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Inhibition of insulin/IGF-1 receptor signaling enhances bile acid toxicity in primary hepatocytes

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Abstract

Modulation of ERBB and insulin-like growth factor 1 (IGF-1) receptor function is recognized as a potential mechanism to inhibit tumor growth. We and others have shown that inhibition of ERBB1 can enhance bile acid toxicity. Herein, we extend our analyses to examine the impact of insulin/IGF-1 receptor inhibition on primary hepatocyte survival when exposed to the secondary bile acid deoxycholic acid (DCA) and compare the impact inhibition of this receptor has on bile acid toxicity effects to that of ERBB1/MEK1/2 inhibition. The insulin/IGF-1 receptor inhibitor NVP-ADW742 at concentrations which inhibit both the insulin and IGF-1 receptors had a modest negative impact on hepatocyte viability, and strongly potentiated DCA-induced apoptotic cell death. Identical data were obtained expressing a dominant negative IGF-1 receptor in hepatocytes; a receptor which acts to inhibit both the IGF-1 receptor and the insulin receptor in trans. Inhibition of ERBB1 function using Iressa (gefitinib) or the tyrphostin AG1478 had more modest effects at enhancing DCA lethality than inhibition of the insulin/IGF-1 receptor function. In contrast, over-expression of a dominant negative ERBB1 protein had pleiotropic effects on multiple signaling pathways in an apparently non-specific manner. These findings suggest that novel therapeutic kinase inhibitors, targeted against growth factor receptors, have the potential to promote bile acid toxicity in hepatocyte when bile flow may be impaired.

Keywords: Bile acid; Kinase; Receptor; Apoptosis; Dominant negative; Insulin

1. Introduction

Bile acids are detergent molecules, synthesized from cholesterol in the liver, that are released into the gut upon feeding and are essential for digestion. Bile acids, post-feeding, re-enter the liver via the portal vein together with digested nutrients and are re-circulated back into the gall bladder for use during the next feeding cycle as part of the enterohepatic circulation [1]. Individually, when retained within the liver because of impaired secretion into the bile canaliculi, bile acids are also known to have hepatocellular-toxicity both in vivo and in vitro [2–4].

Recently, we reported that treatment of primary rodent or human hepatocytes with the secondary bile acid deoxycholic acid (DCA) caused activation of ERBB1 (the epidermal growth factor (EGF) receptor), which was responsible for activation of the extracellular regulated kinase (ERK1/2), and to a lesser extent AKT, pathways [5–7]. In addition to ERBB1, bile acids have also been shown to induce activation of the insulin receptor, *but not* the insulin-like growth factor 1 (IGF-1) receptor, in primary rodent hepatocytes [8]. Activation of the phosphatidyl ino-

Abbreviations: AG, AG1478; ASM, acidic sphingomyelinase; ca, constitutively active; CD533, dominant negative ERBB1; DCA, deoxycholic acid; dn, dominant negative; EGF, epidermal growth factor; ERK, extracellular regulated kinase; FADD, FAS associating death domain protein; IGF, insulin-like growth factor; JNK, c-Jun NH_2-terminal kinase; JNK I, JNK1/2 inhibitory peptide derived from JIP1; LY, LY294002; MAPK, mitogen activated protein kinase; MEK, mitogen activated extracellular regulated kinase; NVP, NVP-ADW742; P, phospho-; PD, PD184352; R, receptor; WT, wild type; −/−, null/gene deleted

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sitol 3 kinase (PI3K)/AKT pathway by DCA was more strongly linked to activation of the insulin receptor than to ERBB1. Reactive oxygen species (ROS) scavengers and inhibitors of mitochondrial ROS generation blocked DCA-induced inhibition of protein tyrosine phosphatases (PTPase) activity as well as activation of ERBB1 and the insulin receptor; several studies by other laboratories have argued that PTPase activity is sensitive to cellular redox status [9,10]. Blockade of DCA-induced ERK1/2 or PI3K activation, with inhibitors of Ras, PI3K or mitogen activated extracellular regulated kinase (MEK1/2), increased hepatocyte apoptosis ~10-fold within 6 h of exposure [6,7]. In these studies, apoptosis was dependent on bile acid-induced, ligand-independent, activation of the FAS death receptor (CD95). Recent studies by our group have demonstrated that FAS receptor and c-Jun NH_2-terminal kinase (JNK1/2) pathway activation was independent of bile acid-induced ROS generation [11]. Other groups have also discovered that bile acids can activate ERBB1, the membrane associated tyrosine kinase Src, and the FAS receptor [12,13]. At present, no studies have yet been performed to compare the ability of bile acid-induced insulin/IGF-1 receptor signaling to modulate apoptotic/survival responses of hepatocytes versus the ability of ERBB1 signaling to block hepatocyte killing.

Growth factor receptors are often highly activated in tumor cells (e.g. [14,15]). Indeed, modulation of ERBB family and/or IGF-1 growth factor receptor function is recognized as a potential mechanism to inhibit tumor growth. Furthermore, inhibition of chemotherapeutic drug and radiation-induced growth factor receptor activation by novel inhibitors of receptor kinase domains enhances the toxicity of chemotherapy/radiation [14–16]. Based on our prior findings in bile acid treated hepatocytes, a significant possibility remains that therapeutic receptor kinase inhibitors may also negatively impact on the viability of non-transformed cells in the liver and the colon, in part due to their location in a bile acid rich/ROS generating environment. For example, we and others have argued that bile acid-induced activation of ERBB1 can play a vital role in the activation of the ERK1/2 pathway and that this pathway protects against cell death [7,12]. Herein, we examine the impact of insulin/IGF-1 receptor inhibition on primary hepatocyte survival when exposed to the secondary bile acid deoxycholic acid.

2. Materials and methods

2.1. Materials

All bile acids and CHAPS were obtained from Sigma Chemical Co. (St. Louis, MO). Phospho-/total-ERK1/2, phospho-/total-JNK1/2, anti-S473 AKT and total AKT antibodies were purchased from Cell Signaling Technologies (Worcester, MA). Phospho-ERBB1 (Y1173) and P-IGF-1/insulin receptor (Y1136-1137/Y1150-1151) were purchased from Cell Signaling Technologies. All the secondary antibodies (anti-rabbit-HRP, anti-mouse-HRP and anti-goat-HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Src family kinase inhibitor (PP2), JNK peptide inhibitor (JNK I) and ERBB1 inhibitor (AG1478) were supplied by Calbiochem (San Diego, CA) as powder, dissolved in sterile DMSO and stored frozen under light-protected conditions at −80 °C. Enhanced chemiluminescence (ECL) kits were purchased from Amersham Enhanced Chemi-Luminescence system (Bucks, England) and NEN Life Science Products (NEN Life Science Products, Boston, MA). Trypsin–EDTA, Williams Medium E, penicillin–streptomycin were purchased from GIBCOBRL Life Technologies (Grand Island, NY). NVP-ADW742 was obtained from Novartis Institutes for BioMedical Research, Novartis Pharma AG, Basel, Switzerland, and stored in DMSO at a stock concentration of 50 mM. Iressa (gefitinib) was obtained from the VCU/MCV Hospital pharmacy and stored in DMSO at a stock concentration of 50 mM. Other reagents were of the highest quality commercially available [5–7,17–19].

