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Protein Kinase C Signaling as a Survival Pathway Against CYP2E1-Derived Oxidative Stress and Toxicity in HepG2 Cells*

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Running title: PKC Activation Prevents CYP2E1-Related Toxicity in E47 Cells

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Abbreviations: AA, arachidonic acid; CYP2E1, cytochrome P450 2E1; E47 cells, transfected HepG2 hepatoma cells overexpressing CYP2E1; Fe-NTA, ferric-nitrilotriacetate; GSH, glutathione; MAPK, mitogen-activated protein kinase; MTT, thiazolyl blue tetrazolium bromide; PBS, phosphate-buffered saline; PKC, protein kinase C; ROS, reactive oxygen species; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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Abstract

Hepatic induction of cytochrome P450 2E1 (CYP2E1) is a major pathway involved in oxidative stress and damage caused by chronic ethanol consumption; CYP2E1 also promotes the activation of a variety of hepatotoxins to reactive intermediates. Phorbol esters activate protein kinase C (PKC) thereby blocking cell differentiation and promoting tumor growth. In this study we examined the possible role of PKC signaling as a survival pathway against CYP2E1-mediated toxicity using transfected HepG2 hepatoma cells stably overexpressing CYP2E1 (E47 cells). Cells were exposed to arachidonic acid plus iron (AA+Fe), which has been previously reported to cause a synergistic toxicity in E47 cells by a mechanism dependent on CYP2E1 activity, and involving oxidative stress and lipid peroxidation. Phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), but not the inactive analogue 4-α-TPA, prevented lipid peroxidation, glutathione depletion, and loss of viability produced by AA+Fe in E47 cells. TPA also protected against the toxicity caused by AA alone, or by iron alone, in the E47 cells. TPA did not lower but instead induced catalytically-active CYP2E1 in these cells. The protective effect of TPA on CYP2E1-dependent AA+Fe toxicity seemed to involve a PKC-related survival mechanism, since PKC inhibitors such as Ro 31-8425 or staurosporine abolished that protection, and activation of PKC by TPA was an early event which occurs prior to the developing toxicity. In conclusion, PKC activation by TPA prevents CYP2E1-derived acute oxidative stress and toxicity in HepG2 cells, and this appears to involve maintenance of the intracellular redox homeostasis *via* PKC signal transduction.

Introduction

Several studies have described a synergistic hepatotoxic effect between alcohol ingestion and nutritional factors such as polyunsaturated fat and iron that may facilitate the developing liver damage in patients with alcoholic liver disease (Bonkovsky et al., 1996; French, 2001). Hepatic induction of CYP2E1 is an important pathway involved in oxidative stress and damage caused by chronic ethanol consumption; CYP2E1 also catalyzes the activation of a variety of hepatotoxins to reactive products (Caro and Cederbaum, 2004). Furthermore, cytochrome P450-related oxidative stress appears to be involved in ischemia-reperfusion injury during myocardial infarction (Granville et al., 2004) and to be a risk factor for cancer (Stickel et al., 2002). There is increasing evidence that oxidative stress and a decrease in antioxidant defense contribute to ethanol-induced liver injury (Arteel, 2003; Nordmann et al., 1992). Recent findings in our laboratory suggest a link between CYP2E1 induction by ethanol, reactive oxygen species (ROS) generation and alcohol-induced cell injury (Caro and Cederbaum, 2004; Cederbaum et al., 2001).

Phorbol esters have been widely used as agents to block cellular differentiation and promote tumor growth, and their mechanism of action mainly involves a direct and potent activation of PKC (Nishizuka, 1984). Activation of PKC is one of the earliest events in signal transduction leading to a variety of cellular responses including gene expression, cell growth, differentiation, secretion and muscle contraction (Liu, 1996; Nishizuka, 1988). It has been reported that physiological activation of PKC may act as a survival signaling pathway against oxidant damage induced, for example, by ischemia-reperfusion during myocardial infarction (Zhou et al., 2002) or neurotoxins (Levites et al., 2002).

ROS are known to activate members of the PKC family, which comprises at least eleven mammalian Ser/Thr kinase isozymes with conserved catalytic domains that differ in structure, co-factor requirement and substrate specificity (Buchner, 2000; Nishizuka, 1984). These ubiquitous enzymes play a central role in the regulation of many cellular signal transduction pathways. PKC contains two functional domains: an amino-terminal regulatory domain that interacts with Ca^{2+}, acidic phospholipids such as phosphatidylserine and diacylglycerol/phorbol ester, and a carboxyl-terminal catalytic domain containing the ATP and substrate-binding sites. PKC isoforms can been classified into three groups: classical or conventional PKCs (α, βI, βII, γ), which are Ca^{2+} dependent and are activated by phospholipid, diacylglycerol and phorbol ester; novel PKCs (δ, ε, η, θ), which are Ca^{2+} independent but are still regulated by phospholipid, diacylglycerol and phorbol ester; and atypical PKCs (ζ, ι /λ), which do not require phospholipid, diacylglycerol or phorbol ester, but certain lipid cofactors such as phosphoinositides for their activation (Buchner, 2000; Nishizuka, 1988). These three PKC groups, in their regulatory domain, contain an autoinhibitory pseudo-substrate region that binds to the substrate-binding site in the catalytic domain preventing its activation in the absence of cofactors or activators. PKC isozymes are subject to functional control through distinct phosphorylation events before responding to effectors (Keranen et al., 1995; Liu, 1996). Other members have been added to the PKC family based on homology within the catalytic domain; e.g., PKCμ (PKD) is regulated by diacylglycerol and phorbol ester. Moreover, the PKC family may be further enlarged by including the PKC-related kinases.

We have recently shown that the CYP2E1-mediated toxicity by AA is mediated in part *via* activation of the mitogen-activated protein kinase (MAPK) family member p38 in CYP2E1-overexpressing rat hepatocytes and HepG2 cells (Wu and Cederbaum, 2003). It seemed of interest to evaluate whether CYP2E1-induced oxidant stress could induce expression of cell survival signal transduction pathways such as PKC. The aim of the current work was to investigate the possible modulation of CYP2E1-dependent oxidative stress and toxicity by PKC signaling in E47 cells, a transfected HepG2 hepatoma cell line overexpressing CYP2E1 (Chen and Cederbaum, 1998). The phorbol ester TPA was employed as a PKC activator, and potential mechanisms implicated in the actions of TPA were evaluated. In particular, levels and activity of CYP2E1 in the absence and presence of TPA were determined. Treatment with AA+Fe (Caro and Cederbaum, 2001) was used as a model of toxicity, because this system appears to reproduce several of the key features associated with ethanol hepatotoxicity in the intragastric infusion model of ethanol treatment (Fernandez-Checa et al., 1993; French, 2001; Morimoto et al., 1993; Tsukamoto et al., 1995) such as prominent induction of CYP2E1, toxicity by polyunsaturated fatty acids such as AA (but not by saturated fatty acids) or by iron or by glutathione (GSH) depletion, and elevated lipid peroxidation that correlates with the CYP2E1 levels.

Materials and Methods

Chemicals. Geneticin was from Invitrogen (Carlsbad, CA). Glutathione reductase was purchased from Roche (Indianapolis, IN). Other chemicals used were from Sigma (St. Louis, MO).

Cell culture. This study was carried out using as a model the human hepatoma E47-HepG2 cell subline (Chen and Cederbaum, 1998) which constitutively expresses human CYP2E1. Cells were grown in minimal essential medium containing 10% fetal bovine serum 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% CO_2 at 37°C, and were subcultured at a 1:5 ratio once a week.

Cell treatment. Geneticin was omitted from the medium for the various assays. Cells were plated at a density of 3×10^4 cells/cm^2 and maintained in culture medium for 24 h before treatments. Cycloheximide and L-buthionine sulfoximine were dissolved in phosphate-buffered saline (PBS, pH 7.4), and the remaining stock solutions were prepared in dimethyl sulfoxide. Incubation medium was supplemented with dimethyl sulfoxide during the different treatments to reach the same final concentration, typically 0.1% (i.e., 14 mM), which does not inhibit CYP2E1 catalytic activity. Percentage of serum was reduced during the AA (7.5% serum) or iron (5% serum) exposure to enhance toxicity, and serum-free medium was used during the treatment with protein kinase modulators.

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For AA+Fe treatments, cells were initially incubated for 14 h in medium supplemented with 15 μM AA, while untreated cells were used as a no-addition control. After washing with PBS to remove unincorporated AA, cells were incubated with serum-free medium in the absence or presence of a variety of protein kinase modulators as is described in the Figure legends for the various assays. Then, medium with or without ferric-nitrilotriacetate (Fe-NTA, 1:3 complex, pH 7.4) (final concentration of ferric ion, 15 μM) was added to initiate the toxicity phase (t=0), maintaining the corresponding additions in the medium. The iron chelate used, Fe-NTA (1:3) complex was prepared as described previously (Sakurai and Cederbaum, 1998). Cells pre-loaded with AA were incubated for variable periods in the absence (AA-pretreated cells) or presence (AA+Fe-treated cells) of Fe-NTA before the biochemical or analytical analyses. 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, as described previously (Caro and Cederbaum, 2001). Other indexes of cytotoxicity used were the measurement of lactate dehydrogenase leakage (Perez and Cederbaum, 2001) and the change in cell morphology observed under the light microscope.

CYP2E1 catalytic activity assay. CYP2E1 activity was determined by assaying *p*-nitrophenol hydroxylation in microsomes (Chen and Cederbaum, 1998) or in intact cells

(Perez and Cederbaum, 2001), using an extinction coefficient of 9.53 mM^{-1} cm^{-1} for *p*-nitrocatechol.

