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Synergistic toxicity induced by prolonged glutathione depletion and inhibition of nuclear factor-kappaB signaling in liver cells[‡]

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Abstract

TNF- α , GSH depletion and CYP2E1 are factors that play an important role in alcoholic liver disease. Activation of NF- κ B prevents hepatocyte damage caused by TNF- α . This work describes the effect of NF- κ B inhibition on toxicities caused by GSH depletion or arachidonic acid (AA) treatment in liver cells, and evaluates the possible influence of CYP2E1 overexpression. Cells were exposed to the NF- κ B inhibitor BAY11-7082, in the absence or presence of L-buthionine sulfoximine (BSO) to block GSH synthesis. BSO toxicity was higher in CYP2E1-expressing E47 HepG2 cells compared to control cells; the incubation with BAY11-7082 potentiated BSO toxicity in both cell lines comparably. Several other agents which suppress activation of NF- κ B increased BSO toxicity in E47 cells. NF- κ B inhibition, however, did not sensitize E47 cells to AA toxicity. Suppressing activity of NF- κ B also potentiated BSO, but not AA toxicity, in isolated rat hepatocytes. BAY11-7082 plus BSO induced a greater p38 MAPK activation as compared to BAY11-7082 or BSO alone, and a p38 MAPK inhibitor protected against the synergistic toxicity. In summary, inhibition of NF- κ B sensitizes liver cells to toxicity linked to GSH depletion, probably accelerating the processes of thiol homeostasis deregulation and induction of apoptosis through a mechanism mediated by p38 MAPK.

Introduction

A decrease in the intracellular antioxidant defense generates a state of oxidative stress that sensitizes cells to damage by toxins, e.g., ethanol (Arteel, 2003; Nordmann et al., 1992). GSH is the most important endogenous antioxidant, exerting a pivotal role to maintain redox homeostasis (Dickinson & Forman, 2002). Furthermore, selective depletion of mitochondrial GSH has been proposed as a critical factor contributing to alcohol liver injury (Fernandez-

[‡]**Abbreviations:** AA, arachidonic acid; BAY11-7082, (E)-3-(4-methylphenylsulfonyl)-2-propenenitrile; BSO, L-buthionine sulfoximine; C34 cells, HepG2 hepatoma cells transfected with control pCI plasmid and not expressing CYP2E1; CDNB, 1-chloro-2,4-dinitrobenzene; CYP2E1, cytochrome P450 2E1; DCF, 2',7'-dichlorofluorescein; DETA-NONate, (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate; E47 cells, transfected HepG2 cells overexpressing CYP2E1; EGCG, epigallocatechin-3-gallate; FBS, fetal bovine serum; I κ B- α , kappaB inhibitory protein- α ; MAPK, mitogen-activated protein kinase; MTT, thiazolyl blue tetrazolium bromide; NAC, N-acetyl cysteine; NF- κ B, nuclear factor-kappaB; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; ROS, reactive oxygen species; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; SP600125, anthra(1,9-cd)pyrazol-6(2H)-one; TNF- α , tumor necrosis factor-alpha; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; z-VAD-FMK, N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone.

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Checa et al., 1993). Loss of GSH leads to oxidative stress and apoptosis in a variety of cell types under distinct experimental conditions (Hall, 1999; Lu, 1999).

Intragastrically-administered ethanol induces the endotoxin-mediated activation of nuclear transcription factor kappaB (NF- κ B) in Kupffer cells, which accounts for an increased synthesis of the proinflammatory cytokine tumor necrosis factor- α (TNF- α). TNF- α is critically involved in the developing liver injury produced by ethanol in rat models (French, 2001; Uesugi et al., 2001). In hepatocytes, activation of NF- κ B by TNF- α stimulates anti-apoptotic signaling pathways that prevent TNF- α -induced hepatocyte damage; thus, TNF- α toxicity to the hepatocyte only is apparent when the NF- κ B signal transduction cascade is abolished (Uesugi et al., 2001). Acute GSH depletion in murine hepatocytes suppresses the activation of NF- κ B and hence the NF- κ B-dependent synthesis of critical protective/survival factors, e.g., inducible nitric oxide synthase; this results in sensitizing hepatocytes to TNF- α -induced apoptosis (Matsumaru et al., 2003). Indeed, menadione cytotoxicity is enhanced when human HepG2 hepatoma cells are depleted of GSH, most probably due to the prevention of the activation of NF- κ B produced by menadione itself (Chen & Cederbaum, 1997).

