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Selective blockade of mGlu5 metabotropic glutamate receptors is protective against acetaminophen hepatotoxicity in mice

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***Background/Aims*: mGlu5 metabotropic glutamate receptor antagonists protect rat hepatocytes against hypoxic death. Here, we have examined whether mGlu5 receptor antagonists are protective against liver damage induced by oxidative stress.**

***Methods*: Toxicity of isolated hepatocytes was induced by *tert*-butylhydroperoxide (t-BuOOH) after pretreatment with the mGlu5 receptor antagonists, MPEP, SIB-1757 and SIB-1893. The effect of these drugs was also examined in mice challenged with toxic doses of acetaminophen.**

***Results*: Addition of tBuOOH (0.5 mM) to isolated hepatocytes induced cell death (70 ± 5% at 3 h). Addition of MPEP or SIB-1893 to hepatocytes reduced both the production of reactive oxygen species (ROS) and cell toxicity induced by t-BuOOH (tBuOOH = 70 ± 5%; tBuOOH + MPEP = 57 ± 6%; tBuOOH + SIB-1893 = 40 ± 4%). In mice, a single injection of acetaminophen (300 mg/kg, i.p.) induced centrilobular liver necrosis, which was detectable after 24 h. MPEP (20 mg/kg, i.p.) substantially reduced liver necrosis and the production of ROS, although it did not affect the conversion of acetaminophen into the toxic metabolite, *N*-acetylbenzoquinoneimine. MPEP, SIB-1893 and SIB-1757 (all at 20 mg/kg, i.p.) also reduced the increased expression and activity of liver iNOS induced by acetaminophen.**

***Conclusions*: We conclude that pharmacological blockade of mGlu5 receptors might represent a novel target for the treatment of drug-induced liver damage.**

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***Keywords*: Liver; Hepatotoxicity; Cell damage; Oxidative stress; Acetaminophen; mGlu5 receptor antagonist**

1. Introduction

Glutamate (Glu) receptors are present not only in the central nervous system (CNS) but also in peripheral organs, and even in cells that do not originate from the neural crest [2–5]. Glu receptors either form membrane cation channels ('ionotropic' receptors) or couple to G proteins ('metabotropic' or mGlu receptors). mGlu receptors are members of class-3 G-protein coupled receptors and form a family of eight subtypes subdivided into three groups on the basis of their sequence homology, pharmacological profile and transduction mechanisms [6]. Group-I receptors (subtypes mGlu1 and –5) are coupled to polyphosphoinositide hydrolysis, whereas group-II and -III receptors (subtypes mGlu2 and -3 and mGlu4, -6, -7 and -8, respectively) are negatively coupled to adenylyl cyclase [6]. The study of peripheral mGlu receptors moved from the observation that mGlu4 receptors are present in taste buds, and contribute to the process of taste discrimination [4]. We have recently shown that hepatocytes express functional mGlu5 receptors coupled to polyphosphoinositide hydrolysis [1,7]. The presence of micromolar concentrations of glutamate in the portal blood, as well as in the extracellular fluid of isolated

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hepatocytes [1] suggests that mGlu5 receptors are endogenously activated and may therefore be involved in several aspects of liver physiology and pathology. Because mGlu5 receptors contribute to excitotoxic neuronal damage [8], we have initially examined whether mGlu5 receptor agonists or antagonists influence cell damage in isolated hepatocytes subjected to hypoxia. While receptor agonists accelerate cell death, receptor blockade with MPEP (a selective, potent and non-competitive mGlu5 receptor antagonist) protects hepatocytes against hypoxic damage and facilitates their metabolic recovery after re-oxygenation [1]. One way to interpret these results is that endogenous activation of mGlu5 receptors is translated into a death signal in cells that are made vulnerable by hypoxia. We have now extended the study to in vitro and in vivo models of oxidative stress, in which a perturbation of intracellular Ca^{2+} homeostasis is recognized as a causative factor in liver cell damage. Here, we report that pharmacological blockade of mGlu5 receptors is highly protective against liver cell damage induced by either *tert*-butylhydroperoxide (t-BuOOH) in vitro [9]or acetaminophen in vivo [10–12].

2. Materials and methods

Our studies involving animal experimentation were approved by the Italian Ministry of Health and by the University Commission for Animal Care following the criteria of the Italian National Research Council.

2.1. Materials

2-Methyl-6-(phenylethynyl)-pyridine (MPEP), 6-methyl-2-(phenylazo)-pyridin-3-ol (SIB-1757) and (E)-2-methyl-6-styryl-pyridine (SIB-1893) were purchased from Tocris Cookson Ltd. (Bristol, UK). All other drugs and chemicals were purchased from Sigma (Milan, Italy).

2.2. Methods

2.2.1. Hepatocyte isolation, treatments and assessment of cell viability

Hepatocytes were isolated from male Wistar rats (200–250 g) by collagenase perfusion of the liver [13]. Cell viability, estimated at the beginning of the experiments, ranged between 85 and 90%. Cells were suspended in Krebs–Ringer–HEPES (KRH) buffer containing 115 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 12.5 mM HEPES at pH 7.4, and all incubations were performed at a final cell density of 10^6 cells/ml in rotating round-bottomed flasks at 37 °C under 5% CO_2/95% O_2 atmosphere. Hepatocytes were preincubated for 30 min with MPEP 30 μM before exposure to the oxidant *tert*-butylhydroperoxide (t-BuOOH) (0.5 mM). Cell viability was assessed by measuring extracellular LDH activity [14].