2.2. Methods

2.2.1. Primary culture of rodent hepatocytes

Hepatocytes were isolated from adult male Sprague–Dawley rats or C57/BL6 mice by the two-step collagenase perfusion technique. BID null mice were kindly supplied by the late Dr. S. Korsemeyer (Dana Faber Cancer Research Inst., Boston, MA); acidic sphingomyelinase null mice were kindly supplied by Dr. R. Kolesnik (Memorial Sloane Kettering Cancer Center, New York, NY); FAS receptor null mice were purchased from Jackson Laboratories (Bar Harbor, ME). The isolated hepatocytes were plated on rat-tail collagen (Vitrogen)-coated plate at a density of 2×10^5 cells/well and cultured in serum free Williams E medium supplemented with 0.1 μM dexamethasone, 1 μM thyroxine and 100 μg/ml of penicillin/streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2. A medium change was performed 3 h post-plating. Unless otherwise indicated, cells were treated with 100 μM DCA ~24 h after isolation.

2.2.2. Cell treatments, SDS-PAGE and Western blot analysis

Cells were treated with either AG1478 (AG; 10.0 μM), Iressa (10.0 μM), LY294002 (LY; 10 μM), JNK inhibitory peptide derived from JIP1 (JNK I; 10 μM), NVP-ADW742 (NVP; 0.5–10 μM) or PD184352 (PD; 10 μM) 5 min prior to bile acid addition. Cells were then exposed to DCA (100 μM), EGF (10 nM) or insulin (50 nM). One hundred micromolar DCA was chosen for the majority of these studies to observe large statistically significant alterations in hepatocyte biologic behavior. For SDS-PAGE and immunoblotting, hepatocytes were lysed in

either a non-denaturing lysis buffer, and prepared for immunoprecipitation as described in [9–11] or in whole-cell lysis buffer (0.5 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol and 0.02% bromophenol blue), and the samples were boiled for 30 min. After immunoprecipitation, samples were boiled in whole cell lysis buffer. The boiled samples were loaded onto 10–14% SDS-PAGE and electrophoresis was run overnight. Proteins were electrophoretically transferred onto 0.22 μm nitrocellulose, and immunoblotted with various primary antibodies against different proteins. All immunoblots were visualized by ECL. For presentation, immunoblots were digitally scanned at 600 dpi using Adobe PhotoShop 7.0, and their color removed and figures generated in MicroSoft PowerPoint. Densitometric analysis for ECL immunoblots were performed using a Fluorochem 8800 Image System and the respective software (Alpha Innotech Corporation, San Leandro, CA) and band densities were normalized to that of a total protein loading control.

2.2.3. Poly-L-lysine adenoviral vectors and recombinant adenoviral vectors; generation and infection in vitro

Two adenoviral technologies were used. Replication defective adenovirus is conjugated to poly-L-lysine and cDNA plasmid constructs to express a constitutively activated AKT protein (Upstate, Lake Placid, NY), dominant negative IGF-1 receptor, dominant negative caspase 8, dominant negative caspase 9, dominant negative FADD as described in [7,8,10,11]. Second, we generated previously noted recombinant adenoviruses to express dominant negative ERBB1 (COOH-terminal 533 amino acid deletion; CD533) and BCL-XL [7]. Hepatocytes were transfected/infected with these adenoviruses at an approximate m.o.i. of 250 and 30, respectively. Cells were further incubated for 24 h to ensure adequate expression of transduced gene products.

2.2.4. Morphological detection of apoptosis by H-33342 assay

Morphological assessment of apoptosis was performed as follows [7]. Hepatocytes were harvested by trypsinization with trypsin/EDTA for ∼10 min at 37 °C. As some apoptotic cells detached from the culture substratum into the medium, these cells were also collected by centrifugation of the medium at 1500 rpm for 5 min. The pooled cell pellets were resuspended and a fraction of the suspension was centrifuged in a cytospinner (Cytospin 3, Shandon Inc., Pittsburgh, PA). The slides were immediately fixed. The slides were then washed. The fixed cells were stained in Hoechst 33342 washes in PBS to remove excessive dye, air-dried, and mounted in FluroGard™ Antifade. Nuclear morphology was evaluated. Apoptotic cells were identified as those whose nuclei exhibited brightly staining condensed chromatin or nuclear fragmentation or apoptotic bodies. Five hundred cells from several randomly chosen fields were counted and the number of apoptotic cells was counted and expressed as a percentage of the total number of cells counted.

2.2.5. Determination of apoptosis by TUNEL and Wright–Giemsa staining

After hepatocytes were treated with various regimes, cells were collected by trypsinization followed by cytospin onto glass slides, as described above. For Wright–Giemsa, the slides were fixed and stained in Diff-Quik® Stain Set (Dade Diagnostics of P.P. Inc., Aguada, PR, USA), according to the manufacturer's instruction, and viewed under a light microscope. For TUNEL, cells were fixed in methanol:glacial acetic acid (3:1) for 30 min at 4 °C and TUNEL assay was performed on these cells according to the manufacturer's instructions. The slides were viewed under a fluorescence microscope [7].

2.2.6. Data analysis

Comparison of the effects of various treatments was performed using one-way analysis of variance and a two-tailed *t*-test. Differences with a *p*-value of <0.05 were considered statistically significant. Experiments shown are the means of multiple individual points (±S.E.M.).

3. Results

Initial studies examined the impact of expressing either a dominant negative IGF-1 receptor (dnIGF1R) or a dominant negative ERBB1 receptor (dnERBB1) on primary rat hepatocyte survival after DCA exposure, and on DCA-induced activation of ERK1/2, AKT and JNK1/2. In cancer cells dnERBB1 has been shown to inhibit multiple ERBB family receptors and dnIGF1R has been shown to inhibit signaling by both the IGF-1 receptor and the insulin receptor in trans. Expression of dnERBB1 or of dnIGF1R enhanced cell killing following DCA exposure (Fig. 1A). Treatment of hepatocytes expressing dnERBB1 or dnIGF1R with the small molecule inhibitors of these receptors, Iressa (gefitinib) and NVP-ADW742, respectively, did not modify the apoptotic response of hepatocytes above that caused by expression of the dominant negative receptor protein (Fig. 1A).