Cytochrome P450 2E1 (CYP2E1) activates many toxicologically important compounds to reactive intermediates (Gonzalez, 2005). The biochemical and toxicological properties of CYP2E1 have been characterized in HepG2 cells developed to express CYP2E1 (Caro & Cederbaum, 2004). L-buthionine sulfoximine (BSO) blocks GSH production by inhibiting the rate-limiting enzyme in the pathway of GSH synthesis, glutamate-cysteine ligase. Prototoxins such as BSO or arachidonic acid (AA) were more toxic in CYP2E1-expressing E47 HepG2 cells than control C34 HepG2 cells which do not express CYP2E1. One important mechanism for BSO and AA toxicities in CYP2E1-overexpressing liver cells likely involves an activation of the mitogen-activated protein kinase (MAPK) family member p38, which leads to a reduction in the DNA-binding activity of NF- κ B (Wu & Cederbaum, 2003; Wu & Cederbaum, 2004).

The current study examines how different chemicals which can suppress NF- κ B activity influence BSO or AA toxicity in liver cells, i.e., HepG2 cells and isolated rat hepatocytes, both in the absence or presence of CYP2E1 overexpression. Treatment with BSO or AA plus the selective NF- κ B inhibitor (E)-3-(4-methylphenylsulfonyl)-2-propenenitrile [BAY11-7082; (Hansson et al., 2005)] was used as a reference model. Suppression of NF- κ B activity strongly increased toxicity produced by long-term BSO exposure, however it did not potentiate AA toxicity in these cells. Toxicity related to inhibition of NF- κ B signaling does not appear to be affected by CYP2E1-dependent oxidant stress, as both CYP2E1-expressing cells (E47) and cells not expressing CYP2E1 (C34) showed similar sensitization to NF- κ B inhibition, although overall toxicity was higher in the E47 cells.

Materials and Methods

Chemicals

Geneticin was from Invitrogen (Carlsbad, CA). 2',7'-dichlorofluorescein diacetate was acquired from Molecular Probes (Eugene, OR). Other chemicals used were from Sigma (St. Louis, MO).

Cell culture

Experiments were carried out using as a model E47 cells, a human hepatoma HepG2 cell subline which constitutively expresses human CYP2E1 (Chen & Cederbaum, 1998), and their corresponding control cells (C34 subline). In order to extend the results to non-transformed cells, some studies were conducted using primary hepatocytes from pyrazole-or chronically ethanol-treated rats, treatments known to induce the expression of CYP2E1 (Wu & Cederbaum,

2000), and their corresponding control hepatocytes. CYP2E1 levels were validated by catalytic activity with *p*-nitrophenol. Transfected E47 and C34 cells were grown in minimal essential medium containing 10% fetal bovine serum (FBS) and 0.5 mg/ml geneticin, supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, in a humidified atmosphere with 5% CO₂ at 37°C, and were subcultured at a 1:10 ratio once a week. Rat hepatocytes were isolated by a two-step collagenase perfusion method as described previously (Wu & Cederbaum, 2000) and were cultured as above but without geneticin.

Cell treatment

Geneticin was omitted from the medium for the various assays. Cells were plated at a density of 2.5×10^4 cells/cm² and maintained in culture medium for 24 h before treatments. BSO and AA were dissolved in PBS (pH 7.4) or FBS, respectively. Most other reagents were prepared in DMSO, therefore the incubation medium (containing 10% FBS) was supplemented with DMSO during the distinct treatments to reach the same final concentration, typically 0.1% (i.e., 14 mM), which does not inhibit CYP2E1 catalytic activity.

Cells were preincubated with the NF-κB inhibitor BAY11-7082, or other agents, for 1 h. Afterwards, culture medium was supplemented with BSO or AA, whereas untreated cells were used as control; treatments were renewed every 24 h. Protein concentration was measured using the Bio-Rad DC Protein Assay Kit (Hercules, CA).

Cytotoxicity assays

Cells were seeded onto 24-well plates, and after the corresponding treatment the medium was removed and cell viability was evaluated by assaying for the ability of functional mitochondria to catalyze the reduction of thiazolyl blue tetrazolium bromide (MTT) to a formazan salt by mitochondrial dehydrogenases.

Lipid peroxidation and reactive oxygen species (ROS) analysis

After AA treatment, generation of malonaldehyde was determined in cell lysates by assaying for thiobarbituric acid reactive substances (Niehaus & Samuelsson, 1968). Production of ROS after BSO treatment was assayed by a 2',7'-dichlorofluorescein (DCF) fluorescence method.