Immunoblot analysis of total and phospho-PKC (pan), and CYP2E1 expression. The cells (10-cm culture dishes) were washed with PBS and scraped into hypotonic buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and 20 µg/ml aprotinin, and rapidly sonicated at 4°C. Cell extracts were centrifuged at 105,000 *g* for 1 h at 4°C. The total particulate fractions were suspended in lysis buffer consisting of 50 mM HEPES (pH 7.4), 150 mM NaCl, 250 mM sucrose, 10% glycerol and 1% IGEPAL CA-630, and stored at −80°C until use; an aliquot was taken out to determine protein concentration. Equal amounts of protein (30 µg) were separated by 8% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked in 20 mM Tris (pH 7.6), 140 mM NaCl, 0.1% Tween-20 with 5% (w/v) nonfat dry milk and probed with rabbit anti-phospho-PKC (pan) (*βII* Ser660) polyclonal antibody (1:1,000, Cell Signaling Technology, Beverly, MA), a PKC phosphorylation state-dependent primary antibody that detects endogenous levels of PKC*α*, *βI*, *βII*, *δ*, *ε*, *η* and *θ* isoforms (78-85 kDa) only when they are phosphorylated at a carboxyl-terminal residue homologous to Ser660 of human PKC*βII*, i.e., an autophosphorylation site at the hydrophobic region of PKC (Keranen et al., 1995). The relative content of total PKC in cell lysate samples was determined by incubating the membranes with rabbit anti-PKC (pan) (H-300) polyclonal antibody (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), which broadly reacts with all PKC family members of diverse origins, including human. Following incubation with horseradish peroxidaseconjugated goat anti-rabbit IgG as secondary antibody (1:10,000, Sigma), immunoreactive proteins were detected using the enhanced chemiluminiscence (ECL) detection system (Amersham Biosciences, Piscataway, NJ) and exposure to Kodak Biomax Light film (Sigma). After using an antibody stripping solution (Chemicon International, Temecula, CA), blots were re-probed with rabbit anti-human CYP2E1 (54 kDa) polyclonal antibody (1:20,000) kindly provided by Dr. J. Lasker (Hackensack University Medical Center, NJ). Following ECL detection, the blots were again stripped and probed with rabbit anti-Gβ (T-20) polyclonal antibody (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), which recognizes the four β-subunit types (36 kDa) of the heterotrimeric G protein family (including those of human origin) to monitor loading and transfer of blotted samples. Densitometric analysis was carried out using the UN-SCAN-IT gel digitizing software (Silk Scientific, Orem, Utah).

Lipid peroxidation analysis. Cells were plated onto 15-cm culture dishes and at the end of the treatment were harvested as previously described (Caro and Cederbaum, 2001). Generation of malonaldehyde was determined in cell lysates by assaying for thiobarbituric acid reactive substances (Niehaus and Samuelsson, 1968). The amount of malonaldehyde equivalents was calculated from a standard curve prepared using malonaldehyde bis(dimethyl acetal) (Esterbauer and Cheeseman, 1990).

Determination of glutathione levels. Cells were seeded onto 6-well plates and collected after the corresponding treatment. The total GSH content (mainly in reduced form) of samples was assayed by the enzymatic recycling procedure of Tietze (Tietze, 1969). The rate of 2-nitro-5-thiobenzoic acid production was converted to total GSH concentration by using a standard curve with known amounts of GSH.

Statistics. Results are expressed as means ± SEM. One-way analysis of variance with subsequent *post hoc* comparisons by Scheffe's test was performed (SPSS 12.0). *P* values < 0.05 were considered as statistically significant.

Results

Protective effect of TPA on the synergistic toxicity of AA+Fe in E47 cells. AA+Fe produce a synergistic toxicity in E47 cells which is greater than that found in control C34-HepG2 cells which do not express CYP2E1, or in CYP3A4-overexpressing HepG2 cells (Caro and Cederbaum, 2001). We used 15 µM ferric ion in order to generate oxidant stress and cell death in AA-pretreated cells after short incubation periods, i.e., typically 3 h (Jimenez-Lopez and Cederbaum, 2004). To characterize the effect of TPA on this toxicity in E47 cells, dose and time response experiments were carried out using a range of concentrations of TPA. Fig. 1*A* shows that toxicity caused by 15 µM AA plus 15 µM ferric ion in E47 cells was prevented by TPA in a dose-dependent manner, e.g., 1 and 5 ng/ml TPA increased cell viability by 16% and 56%, respectively, after 3 h of AA+Fe treatment when compared to AA+Fe alone, while a concentration equal or higher to 25 ng/ml (i.e., 40 nM) TPA completely maintained viability after the addition of the pro-oxidant Fe-NTA to the cells pre-loaded with AA (Fig. 1, *A* and *B*). An apparent IC_{50} of 6 ng/ml (10 nM) was estimated for the protective effect of TPA against CYP2E1-dependent AA+Fe toxicity in E47 cells (Fig. 1*B*). TPA treatment was not toxic under the assayed conditions; thus, exposure to up to 1,000 ng/ml TPA for 3 h did not affect cell viability of the untreated, or AA-pretreated E47 cells (Fig. 1*A* and data not shown). Treatment of E47 cells with TPA also prevented the CYP2E1-dependent AA+Fe toxicity in a time-dependent manner, as determined both by measurement of cytosolic lactate dehydrogenase leakage into the medium (Fig. 1*C*) and the MTT test (data not shown). A similar protective effect was detected when C34 cells were exposed to AA+Fe in the presence of TPA, although that toxicity was much lower due to the lack of CYP2E1 expression (data not shown). A biologically inactive stereoisomer of TPA, 4-α-TPA, was used as a negative control for the effects of TPA. 4-α-TPA, which was not toxic under the tested conditions, did not protect the E47 cells against the AA+Fe toxicity (Fig. 1, *A* and *C*). Results obtained from the MTT and lactate dehydrogenase assays directly correlated with the extent of cell death as detected by changes in morphology observed under a light microscope (data not shown).

Inhibition of PKC by Ro 31-8425 or staurosporine abolishes the protective action of TPA against CYP2E1-dependent AA+Fe toxicity. Phorbol esters are known to strongly activate PKC. To validate the possible role of PKC activation as an upstream signal implicated in the protective action of TPA, we exposed the E47 cells to this phorbol ester plus AA+Fe in the presence of a variety of cell-permeable PKC inhibitors, i.e., Ro 31-8425 (bisindolylmaleimide X hydrochloride), staurosporine (from *Streptomyces sp.*), calphostin C (from *Cladosporium cladosporioides*), or D-sphingosine. The selective PKC inhibitor Ro 31-8425, which specifically binds to the catalytic region of PKC and acts as a competitive inhibitor with respect to ATP (Merritt et al., 1997; Toullec et al., 1991), abrogated in a concentration-dependent manner (apparent IC_{50} estimated around 60 nM) the protective effect of TPA on AA+Fe toxicity in E47 cells (Fig. 2*A*, see also inset). Similarly, the broad range Ser/Thr protein kinase inhibitor staurosporine, that also interacts with the ATP-binding site of PKC, at a concentration of 200 nM totally abolished the protective effect of TPA (Fig. 2*B*). However, photoactivated calphostin C or D-sphingosine, which specifically interact with the lipid-binding regulatory moiety of PKC (Jarvis and Grant, 1999; Tamaoki, 1991), at doses up to 1 μM had no effect on the protection exerted by TPA (data not shown). The distinct inhibitors of PKC tested in this study were themselves not significantly toxic at the concentrations and times used in the E47 cells (not shown). In the absence of TPA, the short-term inhibition of basal PKC activity by these inhibitors did not potentiate the AA+Fe toxicity in E47 cells (Fig. 2).

TPA also protects against CYP2E1-dependent AA and iron toxicities in E47 cells. The effect of TPA was determined in other CYP2E1-related toxicity models in E47 cells: AA alone in the absence of added iron (Chen et al., 1997), and Fe-NTA (1:3) complex alone in the absence of added AA (Sakurai and Cederbaum, 1998). After an overnight pretreatment with a low dose (10 µM) of AA, E47 cells were exposed to higher concentrations of AA, i.e., 10, 25, or 50 µM, which caused a 9, 14, and 23% loss of viability, respectively, when compared to the AA-pretreated cells (Fig. 3*A*). TPA treatment (50 ng/ml) prevented the AA-induced oxidant toxicity at similar concentrations as those used to prevent the larger toxicity caused by AA+Fe, and 0.5 µM Ro 31-8425 suppressed this preventive effect (Fig. 3*A*). TPA (50 ng/ml) was also able to protect against the Fe-NTA-induced oxidant toxicity in the E47 cells; e.g., incubation with medium containing 25 µM ferric ion (Fe-NTA, 1:3) for 4 h produced a 38% loss of viability in the absence of TPA, but toxicity was negligible in its presence (Fig. 3*B*).

Effect of TPA on phospho-PKC and CYP2E1 expression. TPA is known to transiently induce the translocation of PKC from the cytosol (inactive form) to the membranes (active, phosphorylated form) in intact cells (Chida et al., 1986; Liu, 1996).

To further validate a role for PKC in the protective actions of TPA, we analyzed by immunoblotting the effect caused by TPA on the expression of phospho-PKC and CYP2E1 in the E47 cells. Incubation of E47 cells with TPA produced a time (Fig. 4*A*) and dose dependent (Fig. 4*B*) increase of phosphorylated PKC (pan) in the particulate fraction isolated from the cells. High expression levels of phospho-PKC were observed after 30 min treatment with TPA as compared to basal levels in untreated cells; phospho-PKC levels remained elevated up to 8 and even 24 h in the presence of TPA in the culture medium (Fig. 4*A*). Total content of PKC was not modified after 30 min treatment with TPA, indicating that an increased phosphorylation of the existing pool of PKC molecules occurs in the presence of phorbol ester (data not shown). 4-α-TPA at a 50 ng/ml concentration did not increase phospho-PKC levels (Fig. 4*B*). It is noticeable that high concentrations of Ro 31-8425 itself induced the expression of phospho-PKC after 4 h (Fig. 4*B*), but not 0.5 h (not shown), suggesting a positive feedback mechanism in response to the inhibition of PKC activity. Presumably, in the presence of Ro 31-8425 the PKC isoforms remained catalytically inactive so as not to prevent the toxicity by AA+Fe in the E47 cells (See also Fig. 2*A*). In the absence of TPA, treatment of E47 cells with AA+Fe produced a time-dependent increase in the levels of phospho-PKC (Fig. 4*C*, *left*); however, the presence of TPA caused a higher induction of phospho-PKC expression in the absence or presence of Fe-NTA (Fig. 4*C*, *right*).

