline and OLT with GABAR antagonist (1.67 ± 0.38 vs. 1.82 ± 0.42 , $p=0.5732$). ATM – ataxia-telangiectasia mutated kinase; GABAR – γ -aminobutyric acid receptor; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; NS – not significant ($p \geq 0.05$); OLT – orthotopic liver transplantation; * $p < 0.05$.

Figure 7.

Western blot analyses of PI3K and Akt. (A) Changes in the intensity of PI3K, Akt and GAPDH. (B) Changes in quantitative PI3K/GAPDH. There was a significant difference in quantitative PI3K/GAPDH between laparotomy and OLT with saline (1.00 ± 0.08 vs. 0.86 ± 0.06 , $p=0.0166$). There were no significant differences in quantitative PI3K/GAPDH between OLT with saline and OLT with GABAR agonist (0.86 ± 0.06 vs. 0.94 ± 0.18 , $p=0.7658$) and between OLT with saline and OLT with GABAR antagonist (0.86 ± 0.06 vs. 0.90 ± 0.30 , $p=0.3360$). (C) Changes in quantitative Akt/GAPDH. There was a significant difference in quantitative Akt/GAPDH between laparotomy and OLT with saline (1.00 ± 0.08 vs. 0.86 ± 0.06 , $p=0.0166$). There were no significant differences in quantitative Akt/GAPDH between OLT with saline and OLT with GABAR agonist (0.86 ± 0.06 vs. 0.94 ± 0.18 , $p=0.7658$) and between OLT with saline and OLT with GABAR antagonist (0.86 ± 0.06 vs. 0.90 ± 0.30 , $p=0.3360$). GABAR – γ -aminobutyric acid receptor; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; NS – not significant ($p > 0.05$); OLT – orthotopic liver transplantation; PI3K – phosphatidylinositol-3 kinase; * $p < 0.05$.

Figure 8.

Western blot analyses of SOD1, SOD2 and catalase. **(A)** Changes in the intensity of SOD1, SOD2, catalase and GAPDH. **(B)** Changes in quantitative SOD1/GAPDH. There were no significant differences in quantitative SOD1/GAPDH between laparotomy and OLT with saline (1.00 ± 0.24 vs. 0.81 ± 0.19 , $p=0.2081$), between OLT with saline and OLT with GABAR agonist (0.81 ± 0.19 vs. 1.00 ± 0.17 , $p=0.1350$) and between OLT with saline and OLT with GABAR antagonist (0.81 ± 0.19 vs. 0.79 ± 0.28 , $p=0.9200$). **(C)** Changes in quantitative SOD2/GAPDH. There was a significant difference in quantitative SOD2/GAPDH between laparotomy and OLT with saline (1.00 ± 0.15 vs. 0.64 ± 0.19 , $p=0.0096$). There were no significant differences in quantitative SOD2/GAPDH between OLT with saline and OLT with GABAR agonist (0.64 ± 0.19 vs. 0.73 ± 0.20 , $p=0.4863$) and between OLT with saline and OLT with GABAR antagonist (0.64 ± 0.19 vs. 0.51 ± 0.40 , $p=0.5305$). **(D)** Changes in quantitative catalase/GAPDH. There were no significant differences between laparotomy and OLT with saline (1.00 ± 0.17 vs. 0.90 ± 0.15 , $p=0.3365$), between OLT with saline and OLT with GABAR agonist (0.90 ± 0.15 vs. 0.92 ± 0.17 , $p=0.8488$) and between OLT with saline and OLT with GABAR antagonist (0.90 ± 0.15 vs. 0.86 ± 0.20 , $p=0.7710$). GABAR – γ -aminobutyric acid receptor; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; NS – not significant ($p > 0.05$); OLT – orthotopic liver transplantation; SOD1 – superoxide dismutase 1; SOD2 – superoxide dismutase 2; * $p < 0.05$.

Table 1

Multivariate analyses between multiple groups.

	ANOVA p value	Post-hoc test	
		GABAR agonis statistical differences	GABAR antagonist statistical differences
At 6 h after OLT			
Graft damage score (point)	0.0291 [*]	NS	NS
AST (U/L)	0.0002 [*]	<i>p</i> <0.05	NS
ALT (U/L)	0.0895	NS	NS
T-Bil (mg/dL)	0.9833	NS	NS
PT-INR	0.9777	NS	NS
TUNEL-positive rate	0.0012 [*]	<i>p</i> <0.05	NS
Caspase 3-positive rate	0.0488 [*]	<i>p</i> <0.05	NS
4-HNE/GAPDH	0.3745	NS	NS
ATM/GAPDH	0.0909	NS	NS
H2AX/GAPDH	0.0143 [*]	<i>p</i> <0.05	NS
PI3K/GAPDH	0.8032	NS	NS
Akt/GAPDH	0.6161	NS	NS
SOD1/GAPDH	0.2998	NS	NS
SOD2/GAPDH	0.4833	NS	NS
Catalase/GAPDH	0.8873	NS	NS
At 12 h after OLT			
Graft damage score (point)	0.8873	NS	NS
AST (U/L)	0.0141 [*]	<i>p</i> <0.05	NS
ALT (U/L)	0.0303 [*]	NS	NS
T-Bil (mg/dL)	0.9403	NS	NS
PT-INR	0.9398	NS	NS
TUNEL-positive rate	0.1496	NS	NS
Caspase 3-positive rate	0.1462	NS	NS
At 24 h after OLT			
Graft damage score (point)	<0.0001 [*]	<i>p</i> <0.05	NS
AST (U/L)	0.2647	NS	NS
ALT (U/L)	0.6236	NS	NS
T-Bil (mg/dL)	0.5158	NS	NS
PT-INR	0.4196	NS	NS
TUNEL-positive rate	0.0127 [*]	<i>p</i> <0.05	NS
Caspase 3-positive rate	0.0591	<i>p</i> <0.05	NS

^{*} $p<0.05$; NS – not significant ($p \geq 0.05$).