Expression of dnERBB1 in primary rat hepatocytes blunted basal ERK1/2 and AKT activity and blocked DCA-induced activation of these protein kinases (Fig. 1B; Table 1). Expression of dnERBB1 also blocked activation of JNK1/2 in primary rat hepatocytes. In contrast to findings with dnERBB1, expression of dnIGF1R enhanced basal ERK1/2, AKT and to a lesser extent JNK1/2 activity by 4.3 ± 1.4-, 2.7 ± 0.6- and 1.9 ± 0.3-fold, respectively ($n = 4 \pm$ S.E.M.). Expression of dnIGF1R abolished DCA-induced activation of AKT but not of ERK1/2, and promoted bile acid-induced activation

Table 1
Overview of the inhibitory actions of dominant negative ERBB1, Iressa and dominant negative IGF-1 receptor on the activation of ERK1/2, JNK1/2 and AKT by DCA

	P-ERK1/2	P-AKT	P-JNK1/2
dnERBBl	− − −	− − −	− − −
Iressa (gefitinib) (10 μM)	− − −	− −	Null effect
ERBB1 null (−/−) fibroblast	− − −	− −	Null effect
dnIGFIR	−	− − −	+ + +
NVP-ADW742 (NVP; 10 μM)	− −	− − −	+ + +
NVP-ADW742 (NVP; 0.5 μM)	Null effect	Null effect	+
FAS receptor null (−/−) cell	Null effect	Null effect	− − −
ASM null (−/−) cell	Null effect	Null effect	− −

The table shows whether the small molecule inhibitor, loss of protein expression due to genomic deletion or expression of dominant negative receptor tyrosine kinase promoted activation of the intracellular signaling kinase (+, + +, + + +), had no effect on kinase activation (“null”), or inhibited intracellular kinase activation (−, − −, − − −).

of JNK1/2; JNK1/2 activation in CMV infected cells was enhanced 4.8 ± 1.2-fold and in dnIGF1R infected cells by 6.9 ± 1.4-fold* ($n = 4 \pm$ S.E.M.; $^{*}p < 0.05$ greater than CMV infected cells) 20 min after DCA exposure (Fig. 1B; Table 1).

Previous studies by this group have shown that DCA-induced activation of JNK1/2, but not the activation of ERK1/2 or AKT, is due to ligand-independent, acidic sphingomyelinase (ASM)/ceramide-dependent, stimulation of FAS receptor signaling (see refs. [11,22]). The insulin/IGF-1 receptor inhibitor NVP-ADW742 enhanced the activation of JNK1/2 in DCA-treated rat hepatocytes and in wild type mouse hepatocytes, but not in DCA-treated FAS receptor −/− cells; JNK1/2 activation in rat hepatocytes was enhanced 3.6 ± 1.1-fold in the absence of, and 7.6 ± 1.5-fold* in the presence of, NVP-ADW742 ($n = 4 \pm$ S.E.M.; $^{*}p < 0.05$ greater than cells without NVP-ADW742) 20 min after DCA exposure (Fig. 1C). Thus, DCA-induced JNK1/2 phosphorylation and the facilitation of DCA-induced JNK1/2 phosphorylation by inhibition of insulin/IGF-1 receptor signaling is FAS receptor-dependent.

To confirm our apoptosis findings with respect to signaling by ERBB1 we made use of mouse embryonic fibroblasts lacking expression of ERBB1 but that retain expression of insulin and IGF-1 receptors. DCA is a hydrophobic bile acid that can enter cells through the plasma membrane regardless of bile acid transporter expression. Exposure of wild type fibroblasts to DCA, the MEK1/2 inhibitor PD184352, Iressa or NVP-ADW742 had little impact on cell viability (Fig. 1D). However DCA lethality was enhanced when fibroblasts were co-incubated with PD184352, Iressa or NVP-ADW742. In ERBB1−/− fibroblasts, basal levels of viability were decreased, the toxicity of DCA as a single agent significantly enhanced, and the ability of Iressa to promote DCA lethality abolished (Fig. 1E). In ERBB1−/− cells, basal levels of P-ERK1/2 and P-AKT were lower than in wild type cells, and DCA-induced stimulation of ERK1/2 and AKT phosphorylation was also reduced below those levels induced in wild type cells (Fig. 1D, inset). Thus, DCA-induced activation of ERBB1 is a protective signal versus the toxic effects of DCA exposure in multiple established and primary cell types.

We next performed studies to examine the impact on hepatocyte signaling and survival using NVP-ADW742 [20,21]. This compound has been argued to be a relatively specific inhibitor of the IGF-1 receptor at lower concentrations (∼0.5–1.0 μM) and an inhibitor of the insulin receptor at higher concentrations (∼5–10 μM) [20,21]. At low concentrations, NVP-ADW742 (0.5 μM) did not alter DCA-induced activation of ERK1/2 and AKT (Fig. 2A; cf. data in Fig. 1B using a dnIGF1R). However, higher concentrations of NVP-ADW742 (10.0 μM) abolished DCA-stimulated AKT activity, and blunted DCA-stimulated ERK2 phosphorylation by 77 ± 11% ($n = 4 \pm$ S.E.M.). As previously noted in Fig. 1C, higher concentrations of NVP-ADW742 enhanced DCA-induced JNK1/2 activation (Fig. 2A).

Low concentrations of NVP-ADW742 (0.5 μM) did not alter hepatocyte viability and very weakly enhanced bile acid toxicity (data not shown). High concentrations of NVP-ADW742 (10.0 μM) promoted a more rapid and potent induction of hepatocyte cell death after DCA exposure than inhibition of either ERBB1 (AG1478 and Iressa (gefitinib)), PI3K (LY294002) or MEK1/2 (PD184352) (Fig. 2B). Expression of constitutively activated AKT, but not constitutively activated MEK1, significantly reduced the toxic effects of DCA and NVP-ADW742 treatment in hepatocytes (Fig. 2C, data not shown). Collectively, the studies in Fig. 2 demonstrate that high concentrations of NVP-ADW742 promote JNK1/2 activation and AKT inactivation after bile acid exposure, and that AKT inactivation accounts, at least in part, for the promotion of bile acid lethality by this drug.