Incubation with TPA was previously reported to increase the level of expression of the transduced human CYP2E1 in the MVh2E1-9 cell line (E9 cells) (Dai and Cederbaum, 1995), where CYP2E1 cDNA is under the control of the Moloney murine leukemia virus LTR promoter in the pMV-7 retroviral shuttle vector. E47 cells were selected from

HepG2 cells transfected with a pCI-2E1 plasmid (Chen and Cederbaum, 1998), so the constitutive expression of CYP2E1 in E47 cells is promoted by the human cytomegalovirus immediate-early enhancer/promoter, which as in E9 cells is not its natural promoter. Treatment of E47 cells with TPA induced the expression of CYP2E1 (Fig. 4*A*) in a time-dependent manner; levels of CYP2E1 were significantly higher after 4 h of TPA exposure, increasing further up to 24 h. Levels of CYP2E1 expression directly correlated with the concentration of TPA, i.e., 5, 50, and 500 ng/ml TPA produced a 1.6-, 3.3-, and 4.5-fold induction of CYP2E1 expression (n=2), respectively, when compared to the untreated cells (Fig. 4*B* and data not shown). CYP2E1 induction by TPA in E47 cells appeared to be a PKC-dependent response, since incubation of cells with TPA in the presence of 0.5 μM Ro 31-8425 prevented the increase in CYP2E1, and the negative control 4-α-TPA was not able to increase its expression (Fig. 4*B*). Interestingly, TPA was protecting against CYP2E1-dependent toxicity while paradoxically elevating CYP2E1 content, although the latter occurs at a later time point (e.g., 4 h) compared to the activation of PKC (0.5 h).

To demonstrate that TPA-induced CYP2E1 protein was catalytically active, microsomes isolated from TPA-treated E47 cells were assayed *in vitro* for *p*-nitrophenol oxidation activity. Production of *p*-nitrocatechol increased as the concentration of TPA was higher in the culture medium, e.g., incubation of cells with medium containing 50 ng/ml TPA for 3 h caused a 2.5-fold increase in microsomal *p*-nitrophenol hydroxylase activity, i.e., 38.7 *versus* 102.9 pmol *p*-nitrocatechol/min/mg protein in untreated and TPA-treated E47 cells (n=2), respectively, whereas 4-α-TPA did not modulate CYP2E1 catalytic activity. Another set of experiments was performed *in situ* by assaying the metabolism of *p*-nitrophenol in intact cells incubated in the presence of various doses of TPA, or 4-α-TPA as negative control. Under identical experimental conditions TPA, but not 4-α-TPA, markedly enhanced CYP2E1 activity in a dose-dependent manner in E47 cells; e.g., values of A_{510} after 24 h were 0.037 ± 0.007, 0.254 ± 0.021*, or 0.865 ± 0.023* in untreated, 50 ng/ml TPA-treated, and 500 ng/ml TPA-treated E47 cells (n=3, *$p < 0.05$), corroborating the prior results obtained *in vitro*. Thus, the increase in enzyme activity was correlated with the increase in CYP2E1 protein content by TPA.

Effect of the blockade of a variety of signaling pathways on the protective action by TPA against CYP2E1-dependent AA+Fe toxicity in E47 cells. It is known that TPA-stimulated PKC may activate the extracellular signal-regulated kinase (ERK), c-Jun *N*-terminal kinase (JNK), and p38 MAPK pathways in different cell types (Buchner, 2000). In an attempt to determine if extracellular signal-regulated kinase was involved downstream in the PKC-mediated protective action of TPA against CYP2E1-dependent acute oxidant stress, AA-pretreated E47 cells were incubated with a selective MAPK/extracellular signal-regulated kinase kinase (MEK) inhibitor, PD98059 (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one), followed by treatment with TPA before exposure to Fe-NTA. Shutdown of the MEK/extracellular signal-regulated kinase signaling pathway by this inhibitor was not able to counteract the TPA-mediated suppression of CYP2E1-related AA+Fe toxicity in E47 cells (Fig. 5), indicating that this pathway is unlikely to be required for transduction of the PKC survival signal in this system. Likewise, selective inhibition of c-Jun *N*-terminal kinase or p38 MAPK activities by pretreatment with SP600125 (anthra(1,9-cd)pyrazol-6(2H)-one) and SB203580 (4-(4-

fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole), respectively, did not abolish the protective effect of TPA (Fig. 5).

Selective inhibitors of the cyclic AMP-dependent protein kinase A (PKA) or phosphatidylinositol 3-kinase (PI-3K)/Akt (PKB) survival signaling pathways, i.e., H-89 (N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide hydrochloride), for protein kinase A; and wortmannin (from *Penicillium funiculosum*), LY294002 (2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride) or ML-9 (1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride), for phosphatidylinositol 3-kinase/Akt, did not reverse the protective action of TPA either (Fig. 5). It has been proposed that nuclear factor-κB (NF-κB) signaling-dependent protective factors can promote resistance to apoptotic cell death caused by a variety of insults including TNF family members (Okano et al., 2003) or menadione (Chen and Cederbaum, 1997). Pretreatment with BAY 11-7082 ((E)-3-(4-methylphenylsulfonyl)-2-propenenitrile), a selective and irreversible inhibitor of IκBα phosphorylation that prevents nuclear factor-κB activation, was not able to blunt the protective effect of TPA in E47 cells (Fig. 5), suggesting that the nuclear factor-κB survival pathway does not mediate the protective action of TPA against CYP2E1-dependent AA+Fe toxicity in these cells. A range of concentrations was tested for all these compounds, i.e., ordinarily 0.1-25 µM, taking into account their inhibitory activities in biochemical and cell-based assays.

Effect of TPA on AA+Fe-induced lipid peroxidation. AA+Fe treatment increases lipid peroxidation in E47 cells as assessed by assaying for the production of thiobarbituric acid reactive substances; the increase in lipid peroxidation plays a central role in the developing toxicity (Caro and Cederbaum, 2001). Incubation with AA+Fe produced a 2.4-fold increase in malonaldehyde levels in E47 cells as compared to the AA-treated cells, an effect that was abolished by TPA (Fig. 6). The increment in lipid peroxidation was restored, however, in the cells exposed to AA+Fe plus TPA in the presence of Ro 31-8425 (Fig. 6).

TPA prevents GSH depletion produced by AA+Fe in E47 cells. 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 and Forman, 2002). As expected, AA+Fe-induced oxidative stress in E47 cells reduced the content of GSH (Fig. 7). TPA treatment prevented the AA+Fe-induced decline of intracellular GSH, whereas Ro 31-8425 abrogated this preventive effect, i.e., GSH levels were lowered when AA-pretreated cells were exposed to Fe-NTA in the presence of TPA plus Ro 31-8425 (Fig. 7).

Discussion

In this study, short-term phorbol ester exposure was found to prevent acute AA+Fe (also AA alone or iron alone) oxidative stress and toxicity in the CYP2E1-expressing E47 cells. 4-α-TPA, a TPA-derived biologically inactive molecule unable to activate PKC was not protective. Accordingly, the mechanism by which TPA exerts its protection seems to be related to its ability to activate certain PKC isoform(s) (Nishizuka, 1984), rather than *via* a direct effect as an antioxidant molecule. The protective effect of TPA on CYP2E1-mediated AA+Fe toxicity in E47 cells was dose and time dependent, and occurred simultaneously with an increased translocation of phosphorylated PKC to membranes.

Inhibitors of PKC can interact with the ATP/substrate-binding sites or with regulatory sites of the enzyme. TPA-stimulated PKC isoform(s) involved in the protective action of this phorbol ester on CYP2E1-dependent AA+Fe toxicity in E47 cells were sensitive to the PKC inhibitors Ro 31-8425 and staurosporine, which inhibit PKC in a manner competitive to ATP (Merritt et al., 1997; Toullec et al., 1991), but not calphostin C or D-sphingosine, which bind PKC within the regulatory domain (Jarvis and Grant, 1999; Tamaoki, 1991). These results suggest that PKC-dependent signal transduction is involved, at least in part, in the protective actions of TPA on CYP2E1-related oxidant injury, whereby the involvement of certain TPA-stimulated protective PKC isoforms may be responsible for the ability of some PKC inhibitors such as Ro 31-8425 and staurosporine to repress the preventive effect of TPA. A role of PKC signaling as a survival pathway becomes apparent after inhibition of PKC activity for a long time, which induces apoptosis in distinct cell types and potentiates the activity of diverse cytotoxic agents (Jarvis and Grant, 1999; Wang et al., 2004; Whelan and Parker, 1998). Indeed, as a result of PKC down-regulation by prolonged treatment with TPA, parental HepG2 cells were sensitized to toxicity from menadione (Chen and Cederbaum, 1997).

PKC activation by TPA seems to act as an upstream survival signal that blocks the oxidant state otherwise induced by CYP2E1 plus AA+Fe in the E47 cells, because lipid peroxidation was totally prevented by TPA but could be restored in the simultaneous presence of TPA plus Ro 31-8425. Accordingly, the effect of TPA appears to lie upstream of the CYP2E1 plus AA+Fe-induced lipid peroxidation event, and its antagonistic action on CYP2E1-mediated AA+Fe toxicity could involve the maintenance or upregulation of the cellular antioxidant defense *via* a PKC-dependent mechanism. In fact, levels of GSH were preserved when the CYP2E1-expressing HepG2 cells were exposed to AA+Fe in the presence of TPA, whereas co-incubation with Ro 31-8425 reestablished the GSH decline otherwise produced by AA+Fe in these cells, probably interfering with the PKC-related upkeeping of the intracellular antioxidant homeostasis. As the main antioxidant inside mammalian cells, GSH plays a pivotal role in preventing oxidative stress and mitochondrial function damage caused by numerous toxins (Dickinson and Forman, 2002). Accordingly, the maintenance of intracellular GSH levels by TPA may help in protecting against the oxidant AA+Fe toxicity in E47 cells and avoid cell degeneration and death. Indeed, depletion of GSH by L-buthionine sulfoximine treatment enhanced AA (Chen et al., 1997), Fe-NTA (Sakurai and Cederbaum, 1998), and CYP2E1 itself-derived ROS (Chen and Cederbaum, 1998) toxicities to the HepG2 cells overexpressing CYP2E1.

The acute oxidant stress produced in E47 cells exposed to AA+Fe in the absence of TPA triggered by itself an enhanced expression of phospho-PKC; this may reflect an adaptative mechanism in response to a possible loss of PKC function by the CYP2E1 plus AA+Fe-induced ROS generation and/or a greater requirement of this survival signal to handle the subsequent damage and toxicity, and at least initially to protect the cells against the developing oxidant injury. In agreement with this suggestion, treatment with the PKC inhibitor Ro 31-8425 alone was shown to increase the levels of phospho-PKC. Nevertheless, the modest activation of constitutively expressed PKC by AA+Fe failed to overcome the following CYP2E1-related toxicity in E47 cells, although it is evident that the early and greater activation of some TPA-sensitive PKC isozyme(s) exerted a protective action under similar conditions in this cellular system.