2.2.2. Analysis of radical oxygen species and glutathione levels

Formation of radical oxygen species was monitored as follows. Hepatocytes were preloaded with the cell permeant probe dichlorodihydrofluorescein diacetate at concentrations of 5 μM for 15 min prior to the addition of t-BOOH. At the indicated times, aliquots containing 10^6 cells were withdrawn and centrifuged at $2000 \times g$ for 30 s. Cell pellets were resuspended in 0.5 ml distilled water and immediately frozen. Samples were quickly thawed and 200-μl samples were transferred to a 96-well plate for fluorescence measurements. Fluorescence values were measured on a CytoFluor 4000 (excitation 485 nm, emission 530 nm).

The concentration of total glutathione was measured by the enzymatic recycling method described by Tieze et al. [15], using glutathione reductase and 5,5′-dithio-bis(2-nitrobenzoic acid). Glutathione disulphide (GSSG) was determined after derivatization of reduced glutathione (GSH) with 2-vinylpyridine. GSH levels were calculated from the difference between total glutathione (GSH + GSSG) and GSSG. Protein thiol levels were determined as described by Di Monte et al. and Griffith et al. [16,17]. Proteins were assayed according to the method of Lowry et al. [18]. Lipid-peroxidation was monitored by measuring the formation of malonyldialdehyde (MDA) as described by Gray [19].

2.2.3. In vivo studies

Adult Swiss male mice (Charles River, 30–40 g), were injected intraperitoneally with acetaminophen (300 mg/kg), MPEP (20 mg/kg), SIB-1757 (20 mg/kg) or SIB-1893 (20 mg/kg) alone or in combination. All drugs were dissolved in saline containing 10% ethanol, which was injected in control mice (injection volume = 0.2 ml). In another set of experiments, acetaminophen was dissolved in heated saline brought to pH 8.5 with NaOH without ethanol. The solution containing acetaminophen and/or MPEP, SIB-1757 or SIB-1893 was injected at 37 °C to avoid the precipitation of acetaminophen. Some groups of mice were killed 24 h after a single injection. Additional groups of mice were treated with drugs daily for 6 days, and killed 25 days after the last administration. Animals were killed by decapitation and the liver was washed with saline perfusing the portal vein. The liver was then removed and frozen to −80 °C.

2.2.4. Measurement of N-acetylbenzoquinoneimine (NABQI) and GSH levels in liver

Animals treated with acetaminophen in the absence or presence of mGlu5 receptor antagonists were killed by decapitation 4 h after drugs injection. The liver was excised, rinsed in ice-cold 0.9% NaCl trimmed of adherent tissues, and weighed. An 11% liver homogenate (w/v) was prepared in 4.4% methaphosphoric acid (final concentration, 4%), using an all-glass homogenizer, and kept on ice. After standing for 20–40 min, the homogenate was centrifuged for 1 min ($10\,000 \times g$) and the acid-soluble fraction was collected for measurement of NABQI. Analysis of NABQI was performed by HPLC with electrochemical detection essentially as described by Richie and Lang [20] with slight modifications. GSH levels in liver homogenates were determined as described above.

2.2.5. Histochemical analysis

Serial unfixed cryostat sections were processed for the detection of glucose-6-phosphate dehydrogenase (G6PDH) activity and reactive oxygen species. For the detection of G6PDH activity, incubation media contained 10 mM glucose-6-phosphate (Sigma), 0.8 mM NADP (Sigma), 5 mM $MgCl_2$, 0.44 mM mPMS, 5 mM sodium azide and 5 mM tetranitro blue tetrazolium chloride (TNBT; Sigma) [21]. For the detection of reactive oxygen species, incubation media contained 12.5 mM 3,3′-diaminobenzidine (DAB; Sigma), 2.5 mM $MnCl_2$, and 40 mM $CoCl_2$ [22]. At the end of the incubation, sections were washed in hot distilled water (60 °C) to stop the reaction immediately and to remove the viscous incubation medium. Sections were then mounted in glycerol jelly and studied with a light microscopy. Conventional haematoxylin and eosin staining was used for the assessment of liver morphology.

2.2.6. Western blot analysis of inducible nitric oxide synthase (iNOS)

Livers from control or treated mice were removed, extensively washed in PBS (pH 7.4) and stored frozen at −80 °C. On the day of the experiment, livers were homogenized at 4 °C in ice-cold SDS-lysis buffer containing 1 mM PMSF, pH 7.4, with a motor-driven Teflon-glass homogenizer (1700 rev./min). Five microlitres were used for protein determinations. Seventy micrograms of proteins were resuspended in SDS–bromophenol blue redu-

cing buffer with 20 mM DTT. Western blot analyses were carried out using 8% SDS polyacrylamide gels run on a minigel apparatus (BioRad, Mini Protean II Cell); gels were electroblotted on ImmunBlot PVDF Membrane (BioRad, Italy) for 1 h using a semi-dry electroblotting system (BioRad Trans-blot system SD), and filters were blocked overnight in TTBS buffer (100 mM Tris–HCl, 0.9% NaCl, 0.1% Tween-20, pH 7.4) containing 2% non-fat dry milk. Blots were then incubated for 1 h at room temperature with primary polyclonal antibodies (1 μg/ml) which recognize a specific carboxy-terminal epitope of iNOS (Transduction Laboratories, Lexington, KY). Blots were washed three times with TTBS buffer and then incubated for 1 h with secondary antibodies (peroxidase-coupled anti-mouse, Amersham) diluted 1:3000 with TTBS. Immunostaining was revealed by ECL (Amersham).