Several laboratories have demonstrated that bile acid toxicity in primary hepatocytes is dependent on ligand-independent activation of the FAS receptor followed by: association of FADD and pro-caspase 8 with the FAS receptor; cleavage/activation of pro-caspase 8; cleavage of BID; induction of mitochondrial dysfunction and release of cytochrome *c* into the cytosol; cleavage/activation of

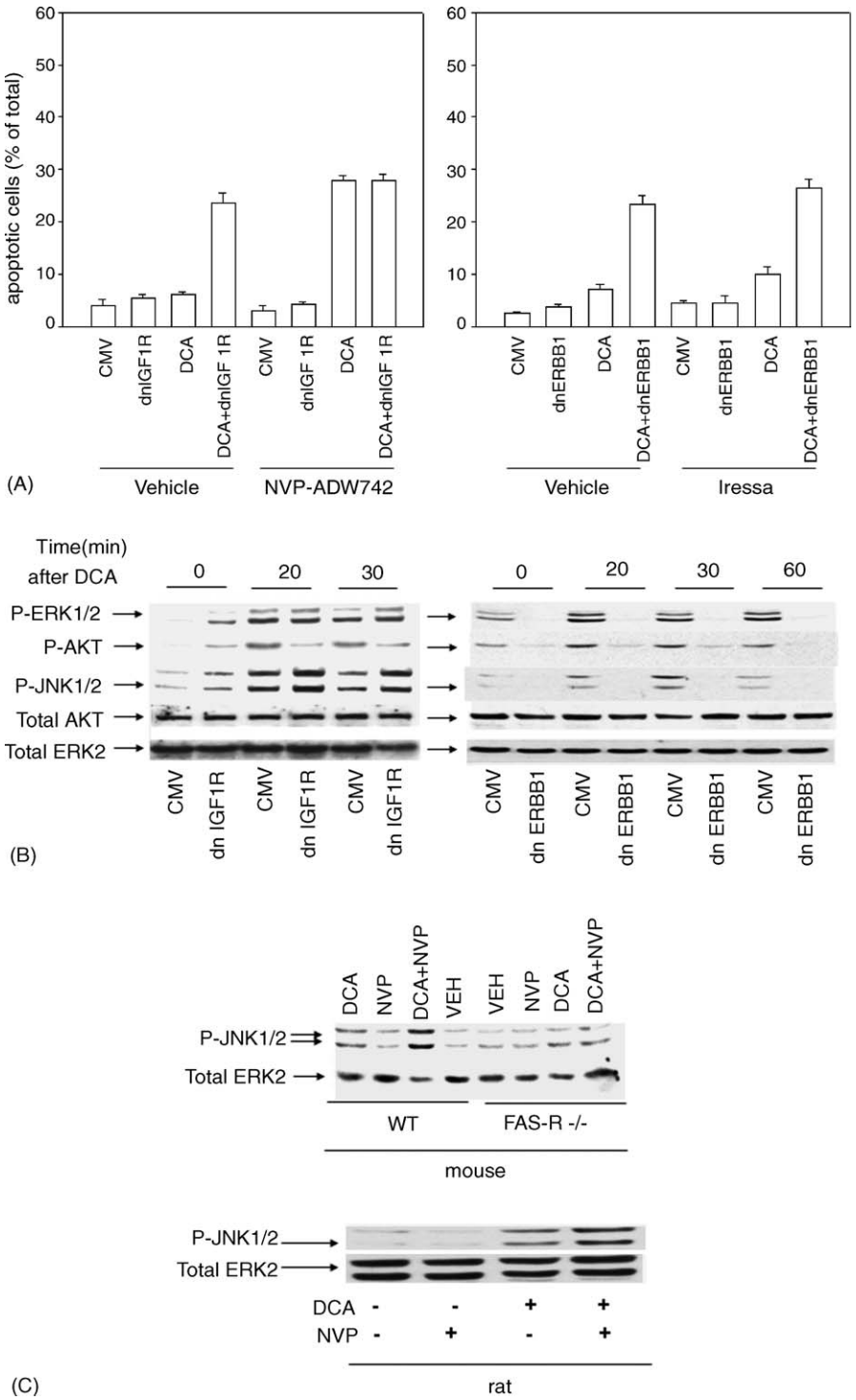

Fig. 1. Inhibition of insulin receptor signaling by NVP-ADW742 or a dominant negative IGF-1 receptor blocks DCA-induced activation of AKT and promotes activation of JNK1/2. (Panel A) Hepatocytes were plated and infected to express: (left section) dominant negative IGF-1 receptor (dnIGF1R) and (right section) dominant negative ERBB1 (dnERBB1), or with control (CMV) virus, as described in Section 2. Hepatocytes were treated 24 h after plating with DCA (100 μM) in the presence or absence of vehicle (VEH; DMSO), NVP-ADW742 (NVP; 10.0 μM) or Iressa (10.0 μM). Cells were isolated 4 h after DCA treatment fixed onto slides, stained with Hoescht dye and counted blind on a fluorescent microscope to determine apoptosis. Data are the means of three independent values per experiment from two independent experiments ± S.E.M. (Panel B) Hepatocytes were plated and infected to express: (left section) dominant negative IGF-1 receptor (dnIGF1R) and (right section) dominant negative ERBB1 (dnERBB1), or with control (CMV) virus, as described in Section 2. Hepatocytes were treated 24 h after plating with DCA (100 μM). Cells were isolated at the indicated time points after bile acid treatment and prepared for SDS-PAGE and immunoblotting performed to determine the phosphorylation of JNK1/2, ERK1/2 and AKT (S473). Data are from a representative experiment ($n = 4$). (Panel C) Wild type (WT) and FAS receptor −/− mouse hepatocytes, and in parallel rat hepatocytes, were treated 24 h after plating with DMSO vehicle or NVP-ADW742 (10.0 μM) 30 min prior to treatment with DCA (100 μM). Cells were isolated 20 min after treatment, and processed to determine the phosphorylation (activity) of JNK1/2. Data for rats and mice are each representative of four separate experiments. (Panels D and E) Mouse embryonic fibroblasts (MEFs) were cultured as