Both extracellular signal-regulated kinase and nuclear factor-κB were found to be involved in the protective action mediated by TPA against Fas-induced apoptosis in Jurkat cells (Engedal and Blomhoff, 2003). Our findings with inhibitors appear to rule out the MAPK, protein kinase A, phosphatidylinositol 3-kinase/Akt, or nuclear factor-κB signaling pathways as essential downstream mediators of the protective actions of TPA on CYP2E1-mediated oxidant toxicity in E47 cells. The possibility exists that PKC signals could cross-talk and thereby down-regulate certain death pathway(s) (Wang et al., 2004) and/or stimulate Ca^{2+} efflux from the cells (Banan et al., 1999) to exert its protective actions; however, antioxidant mechanism(s) must be involved to explain the prevention by TPA of the lipid peroxidation process in the presence of AA+Fe plus CYP2E1. Further studies will be necessary in order to reveal the specific nature of the targets involved dowstream during the PKC-initiated TPA action.

It has been reported that, upon exposure to oxidant insults or TPA, the PKC-directed phosphorylation and subsequent nuclear translocation of NF-E2-related factor 2 (Nrf-2) transcription factor induces antioxidant and phase II detoxifying enzymes *via* the antioxidant response element, including glutamate-cysteine ligase, glutathione *S*-transferase, heme oxygenase-1, and NAD(P)H-quinone oxidoreductase (Huang et al., 2000). Furthermore, TPA has been shown to trigger the translocation of PKC to the nucleus, whereby it may directly regulate transcription (Buchner, 2000). Seeking some possible cell antioxidant(s) involved in the protective actions of TPA, cells were pre-incubated for 3 h with L-buthionine sulfoximine (0.1-1 mM) or chromium mesoporphyrin (20 µM), in order to block the synthesis of GSH and to inhibit heme oxygenase-1 activity, respectively; however, both situations did not prevent the protective effect of TPA on AA+Fe toxicity in E47 cells (data not shown), indicating that GSH *de novo* synthesis and heme oxygenase-1 activity are not essentially required for the protection exerted by this phorbol ester. More efforts will be required to clearly define the precise mechanism of antioxidant defense responsible for the PKC-mediated protective actions of TPA and to further understand how CYP2E1-induced toxicity is suppressed by the PKC cascade in the E47 cells.

In summary, this report describes the involvement of TPA-initiated early PKC activation as an early signal that antagonizes CYP2E1-mediated toxicity in HepG2 hepatoma cells. The mechanism of protection involves prevention of oxidative stress and lipid peroxidation when the CYP2E1-enriched E47 cells are incubated with pro-oxidants such as AA and iron which also act as priming or sensitizing factors for alcohol-induced liver injury (Arteel, 2003; French, 2001; Morimoto et al., 1993; Tsukamoto et al., 1995).

PKC provides a critical upstream signal leading to resistance to death from CYP2E1-dependent acute oxidant stress, which is consistent with previous findings that PKC plays a role in cell survival (Jarvis and Grant, 1999; Whelan and Parker, 1998). The protection is associated with maintenance of endogenous GSH levels, a pivotal antioxidant which also protects against alcohol-induced hepatic damage (Fernandez-Checa et al., 1993). PKC-activating TPA also induces catalytically-active CYP2E1 in E47 cells, therefore its protective action in the presence of AA+Fe overcomes the otherwise toxicity that should result from the higher CYP2E1 expression, which is most likely a consequence of the upkeeping of intracellular antioxidant activities *via* a PKC-dependent mechanism.

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Footnotes

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Legends for Figures

Fig. 1. Dose and time dependent protective effect of TPA against CYP2E1-mediated AA+Fe toxicity in E47 cells. E47 cells were preincubated with medium containing AA for 14 h. Cells were then washed with PBS and incubated with serum-free medium in the absence or presence of different concentrations of TPA (or 4-α-TPA as a negative control) for 1 h before adding medium alone or medium supplemented with Fe-NTA. *A*, Cell viability was determined after 3 h of incubation by the MTT assay and expressed as percentage of the AA-treated cells. *B*, Semi-log plot shows a linear relationship between the concentration of TPA and the percent viability of E47 cells exposed to AA+Fe in the presence of TPA. *C*, Percentage of lactate dehydrogenase activity released from the cells into the medium was determined at different time points as described under Materials and Methods. Data are expressed as means ± SEM and are from a representative experiment repeated once and conducted in triplicate. $^*p < 0.05$ *versus* the corresponding AA-treated cells; $^{\#}p < 0.05$ *versus* the AA+Fe-treated cells in the absence of TPA.

Fig. 2. PKC inhibition by Ro 31-8425 or staurosporine blocks the preventive effect of TPA on AA+Fe-induced toxicity in E47 cells. E47 cells were preincubated with medium supplemented with or without AA for 14 h. The medium was discarded and the cells were washed with PBS and incubated with serum-free medium in the absence or presence of different concentrations of Ro 31-8425 (*A*), or 200 nM staurosporine (*B*) for 1 h. TPA at a final concentration 50 ng/ml was added to the cells (serum-free medium), and after 1 h the toxicity phase was initiated by

adding medium containing Fe-NTA (AA+Fe-treated cells) or medium alone as the reference control. Cell viability was determined after 3 h of incubation by the MTT assay and expressed as percentage of the untreated cells. Inset of Fig. 2*A* shows the percent viability of E47 cells exposed to AA+Fe in the presence of 50 ng/ml TPA plus Ro 31-8425 *versus* the semi-log concentration of Ro 31-8425 (nM). Data are expressed as means ± SEM and are from a representative experiment repeated twice and conducted in triplicate. $^*p < 0.05$ *versus* the corresponding AA+Fe-treated cells in the absence of TPA; $^\#p < 0.05$ *versus* the AA+Fe plus TPA-treated cells in the absence of PKC inhibitor (Ro 31-8425 in *A*; staurosporine in *B*).

Fig. 3. Protective effect of TPA against CYP2E1-mediated AA and iron toxicities in E47 cells. *A*, E47 cells were preincubated with medium containing 10 μM AA for 14 h. The AA-pretreated cells were washed with PBS and incubated with serum-free medium in the absence of any addition, or in the presence of 50 ng/ml TPA, or 0.5 μM Ro 31-8425 (1-h preincubation) plus 50 ng/ml TPA for 1 h, before adding medium supplemented with the indicated concentrations of AA. No iron was added for these experiments. *B*, E47 cells were incubated with serum-free medium in the absence or presence of 50 ng/ml TPA for 1 h, before adding medium alone or medium supplemented with Fe-NTA. No AA was added for these experiments. Cell viability was determined after 4 h of incubation by the MTT assay and expressed as percentage of the control (AA-pretreated in *A*, untreated in *B*) cells. Data are expressed as means ± SEM and are from a representative experiment repeated once and conducted in triplicate. $^*p < 0.05$

versus the corresponding AA-pretreated (*A*) or untreated (*B*) cells; $^{\#}p < 0.05$ *versus* the AA-treated (*A*) or Fe-NTA-treated (*B*) cells in the absence of TPA.

Fig. 4. Induction of phospho-PKC (pan) and CYP2E1 expression by TPA in E47 cells. *A*, The cells were incubated with serum-free medium in the absence or presence of 50 ng/ml TPA. At selected times, cells were harvested by scraping, a microsome-enriched particulate fraction was obtained by centrifugation, and samples were analyzed by immunoblotting to determine the expression of (pan) phospho-PKC, CYP2E1, and Gβ (loading control) as described under Materials and Methods. *B*, The cells were incubated with serum-free medium containing no addition or the indicated concentrations of TPA (ng/ml) plus or minus Ro 31-8425 (nM), or 4-α-TPA (ng/ml) as a negative control. After 4 h, the samples were collected and a total particulate fraction was obtained and analyzed as in *A*. *C*, E47 cells were preincubated with medium containing AA for 14 h. The medium was removed and the cells were washed with PBS and incubated in the absence or presence of 50 ng/ml TPA for 1 h, before adding medium with or without Fe-NTA. At chosen times, cells were harvested by scraping and a total particulate fraction was obtained and analyzed as in *A*. Phospho-PKC and CYP2E1 protein expressions in the samples were normalized to their respective Gβ expression and expressed as arbitrary units of density (relative mean values of immunoreaction intensity are indicated for each condition). The figure shows a representative experiment repeated once and conducted in duplicate.

Fig. 5. Inhibitors of MAPK-, protein kinase A-, phosphatidylinositol 3-kinase/Akt-, or nuclear factor-κB-dependent signaling pathways do not suppress the protective

action of TPA against CYP2E1-mediated AA+Fe toxicity in E47 cells. E47 cells were preincubated with medium containing AA for 14 h. The medium was removed and the cells were washed with PBS and incubated with serum-free medium in the absence or presence of the following inhibitors for 1 h: PD98059 (10 µM), for MEK/extracellular signal-regulated kinase; SP600125 (1 µM), for c-Jun *N*-terminal kinase; SB203580 (5 µM), for p38/MAPK; H-89 (1 µM), for protein kinase A; wortmannin (0.1 µM), LY294002 (25 µM), or ML-9 (25 µM), for phosphatidylinositol 3-kinase/Akt; or BAY 11-7082 (2.5 µM), to block the activation of nuclear factor-κB. Serum-free medium with or without TPA at a final concentration 50 ng/ml was added to the cells, and after 1 h the toxicity phase was initiated by adding medium containing 15-20 µM (final concentration) ferric ion as a Fe-NTA (1:3) complex (AA+Fe-treated cells) or medium alone (AA-treated cells) as the control. Cell viability was determined after 2-4 h of incubation by the MTT assay and expressed as percentage of the AA-treated cells. Viability of the AA-treated cells was not significantly affected by the presence of the distinct inhibitors, with the exception of SP600125 and ML-9 which produced a slightly toxicity themselves. Cell death produced by AA+Fe ranged from 40% to 75%, depending on the assay conditions. Data are expressed as means ± SEM and are from a representative experiment repeated at least once and conducted in triplicate. $^{\#}p < 0.05$ *versus* the AA+Fe plus inhibitor-treated cells in the absence of TPA.