2.2.7. Measurement of iNOS activity in liver homogenates

Activity of iNOS was assessed by measuring the conversion of [L-^{14}C]arginine into [L-^{14}C]citrulline in liver homogenates incubated in the absence of calcium [23]. Frozen tissues were pulverized and placed in 5 volumes of ice-cold homogenization buffer (25 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA). An aliquot of total homogenates was used for protein determination. The reaction was carried out in triplicate; non specific activity was determined in the presence of 1 mM L-NAME. The reaction was started by adding 50 μl of protein homogenates to 50 μl of 50 mM Tris–HCl (pH 7.4), containing 0.3 μM tetrahydrobiopterin, 1 μM flavin adenine dinucleotide, 1 μM flavin adenine mononucleotide, 0.1 μM calmodulin, 1 μM NADPH, 0.5 μCi [^{14}C]arginine (specific activity at 359 mCi/mmol, Amersham), 1 mM EGTA with or without 2 mM L-NAME. The reaction was carried out at 27 °C for 1 h and terminated by adding 400 μl of ice-cold stop buffer (20 mM HEPES, 5 mM EDTA, pH 5.5). Separation of [L-^{14}C]arginine from [L-^{14}C]citrulline was accomplished by using affinity columns containing a resin that retains the charged species of [L-^{14}C]argi-C]arginine while allowing [L-^{14}C]citrulline to pass through. Thus the assay mixture was passed over Poly-Prep Chromatography columns (BioRad) loaded with 1 ml of equilibrated Dowex 50W-X8 Na^+ form 200 mesh molecular biology grade resin (Sigma Aldrich), and the eluate was collected. The column was washed with 4 ml of water during collection of the eluate. An aliquot of each eluate was added to 5 ml of scintillation fluid, and [L-^{14}C]citrulline activity was determined using a Beckman LS6500 liquid scintillation counter. Blank incubation contained 50 μl of protein-free homogenization buffer. NO production by iNOS was calculated as picomoles of [L-^{14}C]citrulline produced per milligram of protein per hour.

3. Results

3.1. mGlu5 receptor antagonists protect isolated hepatocytes against oxidative damage produced by t-BuOOH

Addition of t-BuOOH (0.5 mM) to isolated hepatocytes induced a time-dependent cell death, with about 70% of hepatocytes being damaged after 3.5 h of exposure. Cell damage was substantially delayed when hepatocytes were treated with 30 μM of either MPEP or SIB-1893 [24,25] (both applied 30 min prior to t-BuOOH) (Fig. 1A). These concentrations of MPEP and SIB-1893 are proven to be maximally protective against neuronal death in culture [26]. In another experiment, hepatocytes were exposed to t-BuOOH after pretreatment with different concentrations of MPEP. MPEP was protective at concentrations as low as 30 nM (Fig. 2A,B), which coincide with the IC_{50} value reported for the inhibition of recombinantly expressed mGlu5 receptors [24]. Neither MPEP nor SIB-1893 affected hepatocyte viability per se (Fig. 1). Both MPEP and SIB-1893 reduced the formation of radical oxygen species in hepatocytes treated with t-BuOOH, as shown by the use of the fluorescent probe, dichlorofluorescein diacetate (Table 1, only MPEP), by measurements of MDA (a final product of lipid peroxidation) (Fig. 3A), and by the detection of the free thiol groups of proteins (Fig. 3B). However, neither MPEP nor SIB-1893 prevented the reduction in intracellular GSH levels induced by t-BuOOH (Fig. 3C). MPEP induced per se a small reduction in intracellular GSH levels, which was

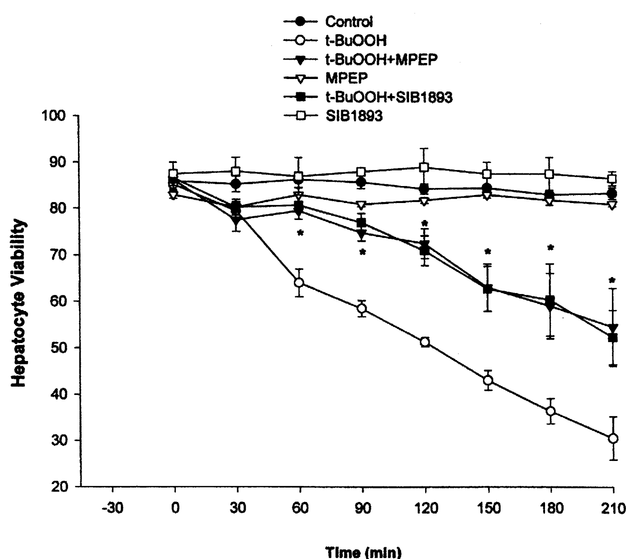

Fig. 1. Protective effect of MPEP and SIB-1893 against tBuOOH-induced cell damage in isolated hepatocytes. Closed circles, untreated hepatocytes; open circles, hepatocytes exposed to t-BuOOH (0.5 mM); closed triangles: hepatocytes pretreated for 30 min with MPEP (30 μM) and then exposed to t-BuOOH; open triangles, hepatocytes exposed to MPEP; closed squares, hepatocytes pretreated for 30 min with SIB-1893 (30 μM) and then exposed to t-BuOOH; open squares, hepatocytes exposed to SIB-1893. Results are mean ± SEM of 4–8 individual determinations. *$P < 0.05$ (One-way ANOVA + Fisher PLSD) vs. t-BuOOH alone.

Table 1
Reactive oxygen species formation in cultured hepatocytes treated with t-BOOH in the absence or presence of MPEP[a]

Time (min)	% of the initial fluorescence t-BOOH	t-BOOH + MPEP
0	92 ± 4.9	87 ± 2
30	122 ± 7.7	84 ± 4.1*
60	136 ± 7.8	84 ± 2.5*
90	141 ± 8.6	86 ± 5.7*
120	160 ± 14	92 ± 13*
150	172 ± 16	104 ± 17*

[a] Values (referred to as the fluorescence generated by dichlorofluorescein diacetate) were calculated from four or five individual determinations. *$P < 0.05$ (Student's *t*-test), vs. values obtained with t-BOOH alone. MPEP alone did not affect fluorescence in hepatocytes which were not treated with t-BOOH (not shown).