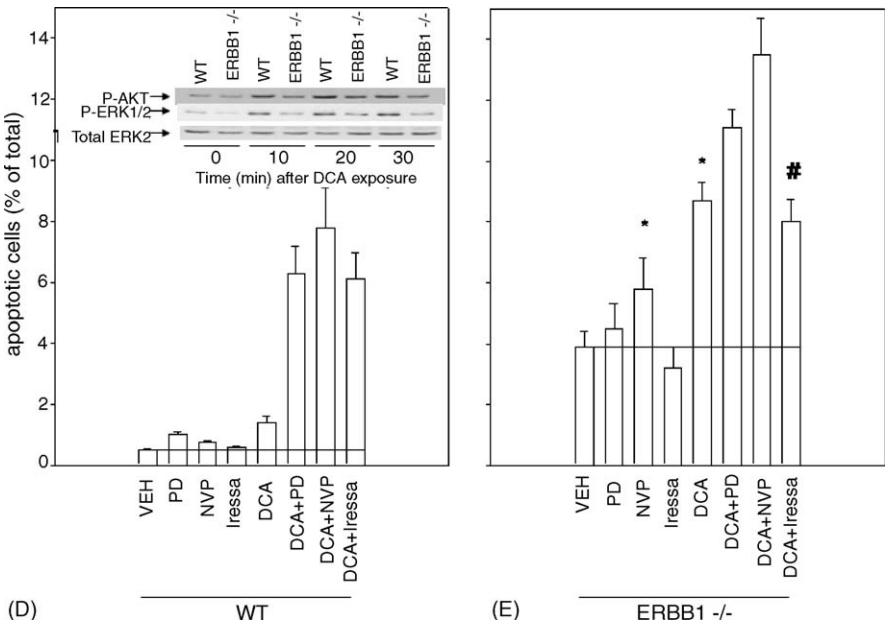

Fig. 1. (*Continued*).

pro-caspase 9; cleavage/activation of pro-caspase 3; the manifestation of nuclear apoptotic morphology [2,6,7,23,24]. Prior findings from this laboratory using DCA and either MEK1/2 or PI3K inhibitors have shown that the potentiation of bile acid toxicity by both kinase inhibitors was FAS receptor-dependent (e.g. [6,7]). Higher concentrations of NVP-ADW742 promoted bile acid toxicity in wild type and FAS receptor null (−/−) cells, albeit that cell killing was induced to a lesser extent in FAS receptor −/− cells than in wild type cells (Fig. 3A). In simultaneous parallel experiments, the potentiation of bile acid lethality by MEK1/2 inhibition or by ERBB1 inhibition was abolished in FAS receptor −/− cells. These data suggest that inhibition of DCA-induced insulin/IGF-1 receptor signaling promotes both FAS receptor-dependent and FAS receptor-independent bile acid toxicity.

As our data using FAS receptor −/− cells suggested that mechanisms in addition to FAS receptor signaling were also responsible for the induction of apoptosis induced by DCA and NVP-ADW742, we performed more detailed analyses to precisely determine how apoptosis was initiated. In agreement with data in other manuscripts [6,7,22], the promotion of DCA toxicity by NVP-ADW742 was blocked by the pan-caspase inhibitor (zVAD), by an inhibitor of caspase 9 (LEHD) and by an inhibitor of caspase 8 (IETD) (data not shown). Similar data to that using LEHD and IETD were obtained when dominant negative caspase 9, dominant negative caspase 8 or BCL-XL were expressed in hepatocytes (Fig. 3B). In general agreement with our findings in FAS receptor −/− cells, expression of dominant negative FADD inhibited the apoptotic response after DCA and NVP-ADW742 treatment by only ∼50% (Fig. 3B). In a similar manner to NVP-ADW742, expression of dnIGF1R enhanced DCA lethality, which was also abolished by LEHD, IETD or expression of BCL-XL and suppressed by ∼50% in the presence of dominant negative FADD (data not shown).

Use of hepatocytes lacking expression of the pro-apoptotic protein BID, a caspase 8 substrate required for causing mitochondrial dysfunction downstream of FAS receptor signaling, abolished DCA lethality and the potentiation of DCA lethality by inhibition of ERBB1 or MEK1/2 (Fig. 3C). However, in concordance with our findings using FAS receptor −/− hepatocytes and hepatocytes expressing dominant negative FADD, loss of BID function only partially reduced the apoptotic response of cells treated with DCA and NVP-ADW742 (Fig. 3C). Collectively, our findings in Fig. 3 argue that DCA and NVP-ADW742 promoted cell death via a caspase 8-dependent mechanism, but which is only partially reliant on the pro-apoptotic function of the FAS receptor, FADD or the

described in Section 2. In Panel D, wild type MEFs and in Panel E, ERBB1−/− MEFs were treated 24 h after plating with DMSO vehicle, NVP-ADW742 (10.0 μM), PD184352 (PD; 10.0 μM) or Iressa (10.0 μM) as indicated, 30 min prior to treatment with DCA (100 μM). Cells were isolated 6 h after treatment, fixed onto slides, stained with Hoescht dye and counted blind on a fluorescent microscope to determine apoptosis. Data are the means of three independent values per experiment from two independent experiments ± S.E.M. $^{\#}p > 0.05$ compared to DCA alone and $^{*}p < 0.05$ compared to control treatment. Inset in Panel D: basal levels and DCA-induced activation of ERK1/2 and AKT are lower in ERBB1−/− fibroblasts. MEFs were cultured as described in Section 2. MEFs were treated 24 h after plating with DCA (100 μM). Cells were isolated 0–30 min after DCA exposure. Data are representative of two separate experiments.