Immunoblot analysis of p38 MAPK activation

E47 cells were first incubated with medium in the absence or presence of 5 µM SB203580 for 1 h. Medium with or without BAY11-7082 at a final concentration of 2.5 µM was then added to the cells and after 1 h incubation, medium was added with or without BSO (at a final concentration of 150 µM); the cells were incubated for 0.5, 1, 2, 4, 8, 12, 24, 36, or 48 h. Immunoblots were carried out to determine the content of p38 and p-p38 MAPK in the cellular lysates collected at the above times as previously described (Wu & Cederbaum, 2004). The p38 and p-p38 MAPK bands were scanned and arbitrary units were assigned by densitometric analysis carried out using the UN-SCAN-IT gel digitizing software (Silk Scientific Inc., Orem, UT).

Statistics

Results are expressed as means ± S.E.M. One-way ANOVA with subsequent post hoc comparisons by Scheffe's test was performed (SPSS 13.0). *P* values < 0.05 were considered as statistically significant.

Results

Synergistic toxicity produced by BAY11-7082 and GSH depletion in HepG2 cells

Experiments were carried out to evaluate the effect of inhibition of NF- κ B on the toxicity of BSO in CYP2E1-expressing E47 cells and control C34 cells which do not express CYP2E1. BSO produced greater toxicity in E47 compared to C34 cells (Fig. 1B); e.g., at 72 h after addition of 200 μ M BSO, C34 cells remained totally viable whereas viability decreased 25% in the E47 cells. Fig. 1A shows that exposure to nontoxic low concentrations of BAY11-7082 (2–3 μ M) greatly increases BSO toxicity in both the E47 and C34 HepG2 cells; e.g., incubation of E47 cells with 100 μ M BSO alone decreased cell viability by 12% after 48 h, but viability decreased 74% in the presence of BSO plus 2.5 μ M BAY11-7082; viability of C34 cells was not affected by BSO alone after 48 h, but decreased 34% in the presence of BSO plus 2.5 μ M BAY11-7082. Thus, BAY11-7082 potentiated BSO toxicity in E47 as well as in C34 cells. Incubation of these cells with doses of BAY11-7082 higher than 3 μ M for 48 h caused a dose-dependent toxic response (Fig. 1A); an apparent IC₅₀ around 5 μ M was estimated for the toxicity produced by BAY11-7082 alone in the E47 cells. The synergistic toxicity induced by BSO in the presence of BAY11-7082 depends on the concentration of BSO and the time of exposure to BSO, as shown in Fig. 1B. BAY11-7082 potentiated BSO toxicity in both E47 and C34 cells after 48 or 72 h; overall toxicity was greater in the E47 cells treated with BAY11-7082 than C34 cells. Results obtained from the MTT assay directly correlated with the extent of cell death as detected by changes in morphology observed under a light microscope (data not shown). Trypan blue exclusion results were: untreated E47 cells, 100% viable; E47 cells treated with 2.5 μ M BAY11-7082, 82% viable; E47 cells treated with 50 μ M BSO, 93% viable; E47 cells treated with 2.5 μ M BAY11-7082 plus 50 μ M BSO, 27% viable after 48 h of incubation.

Effect of BAY11-7082 on AA toxicity in HepG2 cells

Treatment with AA produced a greater loss of viability in the E47 cells than C34 cells (Fig. 2A). Incubation with BAY11-7082 did not synergistically increase AA toxicity either in the E47 or the C34 HepG2 cells (Fig. 2A); thus, coexposure of BAY11-7082 plus AA caused only an additive toxicity, both in the absence or presence of CYP2E1 activity. Accordingly, lipid peroxidation induced by AA was not increased by the presence of BAY11-7082, similar to the lack of potentiation of AA toxicity by BAY11-7082. BAY11-7082 itself did not produce lipid peroxidation (Fig. 2B).