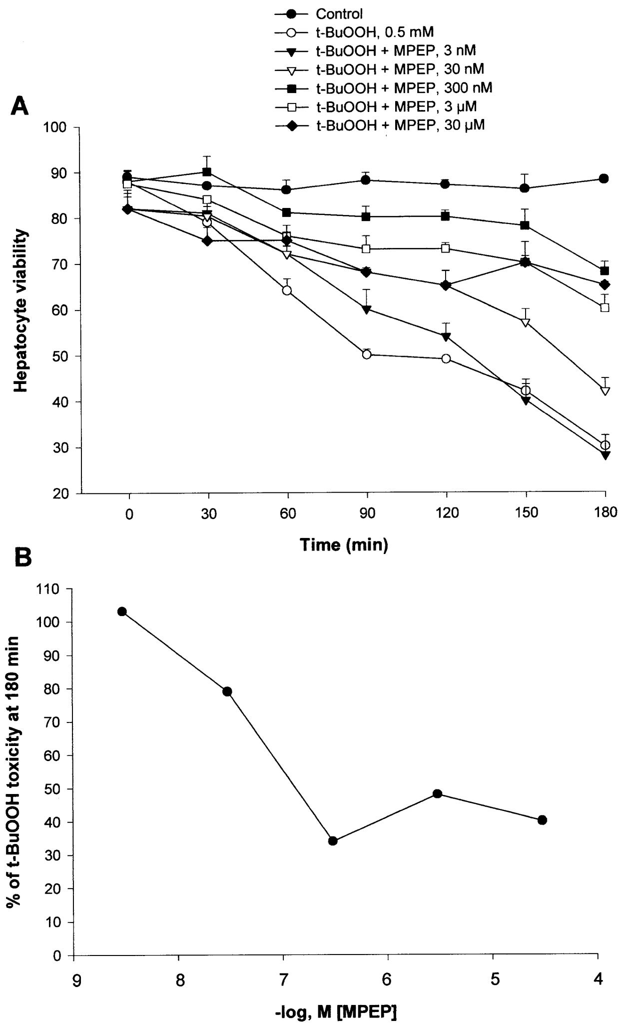

Fig. 2. Concentration dependent protection by MPEP against hepatocyte toxicity induced by t-BuOOH. In (A), different concentrations of MPEP were added to isolated hepatocytes 30 min prior to the addition of 0.5 mM t-BuOOH. Values are means ± SEM of 4 determinations. All points with t-BuOOH + MPEP in a concentration range between 30 nM and 3 μM were significantly different from the respective values with t-BuOOH alone at time points from 60 to 180 min. Values obtained with t-BuOOH + 3 nM MPEP were significantly different from t-BuOOH alone at 60 and 90 min (*P* < 0.05, one-way ANOVA + Fisher PLSD). The concentration-dependent protection by MPEP after 180 min of exposure to t-BuOOH is shown in (B). In this particular case, values are expressed as per cent of t-BuOOH toxicity and were calculated from the means shown in (A). Note that MPEP is maximally effective at 300 nM. At concentrations of 300 μM, MPEP completely lost its cytoprotective activity against t-BuOOH toxicity.

additive to the larger reduction induced by t-BuOOH (Fig. 3C). We also combined t-BuOOH with either glutamate or quisqualate (both at 100 μM), which are known to activate mGlu5 receptors. Measurements of cell viability showed that neither of these drugs potentiated the toxic effect of t-BuOOH (not shown). However, this was expected because isolated hepatocytes release micromolar amounts of glutamate that are sufficient to saturate mGlu5 receptors [1].