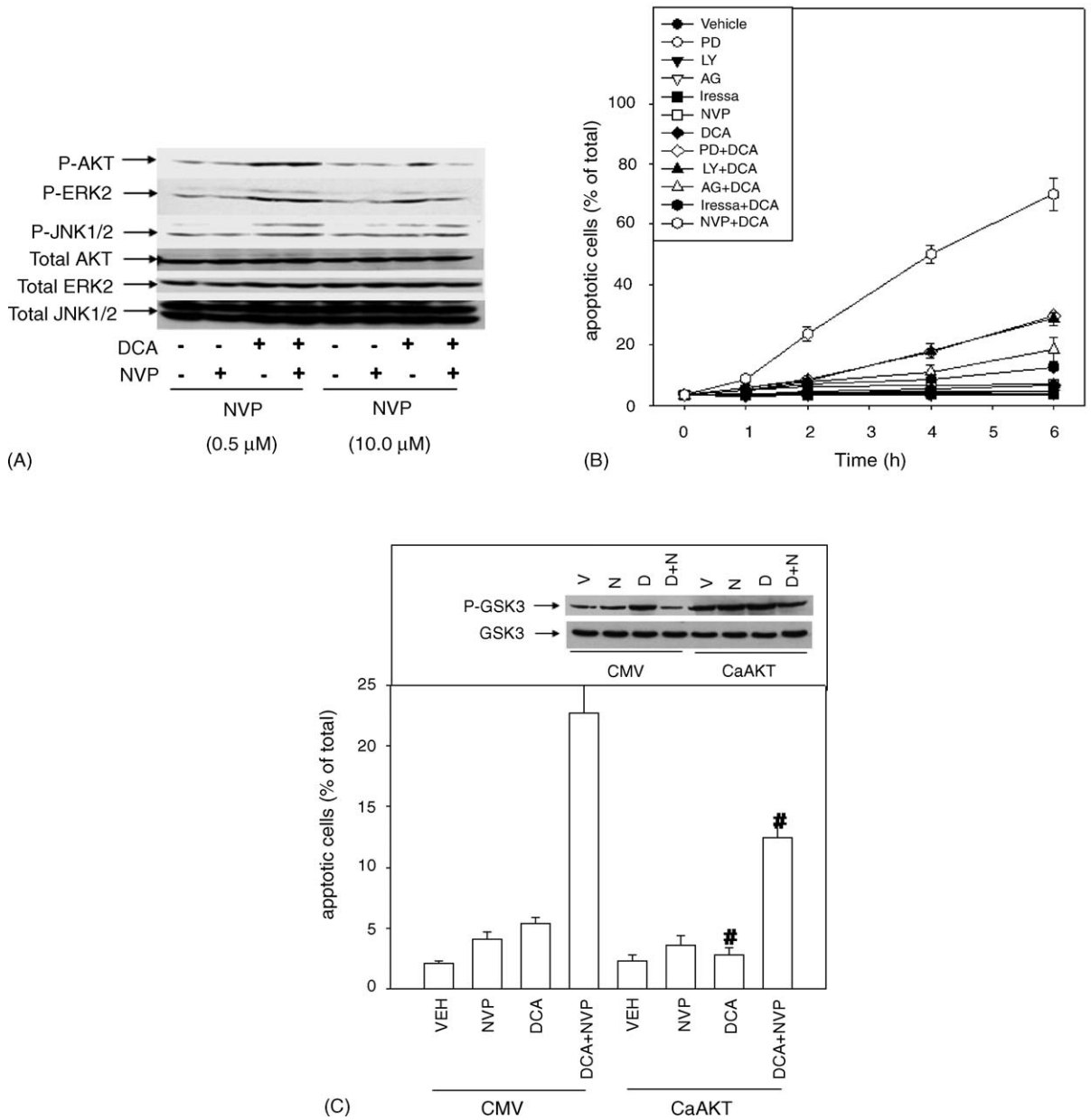

Fig. 2. NVP-ADW742 at concentrations, which inhibit the insulin receptor promotes DCA lethality in primary hepatocytes. (Panel A) Hepatocytes were treated 24 h after plating with DMSO vehicle control or with NVP-ADW742 (NVP; 0.5 and 10 μM) as indicated, 30 min prior to treatment with DCA (100 μM). Cells were isolated at the indicated time points after bile acid treatment and prepared for SDS-PAGE and immunoblotting performed to determine the phosphorylation of JNK1/2, ERK1/2 and AKT (S473). Data are from a representative experiment ($n = 4$). (Panel B) Hepatocytes were treated 24 h after plating with DMSO (VEH), NVP-ADW742 (10.0 μM), PD184352 (PD; 10 μM), LY294002 (LY; 10 μM), AG1478 (AG; 10 μM) and Iressa (gefitinib) (10 μM), 30 min prior to treatment with DCA (100 μM). Cells were isolated 0–6 h after treatment, fixed onto slides, stained with Hoescht dye and counted blind on a fluorescent microscope to determine apoptosis. Data are the means of three independent values per experiment from two independent experiments ± S.E.M. (Panel C) Hepatocytes were plated and infected to express constitutively active (ca) AKT (caAKT; purchased from Upstate Biotechnology, Lake Placid, NY) as described in Section 2. Hepatocytes were treated 24 h after plating with DMSO vehicle, NVP-ADW742 (10.0 μM) and DCA (100 μM). Cells were isolated at the indicated time points after DCA treatment fixed onto slides, stained with Hoescht dye and counted blind on a fluorescent microscope to determine apoptosis. Data are the means of three independent values per experiment from two independent experiments ± S.E.M. $^{\#}p < 0.05$ less than corresponding values in control infected cells. Inset: phosphorylation of glycogen synthase kinase 3 (GSK3) at position S9 in cells isolated 6 h after bile acid treatment—N: NVP-ADW742; V: DMSO vehicle; D: DCA.

actions of the pro-apoptotic caspase 8 substrate protein BID.

In previous studies, we have linked bile acid-induced activation of acidic sphingomyelinase (ASM) and subsequent ceramide generation to the clustering and activation of the FAS receptor, and downstream the JNK1/2 pathway [7,11,22]. ASM null (−/−) hepatocytes exhibited lower levels of apoptosis when treated with either DCA or with DCA and NVP-ADW742, concordant with data in prior publications showing that in ASM −/− hepatocytes bile