Other compounds known to suppress the activation of NF- κ B also potentiate BSO but not AA toxicity in HepG2 cells

Since a variety of stress conditions may cause GSH depletion, e.g., prolonged inhibition of NF- κ B itself, we attempted to further extend the above results by examining the effects produced by other previously described NF- κ B inhibitors on BSO or AA toxicity in HepG2 cells. Thus, the green tea flavanol epigallocatechin-3-gallate [EGCG; (Nishikawa et al., 2006)] (Fig. 3A), the thioredoxin reductase inhibitor 1-chloro-2,4-dinitrobenzene [CDNB; (Heiss & Gerhauser, 2005; Sakurai et al., 2004)] (Fig. 3B), or AA itself (data not shown) synergistically increase BSO toxicity in E47 cells as compared to the cells treated with BSO alone; e.g., a treatment with 25 μ M EGCG for 48 h which did not cause any toxicity, nor did it reduce intracellular GSH levels (not shown), produced a 3.5-fold increase in 100- μ M BSO toxicity (Fig. 3A). Similar to what occurs for the toxicity induced by BAY11-7082 plus BSO (Fig. 1B), the synergistic toxicities of EGCG plus BSO and CDBN plus BSO also depend on BSO concentration (Fig. 3B) and the time of coexposure (data not shown). EGCG and CDBN also increased BSO toxicity in the C34 cells (data not shown), similar to the increase in toxicity produced by BAY11-7082. Thus, potentiation of BSO toxicity by these NF- κ B inhibitors is

independent of CYP2E1 expression. Neither EGCG (not shown) nor CDNB (Fig. 3C) potentiated AA toxicity in these cells, similar to the lack of potentiation by BAY11-7082.

Inhibition of NF- κ B activity potentiates BSO but not AA toxicity in isolated rat hepatocytes

The synergistic toxicities of BAY11-7082 plus BSO and CDNB plus BSO were also observed in primary hepatocytes from pyrazole- (Fig. 4A) or chronically ethanol-treated rats (Figs. 4B and 4C) and their corresponding control hepatocytes. The synergistic toxicity induced by BSO plus the NF- κ B inhibitor (i.e., BAY11-7082 or CDNB) in isolated hepatocytes is not dependent on CYP2E1-related oxidative stress as loss of viability, e.g., induced by CDNB plus BSO occurred comparably in cultured hepatocytes isolated from chronic ethanol- or dextrose-fed rats (Fig. 4B). These results with BSO were confirmed using other NF- κ B signaling inhibitors, such as 2.5 μ M *N*-tosyl-L-phenylalanine chloromethyl ketone [TPCK; (Mellits et al., 1993)] and 2.5 μ M parthenolide (Zhang et al., 2004); accordingly, both agents were able to greatly increase BSO toxicity in cultured primary hepatocytes (Fig. 4C). In contrast to BSO toxicity, the toxicity of AA to the cultured rat hepatocytes was not affected by coexposure to BAY11-7082, TPCK, or parthenolide (Fig. 4D); as a positive control, these three agents slightly potentiated toxicity produced by TNF- α (Fig. 4C), likely due to the suppression of NF- κ B activity (Uesugi et al., 2001).

Inhibition of p38 MAPK abolishes the synergistic toxicity of BAY11-7082 plus BSO in HepG2 cells

In an attempt to reverse the synergistic toxicity induced by BAY11-7082 and BSO coexposure, as well as define some possible mechanism(s) involved, E47 cells were preincubated with the following agents (Fig. 5A): *i*) several antioxidants [the vitamin E-derivative trolox, catalase, *N*-acetyl cysteine (NAC)], *ii*) MAPK inhibitors [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), for p38 MAPK; 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), for extracellular signal-regulated kinase; anthra(1,9-cd)pyrazol-6(2H)-one (SP600125), for c-Jun *N*-terminal kinase], *iii*) *N*-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (z-VAD-FMK), a broad-spectrum caspase inhibitor, *iv*) GSH ethyl ester, to replenish GSH, and *v*) (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate), a nitric oxide donor which inhibits apoptosis.

As shown in Fig. 5A, none of the cell-permeable antioxidants could prevent the BAY11-7082 plus BSO-induced synergistic toxicity. In another set of experiments, while BSO alone increased DCF fluorescence as a measure of ROS production, BAY11-7082 had no effect on the basal production of ROS nor did it further elevate the increase produced by the BSO treatment (Fig. 5B) under conditions in which the NF- κ B inhibitor potentiated cell toxicity. Toxicity was not blocked either by the extracellular signal-regulated kinase or c-Jun *N*-terminal kinase inhibitors (Fig. 5A), indicating that these MAPK pathways are likely not involved in the mechanism(s) of toxicity. Interestingly, the specific p38 MAPK inhibitor SB203580 strongly prevented (around 75%) the toxicity induced by BAY11-7082 plus BSO (Fig. 5A). Replenishment of intracellular GSH by adding GSH ethyl ester totally abolished the toxicity (Fig. 5A). Likewise, a significant protective effect was observed after preincubating the cells with either the pan caspase inhibitor z-VAD-FMK (45% protection) or the nitric oxide donor DETA-NONOate (30% protection) under the conditions assayed (Fig. 5A).