Fig. 6. Effect of TPA on the AA+Fe-induced increase in lipid peroxidation. E47 cells were preincubated with medium containing AA for 14 h. The medium was

removed and the cells were washed with PBS and incubated with serum-free medium in the absence of any addition (AA-treated cells), or in the presence of 50 ng/ml TPA, or 0.5 μM Ro 31-8425 (1-h preincubation) plus 50 ng/ml TPA for 1 h, before adding medium with or without Fe-NTA. After 3 h of incubation, the cells were harvested by scraping and assayed for the production of malonaldehyde as described under Materials and Methods. Results are expressed as means ± SEM and are from a representative experiment repeated once and conducted in triplicate. *$p < 0.05$ *versus* the corresponding AA-treated cells; $^{\#}p < 0.05$ *versus* the AA+Fe-treated cells in the absence of TPA.

Fig. 7. Effect of TPA on the AA+Fe-induced lowering of GSH levels. E47 cells were preincubated with medium containing AA for 14 h. The medium was discarded and the cells were washed with PBS and incubated with serum-free medium in the absence of any addition (AA-treated cells), or in the presence of 50 ng/ml TPA, 0.5 μM Ro 31-8425, or 0.5 μM Ro 31-8425 (1-h preincubation) plus 50 ng/ml TPA for 1 h, before adding medium with or without Fe-NTA. After 3 h of incubation, the cells were harvested by trypsinization and GSH levels were determined as described under Materials and Methods. Results are expressed as means ± SEM and are from a representative experiment repeated once and conducted in triplicate. *$p < 0.05$ *versus* the corresponding AA-treated cells; $^{\#}p < 0.05$ *versus* the AA+Fe-treated cells in the absence of TPA.

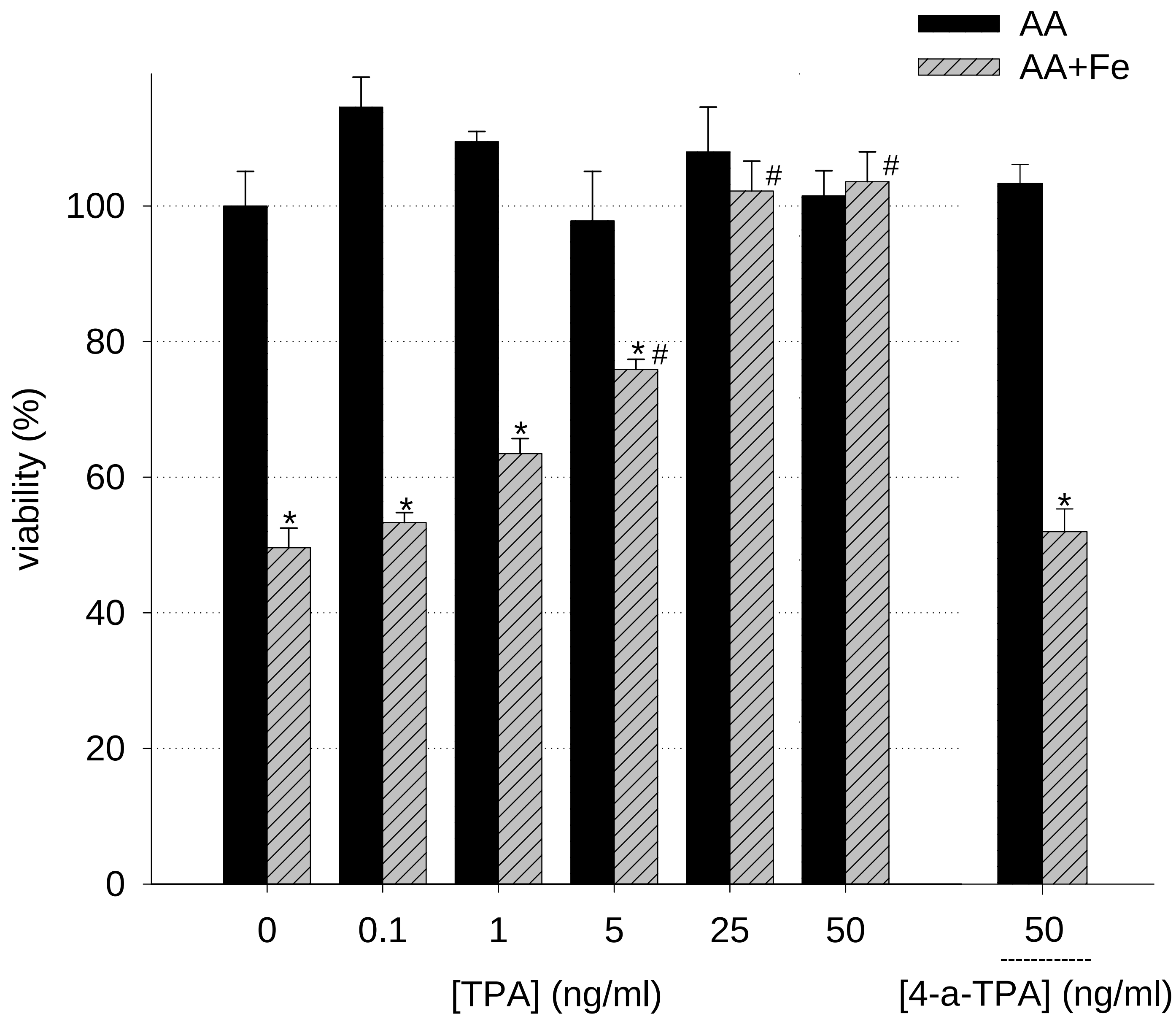

AA
AA+Fe
viability (%)
0
20
40
60
80
100
*
*
*
* #
#
#
*
0
0.1
1
5
25
50
50
[TPA] (ng/ml)
[4-a-TPA] (ng/ml)

Fig.1A

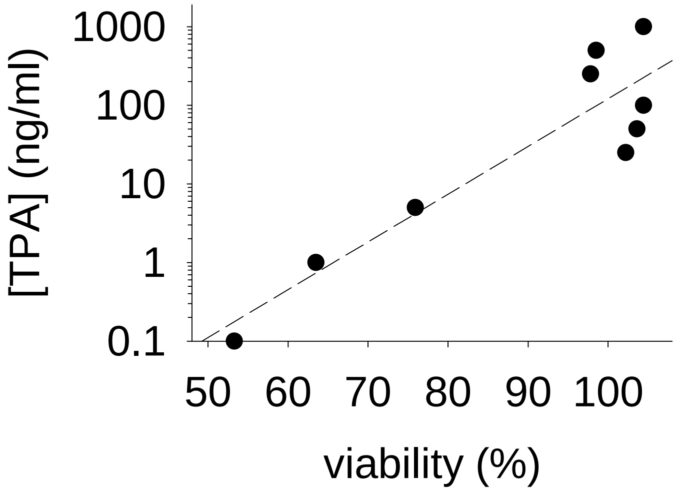


Fig.1B

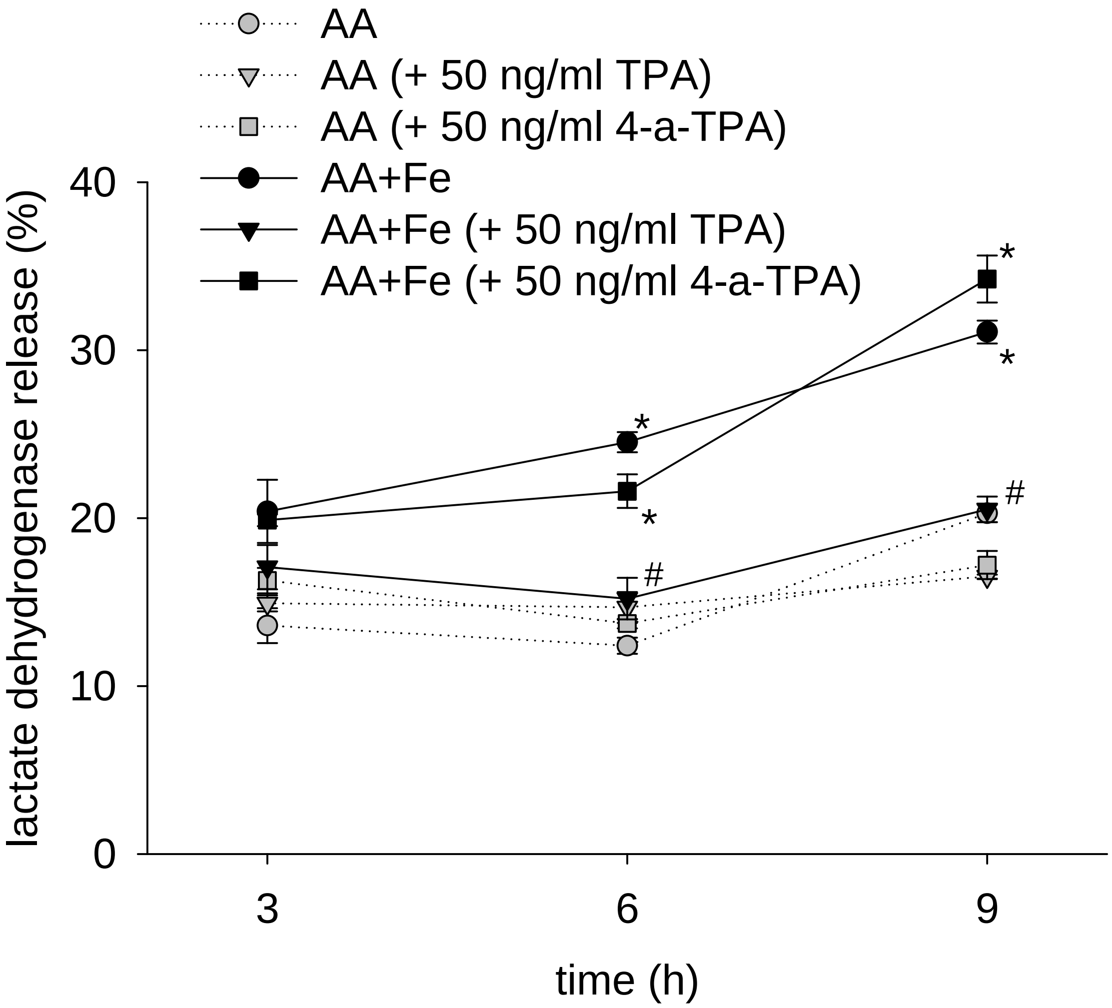

AA
AA (+ 50 ng/ml TPA)
AA (+ 50 ng/ml 4-a-TPA)
AA+Fe
AA+Fe (+ 50 ng/ml TPA)
AA+Fe (+ 50 ng/ml 4-a-TPA)
lactate dehydrogenase release (%)
40
30
20
10
0
3
6
9
time (h)
*
*
*
*
#
#