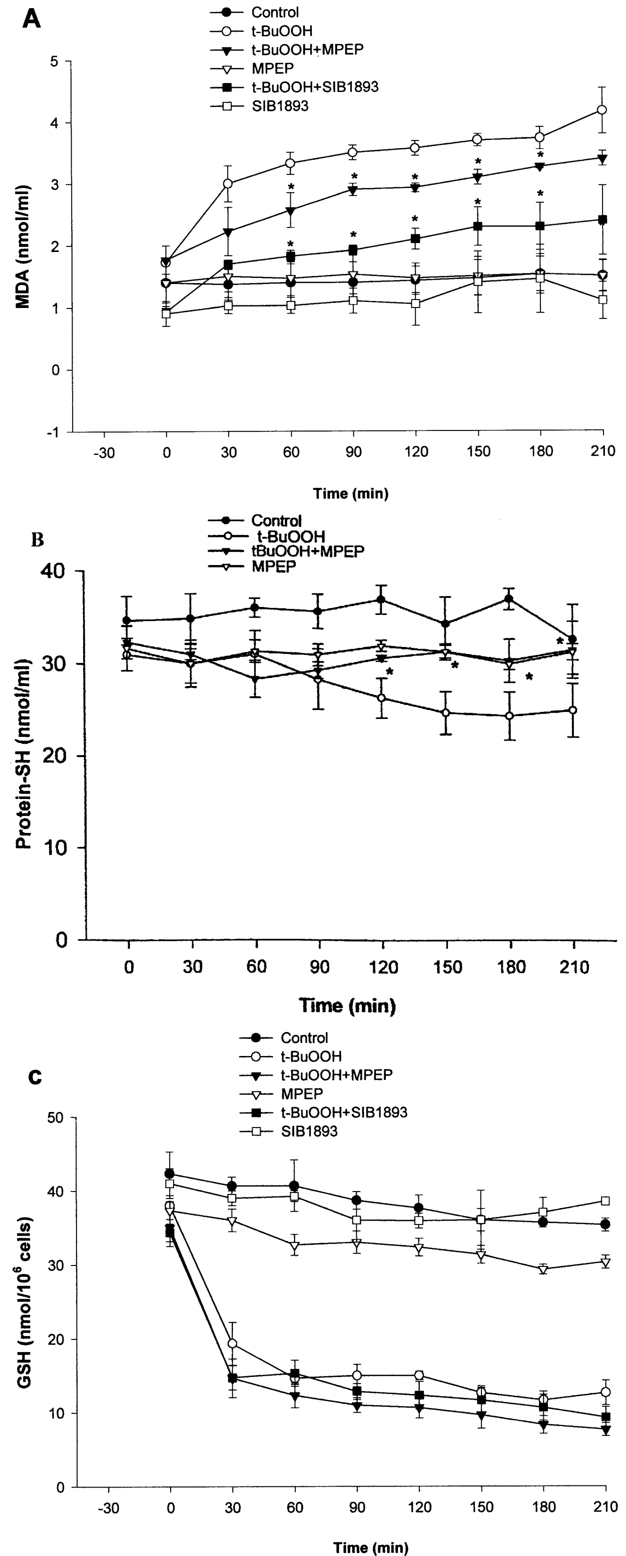

Fig. 3. Same as Fig. 1, but showing MDA formation (A), thiol groups of proteins (B) and GSH levels (C). Results are mean ± SEM of 4–6 individual determinations. **P* < 0.05 (one-way ANOVA + Fisher PLSD) vs. t-BuOOH alone.

3.2. mGlu5 receptor antagonists are protective against liver damage induced by acetaminophen in mice

In a first series of experiments, mice were treated intraperitoneally only once with acetaminophen (300 mg/kg) and examined 24 h later. Acetaminophen treatment induced centrilobular liver necrosis, as shown by Fig. 4A. There was no difference in acetaminophen-induced toxicity as well as in the effect of mGlu5 receptor antagonists (see below) between mice injected with a solution containing 10% ethanol and those injected with an ethanol-free solution at pH 8.5. Co-injection with MPEP (20 mg/kg, i.p.) protected against acetaminophen toxicity (Fig. 4B). MPEP treatments also reduced the formation of reactive oxygen species (Fig.5A–C) and the increase in G6PDH activity (Fig. 6A–C) induced by acetaminophen, although it did not prevent the reduction in liver GSH levels induced by acetaminophen (Table 2). Animals killed 24 h after a single injection of acetaminophen were also used for the detection of iNOS expression and activity in the liver homogenates. Western blot analysis with an anti-iNOS antibody showed the presence of a single band of 130 kDa, which corresponds to the deduced molecular size of iNOS [23]. A single injection with acetaminophen greatly enhanced iNOS expression in the liver. This increase was markedly reduced in mice co-injected with MPEP. MPEP alone had no effect on iNOS expression (Fig. 7A). Similar results were obtained by measuring iNOS activity as the amount of [^{14}C]citrulline formed from [^{14}C]arginine in liver homogenates incubated in the absence of calcium. Acetaminophen treatment increased iNOS activity by more than 3-fold. This increase was largely reduced when acetaminophen was co-injected with MPEP, SIB-1893, or SIB-1757 (all at 20 mg/kg, i.p.) (Fig. 7B). Additional groups of mice were treated intraperitoneally for 6 days with 300 mg/kg of acetaminophen given alone or in combination with MPEP (20 mg/kg), and killed