To further validate a role for p38 MAPK activation in the mechanism resulting in potentiated cell toxicity by exposure to BAY11-7082 plus BSO in the E47 cells, immunoblotting was used to assay for the effect caused by this combination on the expression of phospho-p38 MAPK as referred to total p38 MAPK content (p-p38/p38 MAPK ratio, which reflects p38 MAPK activation). As shown in Fig. 5C, incubation of these cells with BAY11-7082 alone or BSO

alone led to a 2-fold increase of the p-p38/p38 MAPK ratio after 4 h (top panel) that was sustained for an additional 4 h (8 h, bottom panel); SB203580 alone lowered the basal level of p-p38 MAPK according to its inhibitory action against p38 MAPK activation. BAY11-7082 plus BSO treatment induced a greater activation of p38 MAPK as compared to BAY11-7082 alone or BSO alone at both exposure times (Fig. 5C); further, the increased p-p38/p38 MAPK ratio produced by BAY11-7082 plus BSO was strongly decreased by pretreatment with SB203580 (Fig. 5C).

Discussion

The aim of this work was to examine the effect produced by the suppression of NF- κ B activity on the toxicity caused by BSO or AA in cells of hepatic origin, with a special interest in describing a possible role exerted by CYP2E1 activity (Jimenez-Lopez & Cederbaum, 2005) in the developing toxicity.

The rationale for this study was to evaluate how NF- κ B can modulate the toxicity of GSH depletion produced by BSO or addition of AA, whether CYP2E1 alters this modulation by NF- κ B activity, whether BSO and AA toxicities show similar responses to this NF- κ B modulation, and to evaluate possible mechanisms, e.g., oxidative stress, MAPK activation, caspase activation, in the overall pathway of cellular toxicity. Activation of NF- κ B may occur as an adaptive mechanism to protect cells in response to the ensuing oxidative injury produced by GSH depletion, e.g., in human leukemic U937 cells (Filomeni et al., 2005), or after a long-time AA treatment (Wu & Cederbaum, 2003). In fact, synthesis of protective factors stimulated by NF- κ B activation appears to be a critical survival mechanism to protect hepatocytes against apoptotic cell death caused by a variety of insults including TNF family members (Okano et al., 2003; Pikarsky et al., 2004; Uesugi et al., 2001), and vice versa, the inhibition of NF- κ B activity may reduce GSH synthesis by downregulating glutamate-cysteine ligase at the transcriptional level (Lu, 1999; Yang et al., 2005).

Depletion of GSH by BSO treatment was shown to enhance AA (Chen et al., 1997) and CYP2E1 itself-derived ROS (Chen & Cederbaum, 1998) toxicities in the E47 HepG2 cell subline that overexpresses CYP2E1. As the main antioxidant inside mammalian cells, GSH plays a pivotal role in preventing oxidative stress and damage to mitochondrial function produced by numerous toxins (Dickinson & Forman, 2002). Results of the current study indicate that GSH depletion as a result of BSO treatment, but not AA-related lipid peroxidation, sensitizes both HepG2 cells and isolated rat hepatocytes to damage and cell death when inhibition of NF- κ B activity occurs. The observed toxic effect depends on the concentration of BSO, the concentration of the specific NF- κ B inhibitor and the coexposure time.

Treatment with AA increases lipid peroxidation in E47 cells as assessed by assaying for the production of thiobarbituric acid reactive substances (Fig. 2B), and this increase in lipid peroxidation plays a central role in the developing AA toxicity (Caro & Cederbaum, 2004). BAY11-7082 is a well-described selective and irreversible inhibitor of I κ B- α (inhibitory subunit of the cytoplasmic NF- κ B complex) phosphorylation, thereby preventing NF- κ B nuclear translocation and activity (Hansson et al., 2005). BAY11-7082 exposure in AA-treated cells does not further augment lipid peroxidation as compared to cells treated with AA alone, which would explain the absence of higher toxicity, i.e., incubation of cells in the presence of BAY11-7082 plus AA only produces additive but not synergistic toxic effects. Moreover, BAY11-7082 itself does not induce lipid peroxidation, suggesting that there is no causal relationship between this effect and the inhibition of NF- κ B activity.