Fig.1C

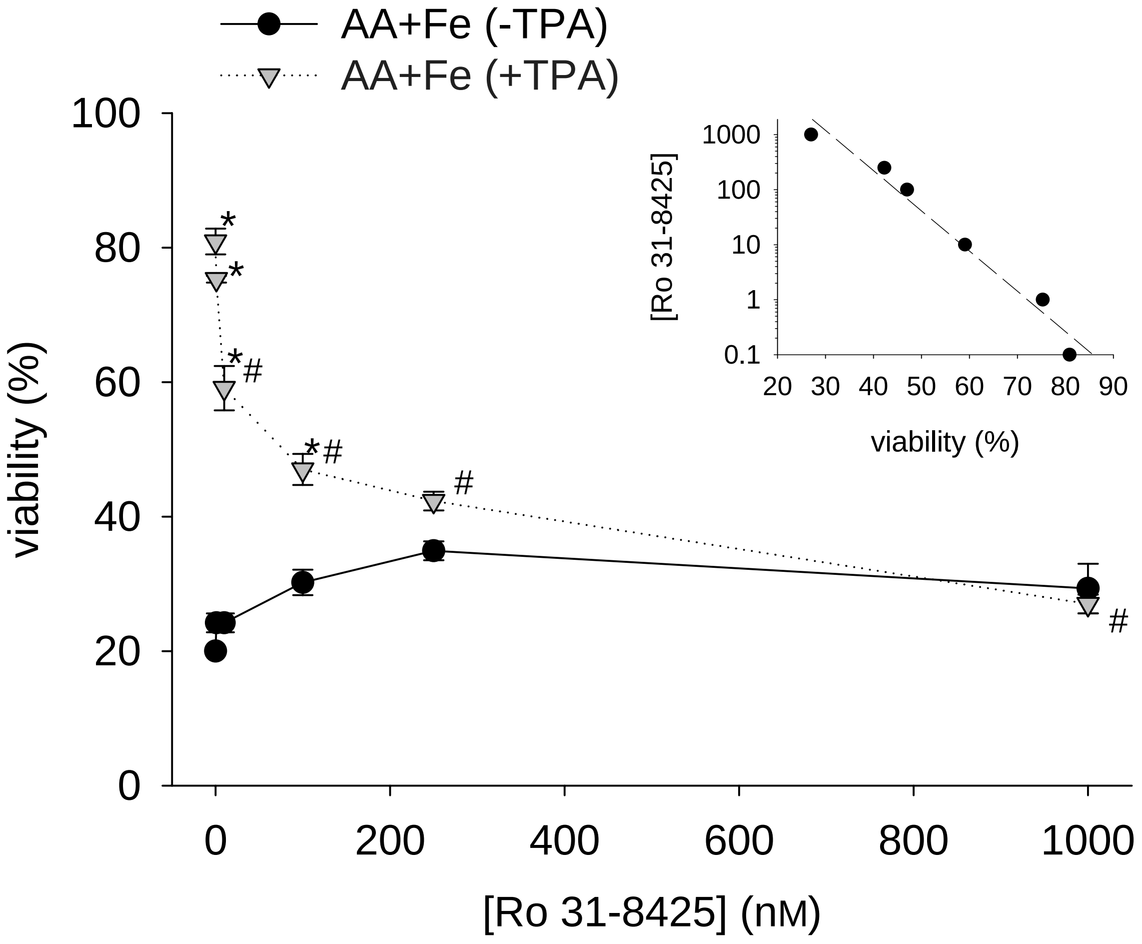

AA+Fe (-TPA)
AA+Fe (+TPA)
100
80
60
40
20
0
viability (%)
0
200
400
600
800
1000
[Ro 31-8425] (nM)
*
*
*#
*#
#
#
1000
100
10
1
0.1
[Ro 31-8425]
20
30
40
50
60
70
80
90
viability (%)

Fig.2A

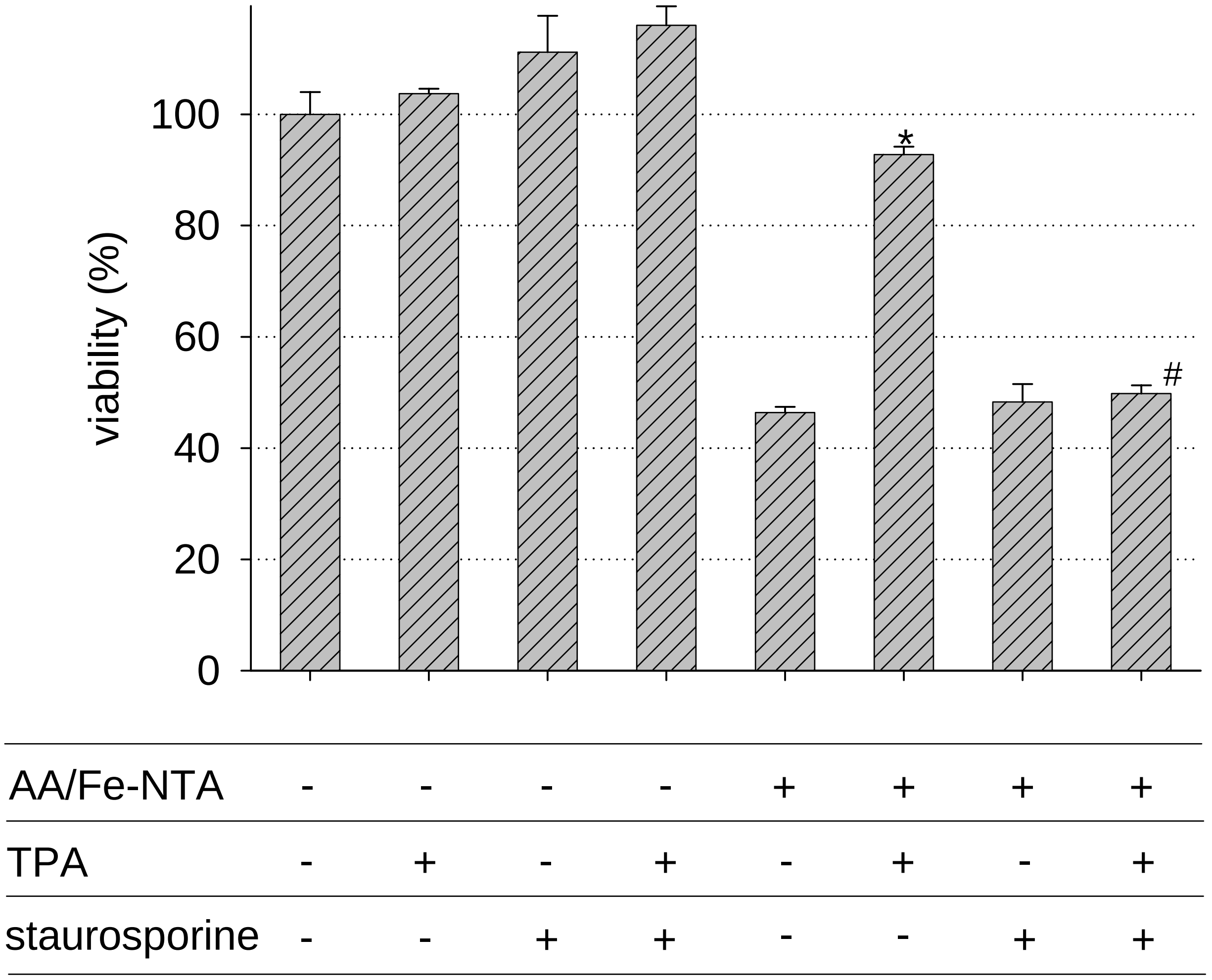


Fig.2B

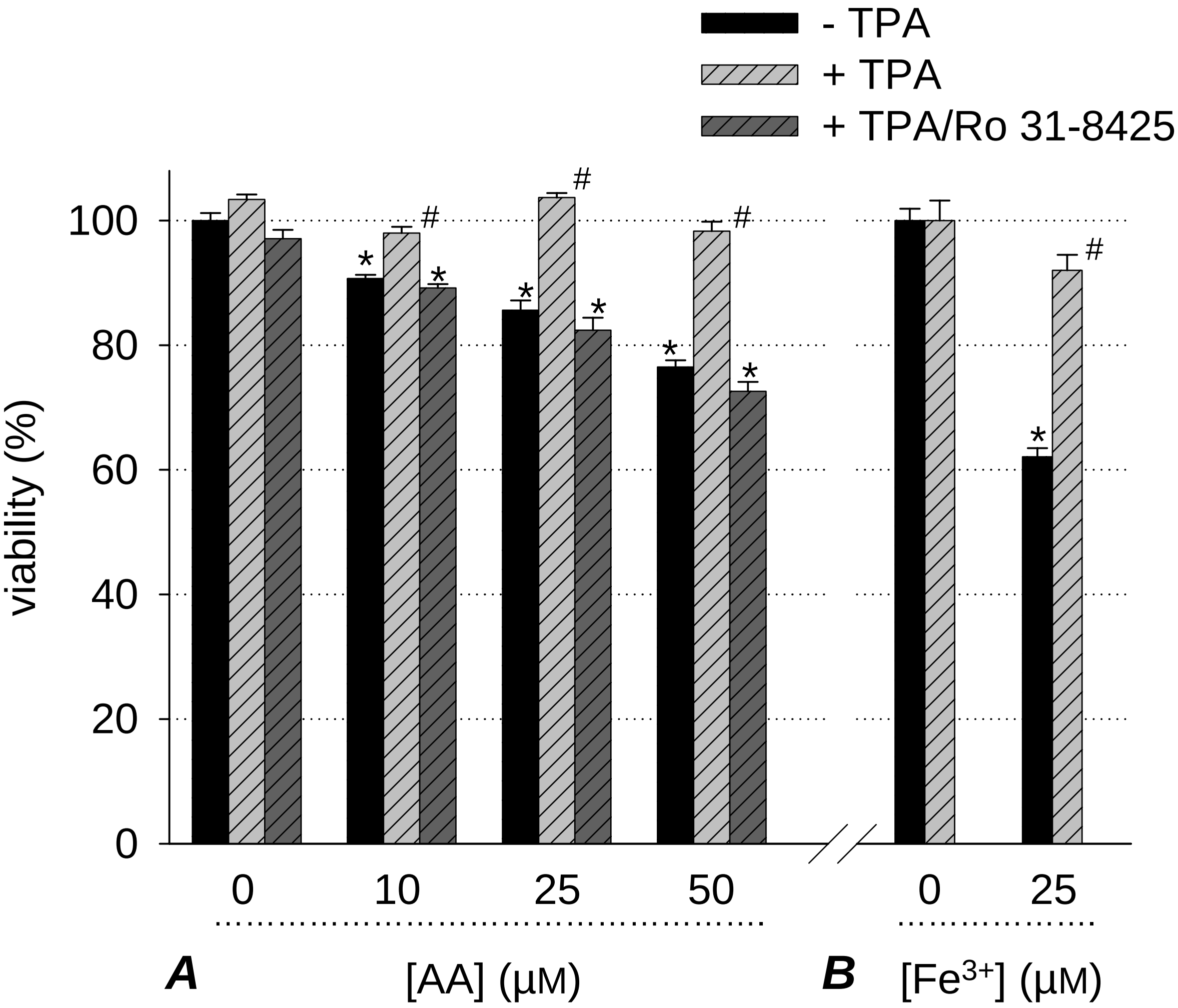

- TPA
+ TPA
+ TPA/Ro 31-8425
viability (%)
0
20
40
60
80
100
0
10
25
50
0
25
A
[AA] (µM)
B
[Fe3+] (µM)
*
#