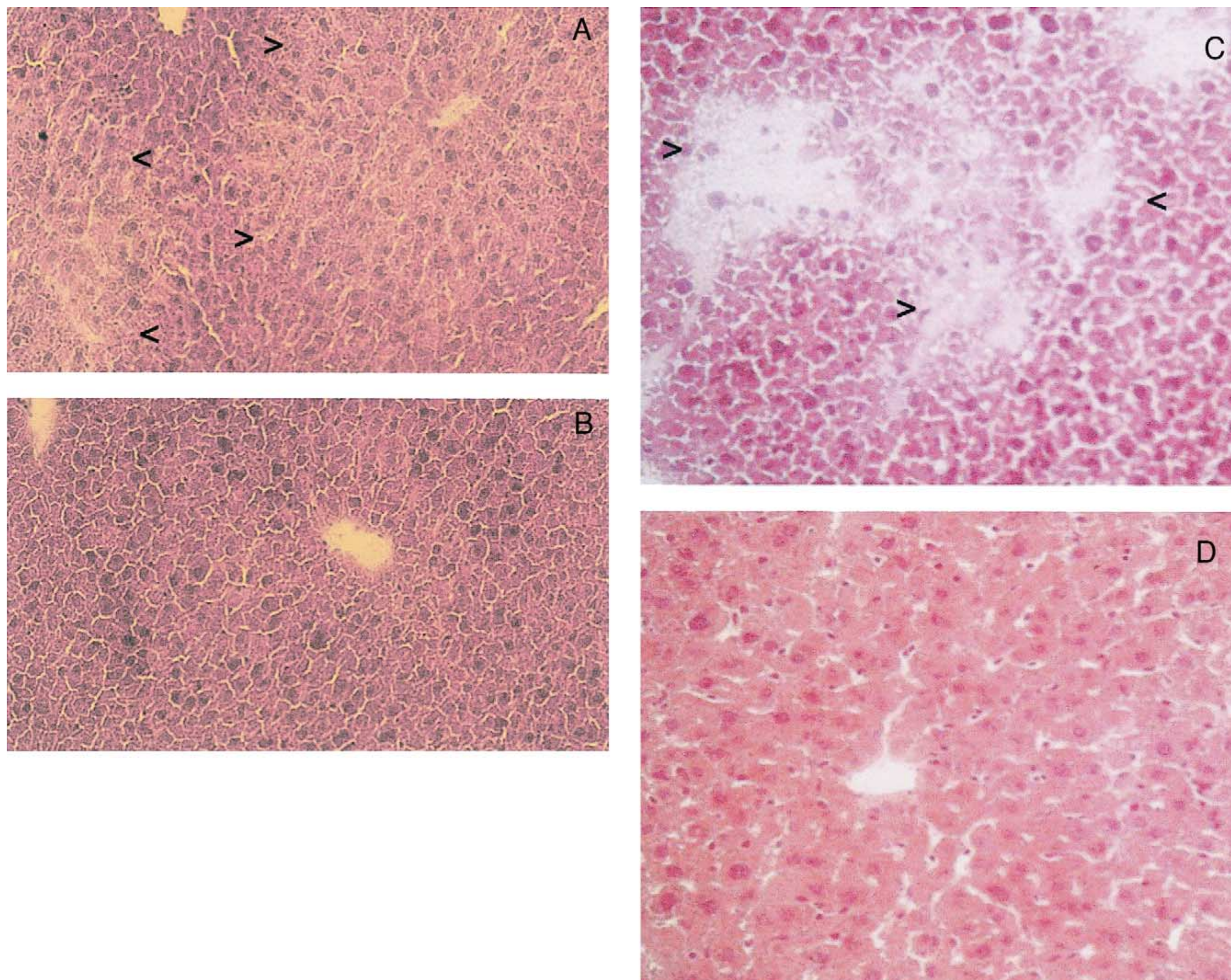

Fig. 4. Haematoxylin–eosin staining in liver sections of mice treated as follows: (A) 24 h after a single injection of acetaminophen (300 mg/kg, i.p.); (B) 24 h after a single injection of acetaminophen + MPEP (20 mg/kg, i.p.); (C) 25 days after a 6-day treatment with acetaminophen (300 mg/kg, i.p., once per day); (D) 25 days after a 6-day treatment with acetaminophen + MPEP (20 mg/kg, i.p., once per day). Arrowheads in (A) and (C) point to areas of centrilobular necrosis. Objective = 40 ×.

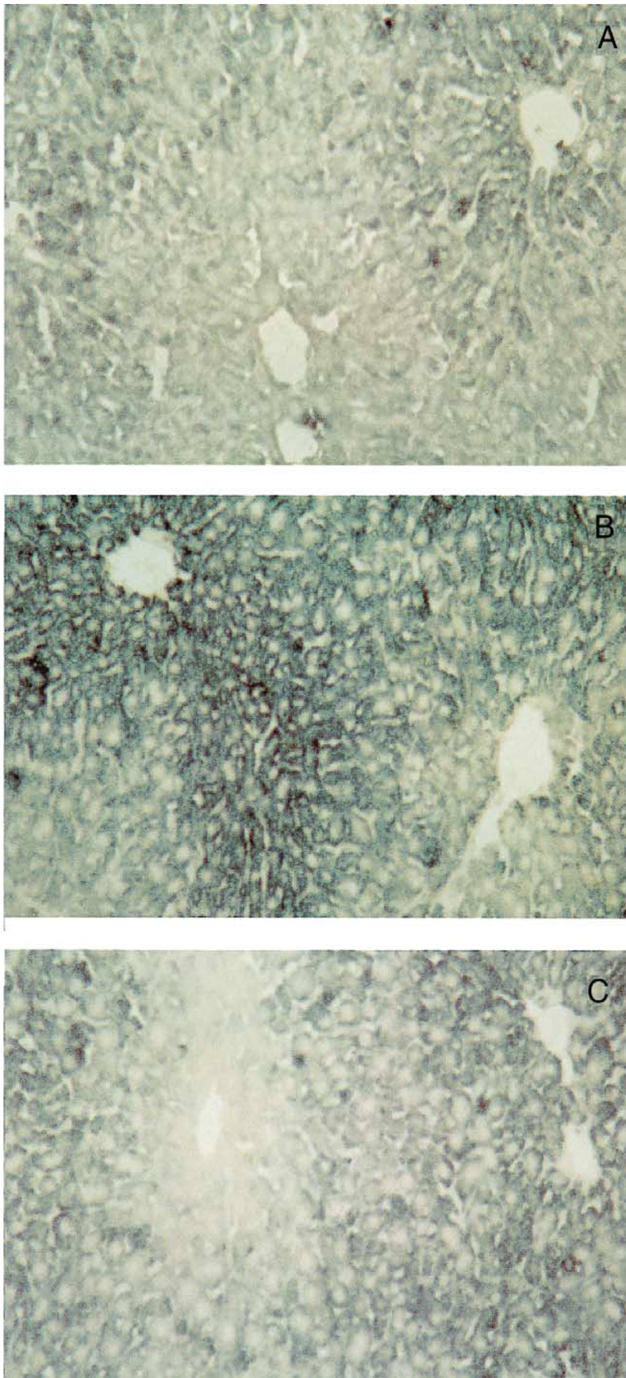

Fig. 5. Histochemical detection of reactive oxygen species in liver sections of mice that received a single injection of: (A) saline; (B) acetaminophen (300 mg/kg, i.p.); and (C) acetaminophen + MPEP (20 mg/kg, i.p.).