Restoration of GSH with GSH ethyl ester completely blocked the BAY11-7082 plus BSO synergistic toxicity in E47 cells, confirming that GSH depletion is critically involved in the

mechanism of toxicity leading to cell death. The protective effect produced by the broad-spectrum caspase inhibitor z-VAD-FMK against BAY11-7082 plus BSO toxicity indicates that cell death depends on caspase activation, at least partially, for the final execution; hence, apoptosis likely accounts for loss of viability after incubating with BAY11-7082 plus BSO, although future studies directly evaluating apoptotic indices are necessary.

ROS generated from CYP2E1 and other sources can be scavenged either by direct reaction with GSH or by the GSH plus glutathione peroxidase reaction (Dickinson & Forman, 2002). To evaluate the possible role of ROS production as a signal involved in the mechanism of potentiated toxicity caused by GSH depletion plus NF- κ B inhibition, the incubation medium was supplemented with a variety of antioxidants before initiating the toxicity phase. Surprisingly, neither trolox- nor catalase- nor NAC-exposed cells overcome the following loss of viability induced by BAY11-7082 plus BSO, indicating that an increased accumulation of ROS caused by these treatments is likely not critical in determining cell death. This is in agreement with results showing that although BSO alone increased ROS production (DCF fluorescence), no potentiation of this BSO-induced increase occurs in the presence of BAY11-7082.

Activation of p38 MAPK seems to be an important signal leading to loss of viability by BAY11-7082 plus BSO treatment, as the p38 MAPK inhibitor SB203580 strongly prevented the synergistic toxicity in E47 cells. Lack of protection by PD98059 or SP600125 suggests that neither extracellular signal-regulated kinase nor c-Jun N-terminal kinase are involved in the mechanism underlying the synergistic toxicity. BSO treatment induced the phosphorylation and hence activation of p38 MAPK in CYP2E1-overexpressing liver cells, which could suppress the activation of NF- κ B. BAY11-7082 treatment was also found to increase the p-p38/p38 MAPK ratio in E47 cells, which may contribute to inhibition of NF- κ B activation by preventing phosphorylation and subsequent degradation of I κ B- α (Schwenger et al., 1998). It is noteworthy that the BAY11-7082 plus BSO combination induced a higher activation of p38 MAPK than BAY11-7082 alone or BSO alone at relatively early time periods prior to the developing toxicity, which might explain, at least in part, the appearance of the synergistic toxicity. In addition, the greater p-p38/p38 MAPK ratio after treatment with BAY11-7082 plus BSO was strongly prevented by SB203580, in accordance to the protection provided by SB203580 against the potentiated toxicity of BAY11-7082 plus BSO in these cells.

Despite the fact that CYP2E1-dependent oxidative stress plays an important role in potentiating loss of cell viability in the above models of toxicity, the current work shows that the expression of CYP2E1 does not appear to change the sensitivity threshold eliciting a toxic response after long-term BSO (but not AA) treatment when cells are coincubated with any of several NF- κ B inhibitors. Together, these results suggest that redox changes caused by profound intracellular GSH depletion are critical enough to sensitize the liver cells to toxicity provoked by inhibition of NF- κ B activity through a mechanism that does not depend on either the accumulation of ROS or lipid peroxidation, but is mediated by p38 MAPK.

Based on the protection by SB203580, pro-apoptotic actions of p38 MAPK can be presumably occurring and likely be responsible for the observed toxicity of BAY11-7082 plus BSO, as p38 MAPK activation has been shown to dysregulate mitochondrial function through distinct mechanisms (Cuenda & Rousseau, 2007). In this respect, it is interesting to notice that provision of exogenous nitric oxide decreased the TNF- α plus acute GSH depletion toxicity in murine hepatocytes (Matsumaru et al., 2003), the BSO plus CYP2E1-dependent toxicity in liver cells (Wu & Cederbaum, 2004), and the toxicity produced by BAY11-7082 plus BSO in E47 cells (Fig. 5A). As previously discussed, the protective role of nitric oxide could be related to its ability to prevent apoptosis through the inhibition of caspases (Matsumaru et al., 2003) as well as by inhibiting cytochrome P450 activity (Wu & Cederbaum, 2004).