Fig.3

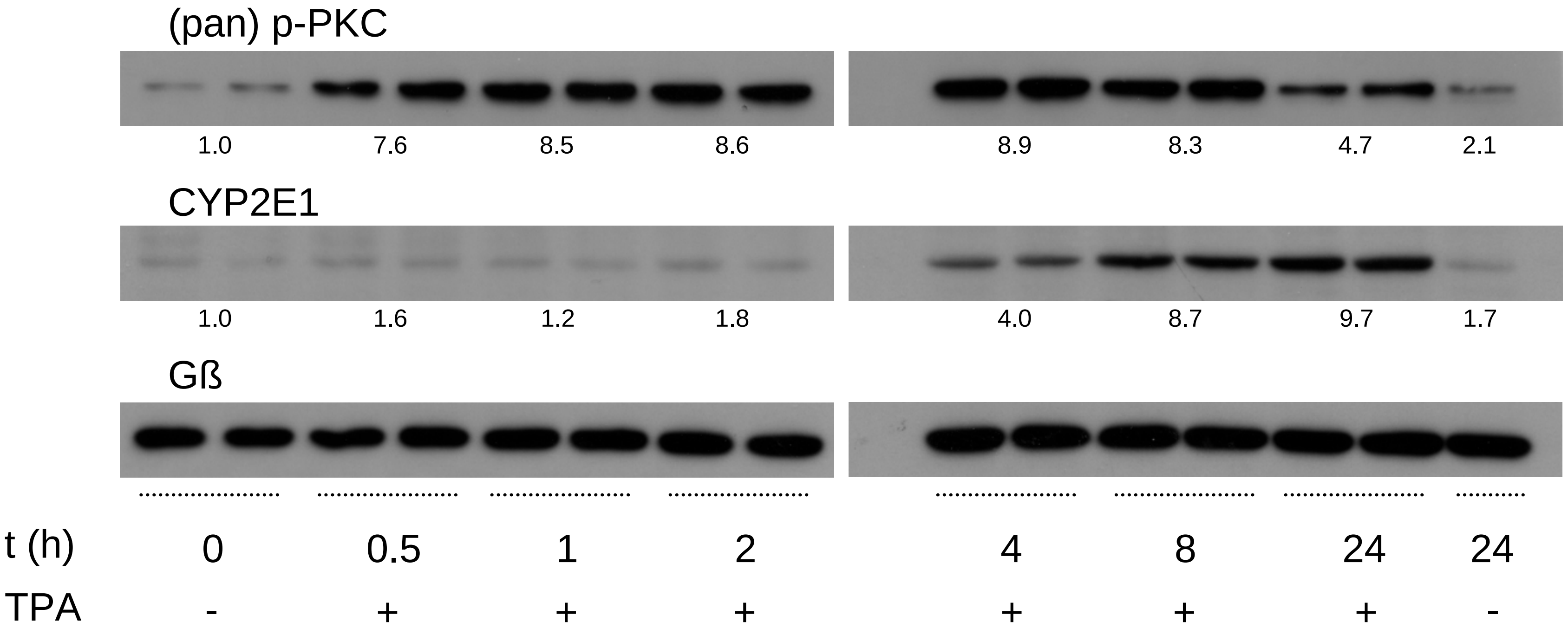

(pan) p-PKC
1.0
7.6
8.5
8.6
8.9
8.3
4.7
2.1
CYP2E1
1.0
1.6
1.2
1.8
4.0
8.7
9.7
1.7
Gß
t (h)
0
0.5
1
2
4
8
24
24
TPA
-
+
+
+
+
+
+
-

Fig.4A

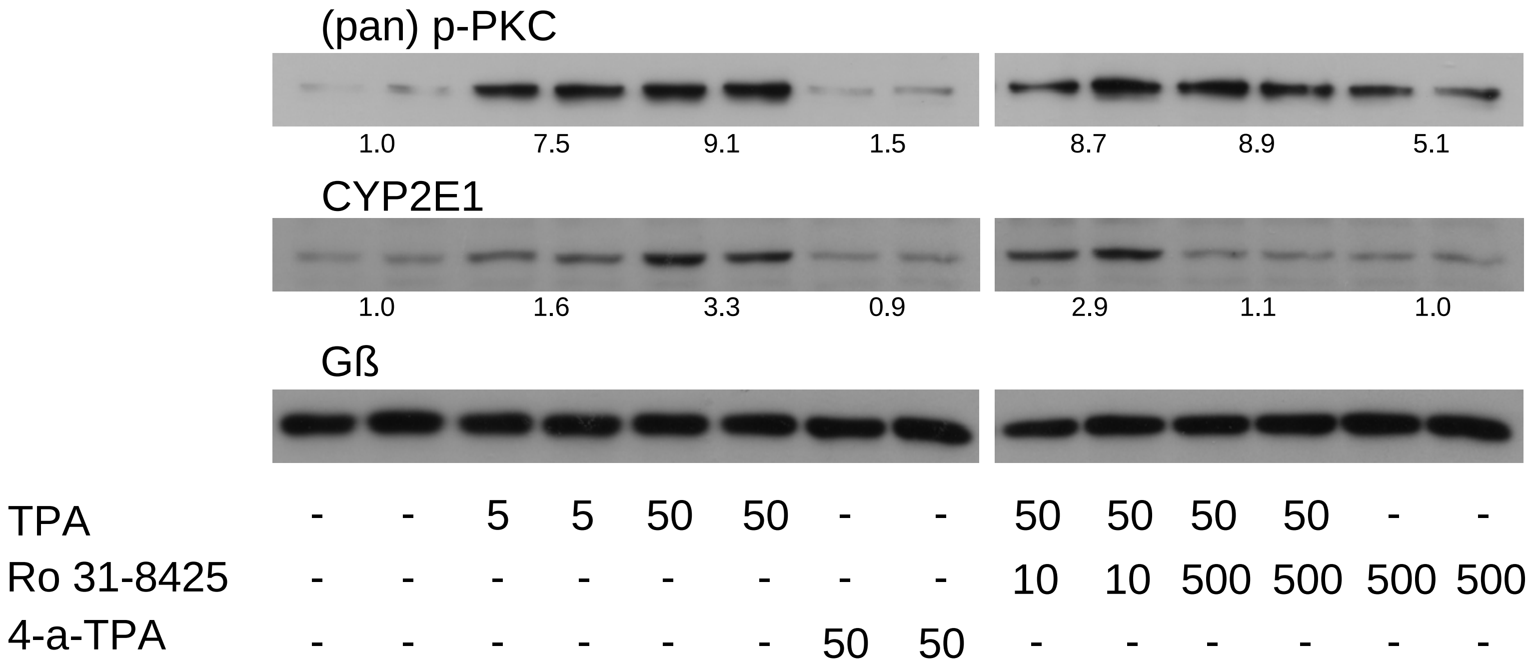

(pan) p-PKC
1.0
7.5
9.1
1.5
8.7
8.9
5.1
CYP2E1
1.0
1.6
3.3
0.9
2.9
1.1
1.0
Gß
TPA - - 5 5 50 50 - - 50 50 50 50 - -
Ro 31-8425 - - - - - - - - 10 10 500 500 500 500
4-a-TPA - - - - - - 50 50 - - - - - -