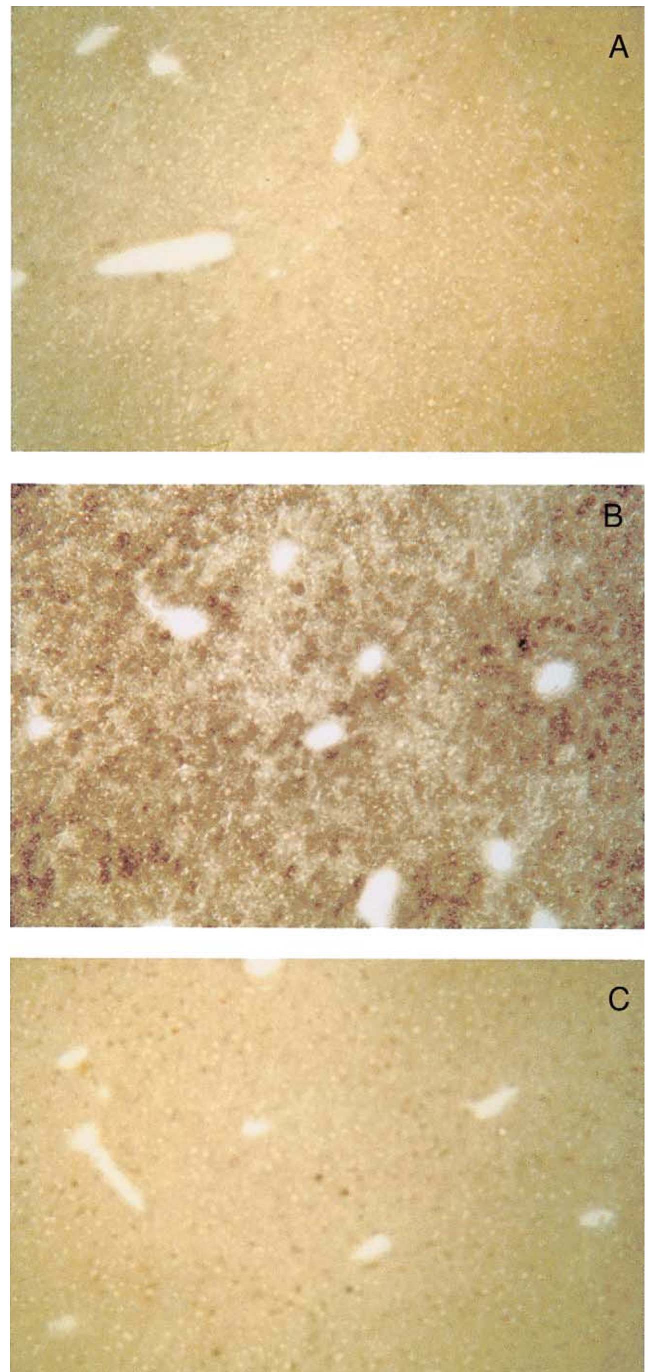

Fig. 6. Histochemical detection of glucose-6-phosphate dehydrogenase (G6PDH) activity in liver sections of mice that received a single injection of: (A) saline; (B) acetaminophen (300 mg/kg, i.p.); and (C) acetaminophen + MPEP (20 mg/kg, i.p.).

25 days after the end of the treatment. Mice treated with acetaminophen alone showed extensive liver necrosis (Fig. 4C) at the time of the sacrifice. Mice co-injected with MPEP were largely protected against acetaminophen toxicity (Fig. 4D). MPEP also prevented the increase in reactive oxygen species and G6PDH activity induced by acetaminophen treatment, as detected by histochemical analysis (not shown). We examine whether mGlu5 receptor antagonists could affect the hepatic conversion of acetaminophen into the reactive metabolite, NABQI. NABQI levels, detected by HPLC, were about 100 ng/g of liver 4 h after a single injection of acetaminophen in mice. These levels did not change in mice co-injected with either MPEP or SIB-1893 (Table 3).

Table 2
Liver GSH levels mice receiving a single injection of acetaminophen in the absence or presence of MPEP[a]

	GSH levels (nmol/mg protein)
Saline	36 ± 2.5
MPEP	32 ± 1.5
Acetaminophen	22 ± 0.8*
Acetaminophen + MPEP	21 ± 1.8*

[a] Values are means ± SEM of four individual determinations. *$P < 0.05$ (One-way ANOVA + Fisher PLSD) vs. the corresponding values obtained in the absence of acetaminophen.