In summary, this report describes a synergistic toxicity induced by GSH depletion and additional suppression of NF- κ B activity, hence loss of NF- κ B-dependent protection, in liver cells. Activation of p38 MAPK is an important early signal leading to toxicity through, at least partially, a caspase-dependent pathway. Differences in response of BSO versus AA toxicity to inhibition of NF- κ B suggests that not all prooxidants induce toxicity by NF- κ B-modulated mechanisms in liver cells, which may complicate therapeutic interventions. Since a reduction of the intracellular GSH levels may occur under diverse prooxidant conditions, which could further interfere with the activation state of NF- κ B, conclusions derived from this study may be of relevance in predicting possible enhanced toxic effects as a consequence of redox homeostasis unbalance in situations involving the inhibition of NF- κ B activity, e.g., after exposure to a variety of drugs.

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Fig.1A

Fig.1B

Fig. 1. Dose and time dependent synergistic toxicity of BAY11-7082 plus BSO in E47 and C34 HepG2 cells

A, E47 and C34 cells were incubated with medium containing different concentrations of BAY11-7082 for 1 h, and then medium was supplemented with or without (control cells) 100 μM BSO. Cell viability was determined after 48 h of incubation by the MTT assay and expressed as percentage of untreated cells. Data are expressed as means $\pm$ S.E.M. and are from a representative experiment repeated twice and conducted in triplicate. * $P < 0.05$ vs the corresponding cells in the absence of BAY11-7082; # $P < 0.05$ vs the corresponding cells in the absence of BSO. Semi-log plots show a linear inverse relationship between the concentration of BAY11-7082 and the percent viability of E47 cells, both in the absence (top) or presence (bottom) of BSO. B, E47 (left panel) and C34 (right panel) cells were exposed to

different concentrations of BSO in the absence or presence of 2.5 μ M BAY11-7082 (1-h preincubation as above). Cell viability was determined after 24, 48 or 72 h of incubation by the MTT assay and expressed as percentage of the corresponding untreated cells. Data are expressed as means $\pm$ S.E.M. and are from a representative experiment repeated twice and conducted in triplicate. * $P < 0.05$ vs the corresponding cells in the absence of BSO; # $P < 0.05$ vs the corresponding cells in the absence of BAY11-7082.

Fig.2A

AA	-	-	+	+
BAY11-7082	-	+	-	+

Fig.2B

Fig. 2. Effect of BAY11-7082 exposure on AA toxicity in HepG2 cells

A, E47 (left panel) and C34 (right panel) cells were incubated with medium containing different concentrations of AA in the absence (control) or presence of 2.5 μ M BAY11-7082 (1-h preincubation). Cell viability was determined after 48 h of incubation by the MTT assay and expressed as percentage of untreated cells. Data are expressed as means $\pm$ S.E.M. and are from a representative experiment repeated twice and conducted in triplicate. * $P < 0.05$ vs the corresponding cells in the absence of AA; # $P < 0.05$ vs the corresponding cells in the absence of BAY11-7082. B, E47 cells were incubated with medium containing 15 μ M AA in the

absence or presence of 3 μ M BAY11-7082. Production of malonaldehyde was assayed after 48 h as described under *Materials and Methods*. Data are the mean value from two independent experiments.

Fig.3A

Fig.3B

Fig.3C

Fig. 3. Synergistic toxicity produced by coexposure of BSO, but not AA, with EGCG or CDNB in E47 HepG2 cells

A, E47 cells were incubated with medium containing different concentrations of EGCG in the absence or presence of 100 μM BSO. Cell viability was determined after 48 h of incubation by the MTT assay and expressed as percentage of untreated cells. Data are expressed as means $\pm$ S.E.M. and are from a representative experiment repeated twice and conducted in triplicate. * $P < 0.05$ vs the corresponding cells in the absence of BSO; # $P < 0.05$ vs the BSO-treated cells in the absence of EGCG. B–C, E47 cells were incubated with medium containing different concentrations of BSO (B) or AA (C) for 24 h, and then medium was supplemented with 0, 2 or 5 μM (final concentration) CDNB. Cell viability was determined after 24 h by the MTT assay and expressed as percentage of untreated cells. Data are expressed as means $\pm$ S.E.M. and are from a representative experiment repeated three times and conducted in triplicate. * $P < 0.05$ vs the corresponding cells in the absence of CDNB.