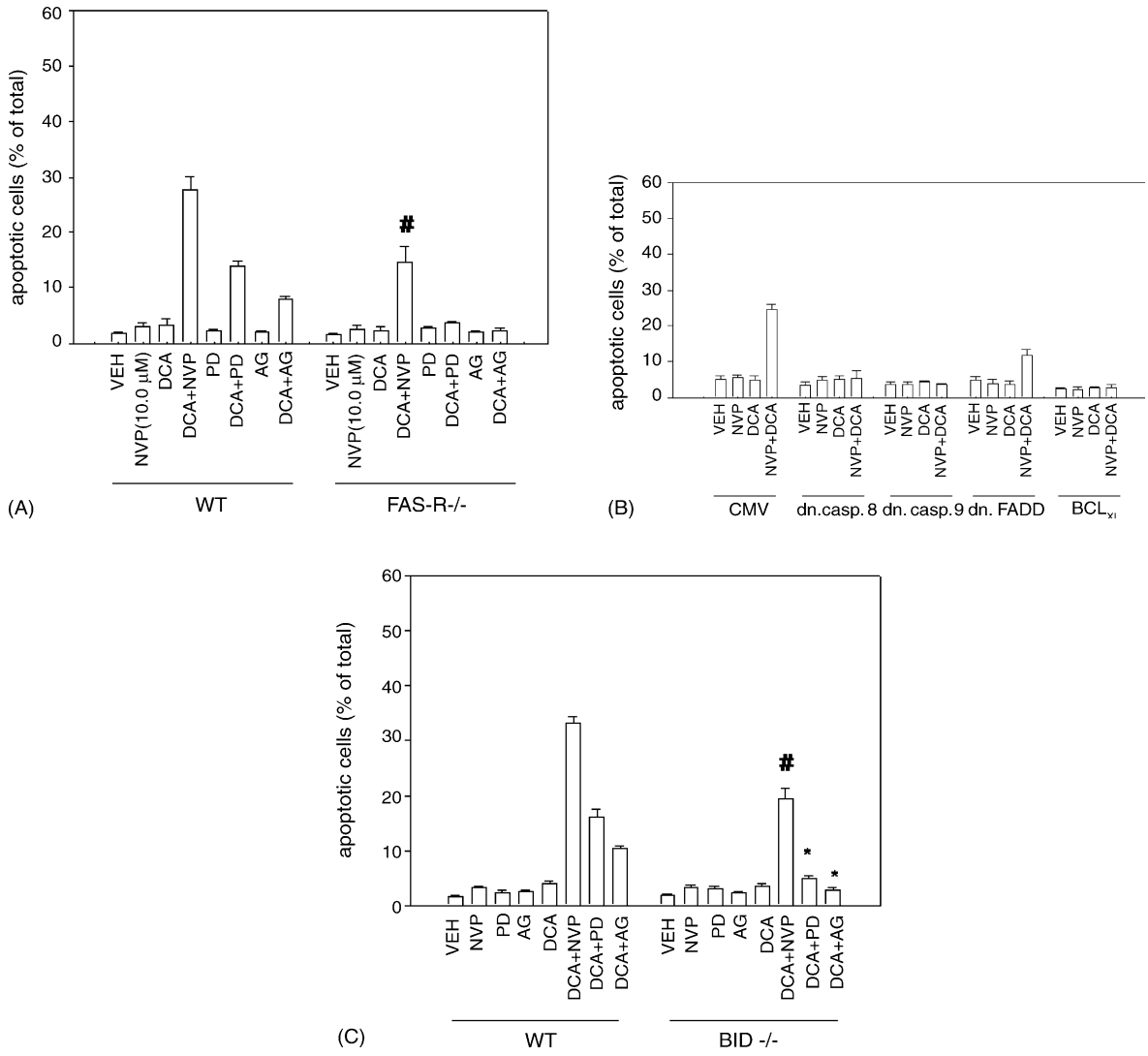

Fig. 3. NVP-ADW742 still promotes DCA lethality in primary hepatocytes lacking either FAS receptor or BID function, but not caspase 8 function. (Panel A) Wild type (WT) and FAS receptor −/− mouse hepatocytes were treated 24 h after plating with DMSO vehicle (VEH), NVP-ADW742 (NVP; 10.0 μM), AG1478 (AG; 10 μM) or PD184352 (PD; 10 μM) 30 min prior to treatment with DCA (100 μM). Cells were isolated 4 h after treatment, fixed onto slides, stained with Hoescht dye and counted blind on a fluorescent microscope to determine apoptosis. Data are the means of three independent values per experiment from two independent experiments ± S.E.M. $^{\#}p < 0.05$ less than corresponding value in wild type cells. (Panel B) Hepatocytes were plated and infected to express dominant negative caspase 8, dominant negative caspase 9 or dominant negative FADD, as described in Section 2. Hepatocytes were treated with DMSO vehicle, NVP-ADW742 (10 μM), AG1478 (10 μM) or PD184352 (10 μM), 30 min prior to treatment with DCA (100 μM). Cells were isolated 6 h after treatment, fixed onto slides, stained with Hoescht dye and counted blind on a fluorescent microscope to determine apoptosis. Data are the means of three independent values per experiment from two independent experiments ± S.E.M. (Panel C) BID −/− and wild type hepatocytes were treated with DMSO vehicle, NVP-ADW742 (10 μM), AG1478 (10 μM) or PD184352 (10 μM), 30 min prior to treatment with DCA (100 μM). Cells were isolated 6 h after treatment, fixed onto slides, stained with Hoescht dye and counted blind on a fluorescent microscope to determine apoptosis. Data are the means of three independent values per experiment from two independent experiments ± S.E.M. $^{\#}p < 0.05$ less than corresponding value in wild type cells, and greater than vehicle treated controls and $^{*}p < 0.05$ less than corresponding value in wild type cells, and equivalent to vehicle treated controls.

acids weakly activate the FAS receptor (Fig. 4A; cf. data in Fig. 3A using FAS receptor −/− cells). Also in agreement with our prior studies, loss of ASM function almost abolished bile acid-induced JNK1/2 signaling in primary mouse hepatocytes (Fig. 4B). To our surprise however, JNK1/2 activation in ASM −/− hepatocytes was enhanced by NVP-ADW742 to 64 ± 8% ($n = 4 \pm$ S.E.M.) of the level found in wild type cells (Fig. 4B). Thus, our findings in Fig. 4 argue that DCA can promote activation of JNK1/2 via a ceramide-independent pathway whose actions are blocked in wild type hepatocytes by signals emanating from the insulin/IGF-1 receptors.

In Figs. 1, 2 and 4, we noted that NVP-ADW742 enhanced DCA-induced JNK1/2 activation, whereas in other publications we have not observed either ERBB1 or MEK1/2 inhibitors enhancing DCA-induced JNK1/2 signaling (e.g. [22]). Because of this data, and our prior findings demonstrating that JNK1/2 signaling is a cyto-