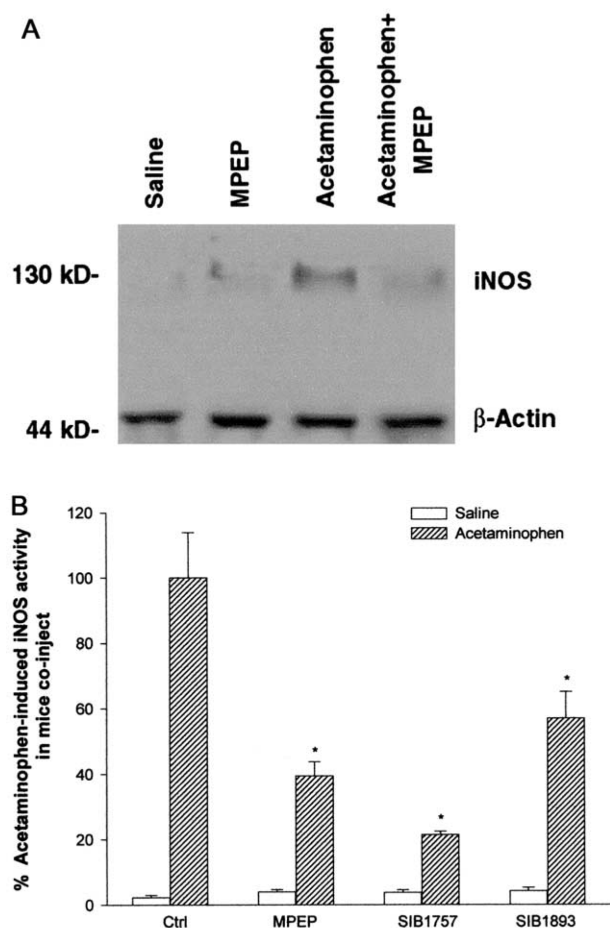

Fig. 7. (A) Western blot analysis of iNOS in protein extracts prepared from the livers of mice treated with a single injection of saline, acetaminophen (300 mg/kg, i.p.), MPEP (20 mg/kg, i.p.), or acetaminophen + MPEP. The band corresponding to iNOS is visible at 130 kDa. (B) iNOS activity in liver homogenates from mice treated with a single injection of saline, acetaminophen (300 mg/kg, i.p.), MPEP (20 mg/kg, i.p.), SIB-1893 (20 mg/kg, i.p.), SIB-1757 (20 mg/kg, i.p.), acetaminophen + MPEP, acetaminophen + SIB-1893, or acetaminophen + SIB-1757. Ctrl = controls, i.e. mice which did not receive any mGlu5 receptor antagonist. Values (means ± SEM) are expressed as % of values obtained in mice treated with acetaminophen alone, and were calculated from 4–8 individual determinations. *$P < 0.01$ (one-way ANOVA + Fisher PLSD) vs. acetaminophen alone. In mice injected with saline and acetaminophen alone, iNOS activity was 330 ± 25 and 1045 ± 96 pmol of [^{14}C]citrulline formed from [^{14}C]arginine/mg protein per hour, respectively.

Table 3
Detection of NABQI in the liver of mice receiving a single injection of acetaminophen in the absence or presence of MPEP or SIB-1893[a]

	NABQI (ng/g tissue)
Acetaminophen	91 ± 31
Acetaminophen + MPEP	102 ± 26
Acetaminophen + SIB-1893	128 ± 42

[a] Values are means ± SEM of four individual determinations.

4. Discussion

We have used the antagonists MPEP, SIB-1757 and SIB-1893 [24,25]to examine how endogenous activation of mGlu5 receptors contributes to oxidative liver damage. These drugs behave as non-competitive antagonists, interacting with a binding pocket lined by the transmembrane domains 3, 6 and 7 [27]. Thus, the effects of these drugs cannot be overwhelmed by the endogenous glutamate. In isolated hepatocytes, MPEP is protective against hypoxic damage and facilitates cell recovery after re-oxygenation [1]. Hence, we have hypothesized that endogenous activation of mGlu5 receptors becomes detrimental in cells that are made vulnerable by a specific insult. mGlu5 receptors are coupled to polyphosphoinositide hydrolysis [7,28,29,30], and their activation generates oscillatory increases in intracellular Ca^{2+} [31]. An increase in intracellular Ca^{2+} is involved in liver cell damage induced by oxidative stress[32]. For example, hepatic cell death induced by t-BuOOH follows a perturbation in intracellular Ca^{2+} homeostasis, which is a direct consequence of t-BuOOH metabolism by the glutathione peroxidase-glutathione reductase system [33,34]. Hence, we have hypothesized that activation of mGlu5 receptors by the endogenous glutamate could act synergistically with oxidizing agents in the induction of liver cell damage. This was supported by the protective effect of MPEP and SIB-1893 against t-BuOOH toxicity in isolated hepatocytes, although the time-dependent profile shown in Fig. 1A suggests that mGlu5 receptor blockade delays rather than preventing cell death. Interestingly, MPEP and SIB-1893 reduced ROS formation without increasing the intracellular levels of GSH. Addition of MPEP even reduced GSH levels, and this may help explain why the drug was less efficient than SIB-1893 in the MDA assay. Our data suggest that mGlu5 receptor antagonists do not interfere with the GSH-mediated t-BuOOH metabolism and that cell protection cannot be entirely ascribed to a reduced ROS formation. We therefore hypothesize that endogenous activation of mGlu5 receptors is permissive to the induction of cell death in hepatocytes challenged with t-BuOOH, although the possibility that MPEP and SIB-1893 act as intracellular free radical scavengers cannot be entirely ruled out. To examine whether a similar scenario occurs in vivo, we have treated mice with toxic doses of acetaminophen combined with mGlu5 receptor antagonists. The doses of MPEP that we have used were

shown to be active in models of neurological disorders [35,36]. Acetaminophen (300 mg/kg, i.p.) induced an extensive centrilobular liver necrosis that was substantial 24 h after a single injection. This effect is due to the bioactivation of acetaminophen by cytochrome P-450 leading to the formation of NABQI, a highly reactive metabolite that forms chemical adducts with proteins after intracellular GSH is depleted [10]. Induction of iNOS, with ensuing formation of $NO^{\cdot}$ and nitration of protein tyrosines also contributes to the development of acetaminophen toxicity [37]. In control mice, acetaminophen induced a substantial increase in ROS formation and in the expression and activity of iNOS. All these effects were reduced when acetaminophen was combined with mGlu5 receptor antagonists. The protective activity of MPEP in vivo was confirmed in a more drastic model of liver toxicity, in which acetaminophen was administered for 6 days. The lack of changes in tissue levels of NABQI in response to MPEP or SIB-1893 suggests that endogenous activation of liver mGlu5 does not affect acetaminophen metabolism but rather enables the engagement of the death pathway in response to acetaminophen. Whether or not mGlu5 receptor antagonists can be used for prophylaxis or treatment of liver toxicity induced cannot be predicted at this time because information on the safety profile of these drugs are still lacking. The possibility that a long-term treatment with MPEP or the SIBs generates some toxic phenylpyridine metabolites raises some major concerns for the use of these drugs, although mice treated with MPEP or the SIBs showed neither signs of liver toxicity nor remarkable changes in spontaneous behaviour. Nonetheless, the possibility that mGlu5 receptors are targeted by novel drugs endowed with protective activity against liver toxicity is certainly exciting and worth to be investigated.

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