Fig.4A

Fig.4B

Fig.4C

Fig.4D

Fig. 4. Synergistic toxicity induced by coexposure of BSO, but not AA, with a variety of NF- κ B signaling inhibitors in isolated rat hepatocytes

A, Primary hepatocytes from pyrazole-treated rats were incubated with medium containing different concentrations of BAY11-7082 (1-h preincubation) in the absence or presence of 200 μ M BSO. Cell viability was determined after 24 h of incubation by the MTT assay and expressed as percentage of untreated cells. Data are expressed as means $\pm$ S.E.M. and are from a representative experiment repeated twice and conducted in triplicate. * P < 0.05 vs the corresponding cells in the absence of BAY11-7082; # P < 0.05 vs the BAY11-7082-treated cells in the absence of BSO. **B**, Isolated hepatocytes from chronically ethanol- or dextrose-fed rats were incubated with medium in the absence or presence of 200 μ M BSO for 72 h. Medium was supplemented with the indicated concentrations of CDNB for the last 24 h. Cell viability

was determined by the MTT assay and expressed as percentage of the corresponding untreated cells. Data are expressed as means $\pm$ S.E.M. and are from a representative experiment repeated once and conducted in triplicate. $*P < 0.05$ vs the corresponding cells in the absence of CDNB; $^{\#}P < 0.05$ vs the corresponding CDNB-treated cells in the absence of BSO. *C*, Isolated ethanol hepatocytes were incubated with medium containing 2.5 μ M BAY11-7082, 2.5 μ M TPCK, or 2.5 μ M parthenolide (1-h preincubation), in the absence (left) or presence of 200 μ M BSO (middle) or 10 ng/ml mouse TNF- α (right). Cell viability was determined after 24 h of incubation by the MTT assay and expressed as percentage of the corresponding untreated cells. Data are expressed as means and are from a representative experiment repeated once. *D*, Isolated pyrazole hepatocytes were incubated with medium containing different concentrations of AA in the absence or presence of 2.5 μ M BAY11-7082, 2.5 μ M TPCK, or 2.5 μ M parthenolide (1-h preincubation). Cell viability was determined after 24 h of incubation by the MTT assay and expressed as percentage of untreated cells. Data are expressed as means $\pm$ S.E.M. and are from a representative experiment repeated twice and conducted in triplicate. $*P < 0.05$ vs the corresponding cells in the absence of AA.

Fig.5A

Fig.5B

Fig.5C

Fig. 5. Reversion of the synergistic toxicity and increased p38 MAPK activation produced by BAY11-7082 plus BSO in E47 HepG2 cells

A, E47 cells were incubated with medium in the absence (control) or presence of the following additions for 1 h: trolox (150 μ M), catalase (300 U/ml), or NAC (2.5 mM), as antioxidants; SB203580 (5 μ M), to inhibit p38/MAPK; PD98059 (3 μ M), to inhibit MEK/extracellular signal-regulated kinase; SP600125 (0.25 μ M), to inhibit c-Jun *N*-terminal kinase; z-VAD-FMK (10 μ M), a broad-spectrum caspase inhibitor; GSH ethyl ester (1 mM), to replenish GSH; DETA-NONOate (50 μ M), a nitric oxide donor. Medium with or without BAY11-7082 at a final concentration of 3 μ M was added to the cells, and after 1 h the “synergistic toxicity phase” was initiated by adding medium in the presence or absence of BSO (final concentration of 120

μM). Cell viability was determined after 48 h of incubation by the MTT assay and expressed as percentage of the untreated cells. Viability of the cells was not significantly affected by the presence of the various agents themselves (“no addition” bars). Potentiated toxicity of BAY11-7082 plus BSO is shown as percent decrease when compared with viability of the corresponding BSO-treated cells. Data are expressed as means ± S.E.M. and are from a representative experiment repeated once and conducted in triplicate. *B*, E47 cells were incubated with medium in the absence or presence of 2.5 μM BAY11-7082 for 1 h, and then medium was supplemented with or without 150 μM BSO. Production of ROS was assayed after 6, 24, 36, or 48 h of incubation as described under *Materials and Methods*. *C*, E47 cells were incubated with medium in the absence (control) or presence of 5 μM SB203580 for 1 h. Medium with or without BAY11-7082 at a final concentration of 2.5 μM was added to the cells, and after 1 h incubation the “synergistic toxicity phase” was initiated by adding medium with or without BSO at a final concentration of 150 μM. Immunoblots were carried out to determine the content of p38 and p-p38 MAPK in the cellular lysates collected at selected times from 0.5 to 48 h (shown only for 4 and 8 h) as described under *Materials and Methods*. The p38 and p-p38 MAPK bands were scanned and arbitrary units were assigned by densitometric analysis. The p-p38/p38 MAPK ratio reflecting activation of p38 MAPK was expressed as - fold increase when compared with the control ratio (1.0).