Fig.4B

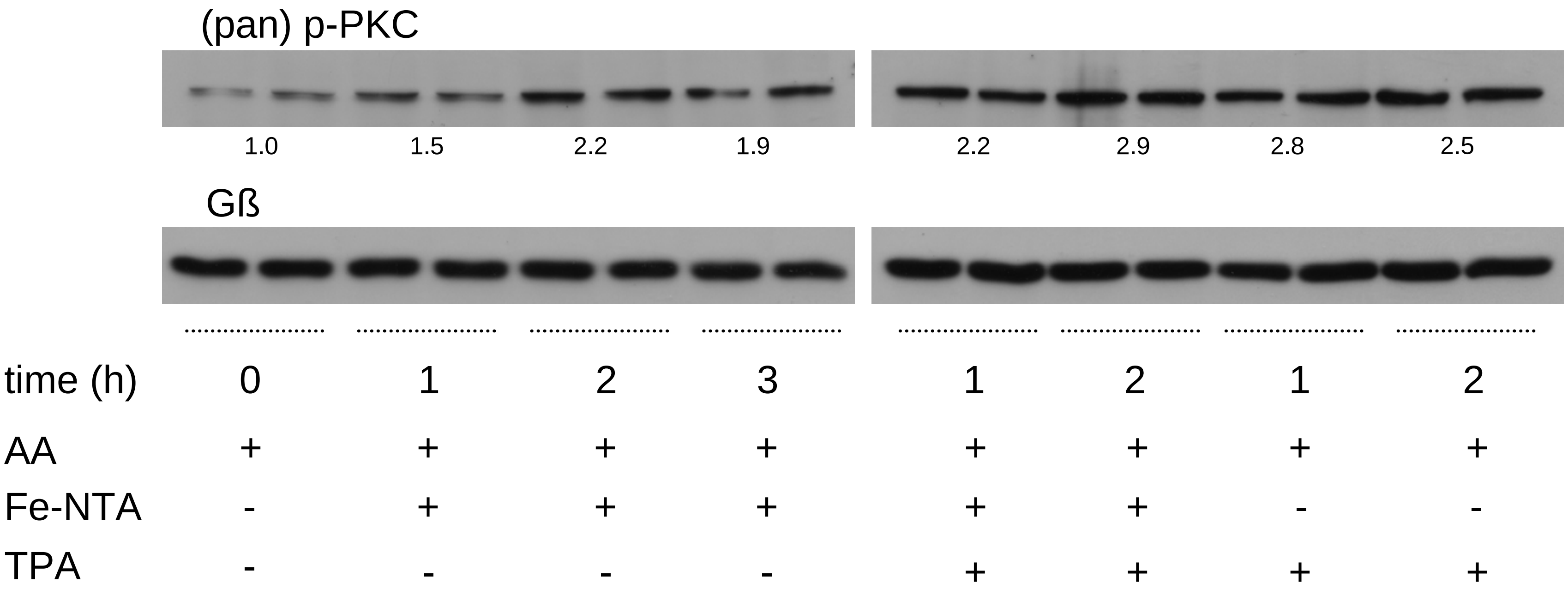


Fig.4C

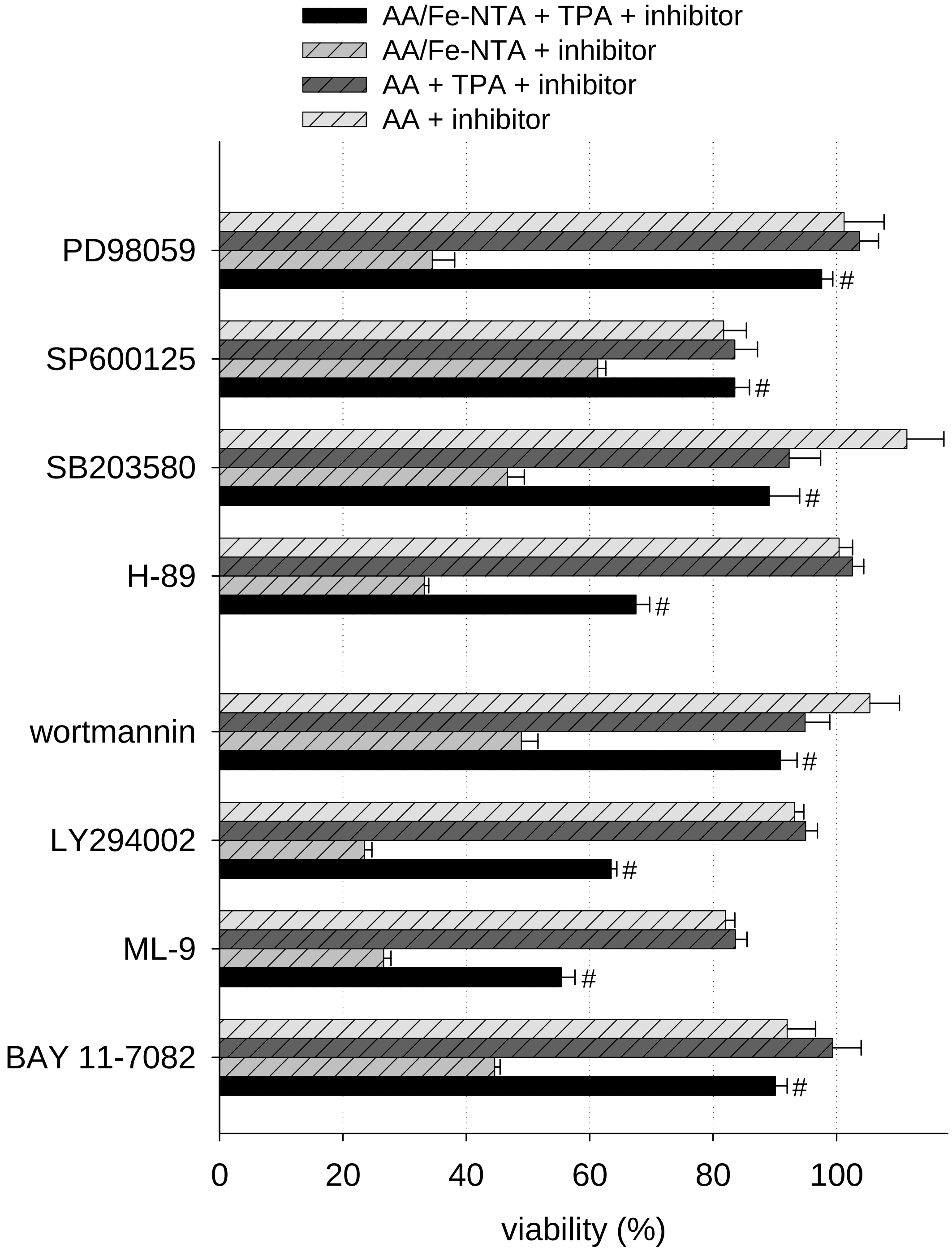
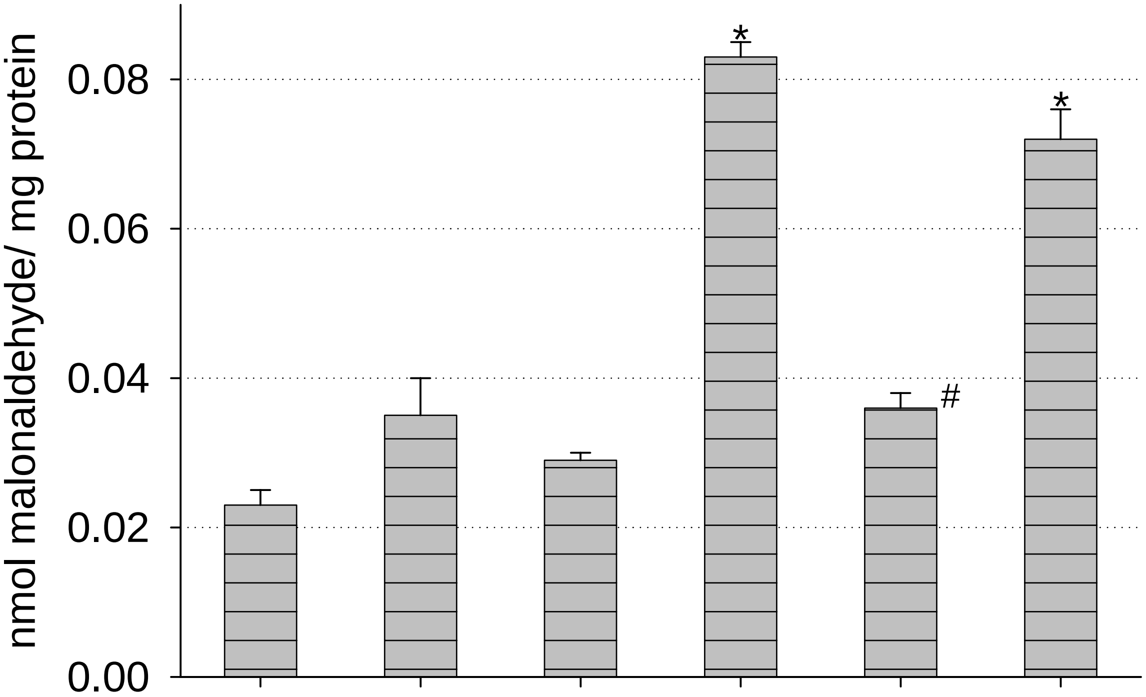
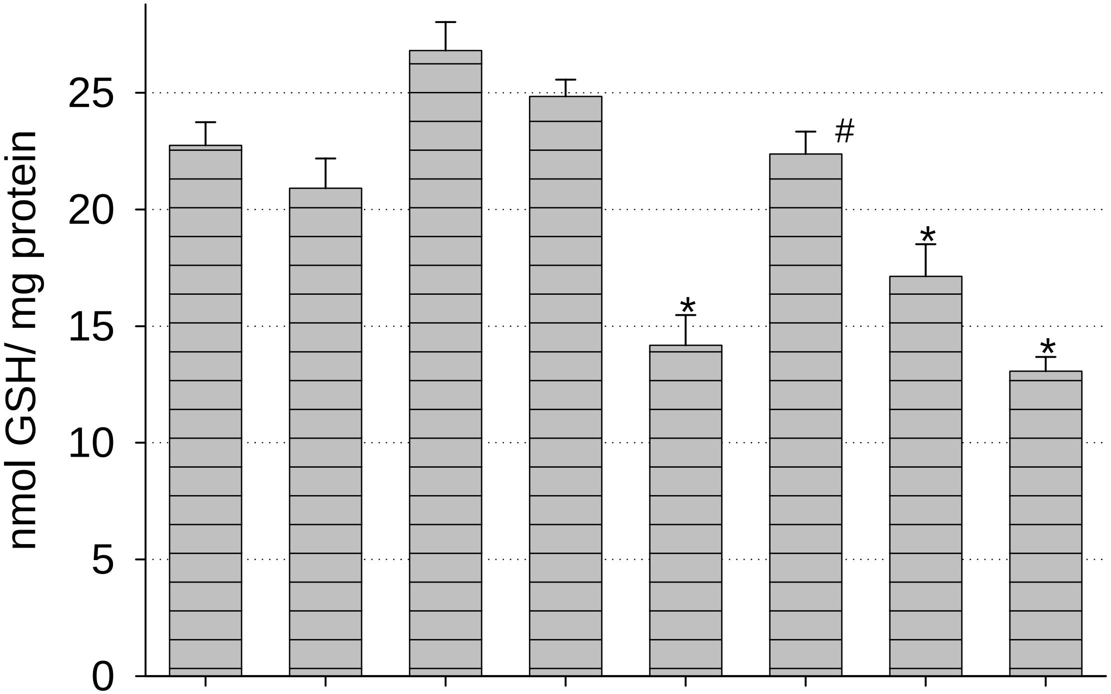

AA/Fe-NTA + TPA + inhibitor
AA/Fe-NTA + inhibitor
AA + TPA + inhibitor
AA + inhibitor
PD98059
SP600125
SB203580
H-89
wortmannin
LY294002
ML-9
BAY 11-7082
#
0
20
40
60
80
100
viability (%)

Fig.5



AA	-	+	+	+	+	+
Fe-NTA	-	-	-	+	+	+
TPA	-	-	+	-	+	+
Ro 31-8425	-	-	-	-	-	+

Fig.6



AA	+	+	+	+	+	+	+	+
Fe-NTA	-	-	-	-	+	+	+	+
TPA	-	+	-	+	-	+	-	+
Ro 31-8425	-	-	+	+	-	-	+	+

Fig.7