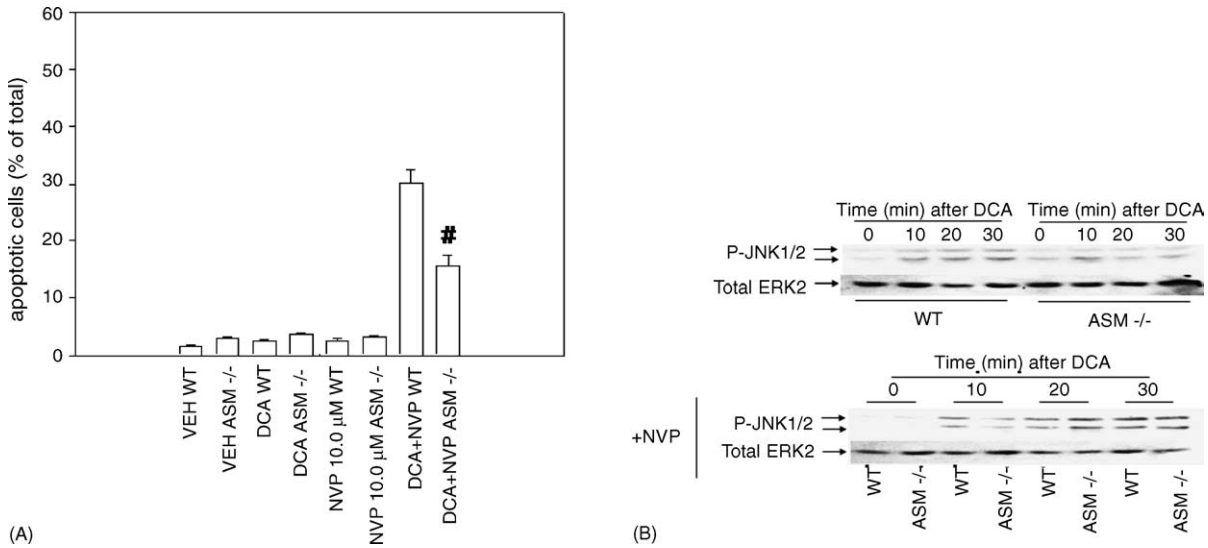

Fig. 4. NVP-ADW742 promotes DCA lethality in primary hepatocytes lacking acidic sphingomyelinase function. (Panel A) Wild type (WT) and acidic sphingomyelinase (ASM) −/− mouse hepatocytes were treated 24 h after plating with DMSO vehicle (VEH), NVP-ADW742 (NVP; 10.0 μM) 30 min prior to treatment with DCA (100 μM). Cells were isolated 4 h after treatment, fixed onto slides, stained with Hoescht dye and counted blind on a fluorescent microscope to determine apoptosis. Data are the means of three independent values per experiment from two independent experiments ± S.E.M. $^{\#}p < 0.05$ less than corresponding value in wild type cells. (Panel B) Wild type and acidic sphingomyelinase −/− mouse hepatocytes were treated 24 h after plating with DMSO vehicle, NVP-ADW742 (10.0 μM) 30 min prior to treatment with DCA (100 μM). Cells were isolated at the indicated time points after bile acid treatment and prepared for SDS-PAGE and immunoblotting performed to determine the phosphorylation of JNK1/2. Data are from a representative experiment ($n = 4$).

protective response following exposure of hepatocytes to DCA and MEK1/2 inhibitors, we compared the role of JNK1/2 signaling in the apoptotic responses of hepatocytes exposed to DCA + NVP-ADW742 or to DCA + PD184352 [22]. Molecular inhibition of JNK1/2 signaling enhanced the apoptotic response of wild type hepatocytes treated with DCA + PD184352 (Fig. 5). Apoptosis induced by DCA + PD184352 treatment was abolished in ASM −/− cells (Fig. 5). In contrast to findings using MEK1/2 inhibitors, inhibition of JNK1/2 signaling *reduced* the apoptotic response of hepatocytes treated with DCA + NVP-ADW742. In ASM −/− cells treated with DCA + NVP-ADW742, inhibition of JNK1/2 function did not modify the total amount of apoptosis induced (Fig. 5). Thus the partial restoration of bile acid-induced JNK1/2 activation by NVP-ADW742 in ASM −/− cells did not change the overall cellular apoptotic response compared to wild type mouse hepatocytes.

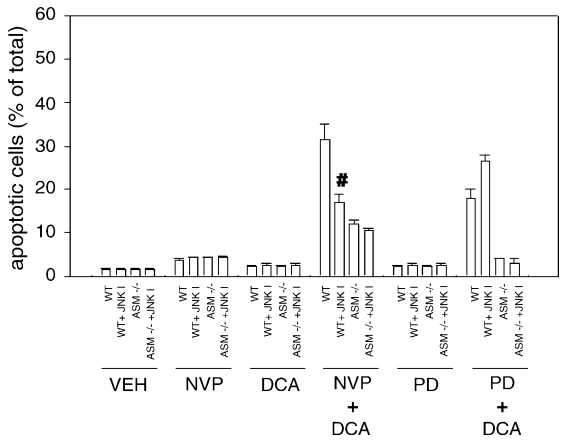

Fig. 5. NVP-ADW742 promotes DCA lethality in primary hepatocytes which is blunted by inhibition of JNK1/2 signaling. Wild type (WT) and acidic sphingomyelinase (ASM) −/− mouse hepatocytes were treated 24 h after plating with DMSO vehicle (VEH), NVP-ADW742 (NVP; 10.0 μM), PD184352 (PD; 10 μM) or with the JIP1 derived JNK inhibitory peptide (JNK I; 10 μM), as indicated, 30 min prior to treatment with DCA (100 μM). Cells were isolated 4 h after treatment, fixed onto slides, stained with Hoescht dye and counted blind on a fluorescent microscope to determine apoptosis. Data are the means of three independent values per experiment from two independent experiments ± S.E.M. $^{\#}p < 0.05$ less than corresponding value in cells lacking JNK inhibitory peptide.

4. Discussion

Modulation of ERBB and IGF-1 growth factor receptor function is recognized as a potential mechanism to inhibit tumor growth. Liver damage, including cholestasis, has been observed in patients treated with conventional cytotoxic therapies [25,26]. Hence, elevated bile acids levels in the liver and gut could have the potential to interact with novel therapeutic modulators of receptor tyrosine kinases, and other signaling molecules, to potentially cause GI damage and toxicity, during combination chemotherapy [27,28]. As ourselves and others have shown that molecular and small molecule inhibition of ERBB1 can modestly enhance bile acid toxicity in liver cells, the present studies were designed to address whether bile acid-induced activation of the insulin receptor could modulate the cell survival responses of primary cultures of rodent hepatocytes exposed to the secondary bile acid deoxycholic acid.

The proprietary IGF-1 receptor inhibitor NVP-ADW742 has been shown to slow tumor cell growth and to enhance the lethality of chemotherapeutic agents [20,21]. At low concentrations (0.1–1.0 μM), NVP-ADW742 has been suggested to be a relatively specific inhibitor of the IGF-1 receptor, becoming at high concentrations an inhibitor of the insulin receptor tyrosine kinase (the IC50 for inhibition of the insulin receptor was noted to be 2.8 μM). Treatment of cells with NVP-ADW742 at concentrations which inhibit both the insulin and IGF-1 receptors for ~60 min abolished DCA-stimulated AKT pathway signaling. Expression of a dominant negative IGF-1 receptor, which inhibits both IGF-1 receptor function and insulin receptor function in trans, abolished the activation of AKT, following DCA treatment, which was similar to our data using NVP-ADW742. NVP-ADW742 did not further enhance bile acid toxicity in the presence of the dominant negative IGF-1 receptor. Collectively, these findings argue that NVP-ADW742 acted in a very similar manner to modify hepatocyte signaling and cell survival as expression of the dominant negative IGF-1 receptor protein. As DCA does not appear to activate the IGF-1 receptor, our findings tend to argue that DCA-induced activation of the PI3K/AKT pathway in primary hepatocytes is largely dependent on activation of the insulin receptor; we have previously noted that activation of the ERK1/2 pathway is primarily dependent on signaling by ERBB1.

We discovered that both NVP-ADW742 and expression of a dominant negative IGF-1 receptor promoted DCA-induced activation of JNK1/2, an effect which was abolished in FAS receptor −/− hepatocytes. In contrast to the effects of blocking IGF-1/insulin receptor signaling, neither the ERBB1 inhibitors AG1478 and Iressa nor expression of a dominant negative ERBB1 receptor promoted JNK1/2 activation. These findings tend to suggest that receptor tyrosine kinases differentially interact with death receptors to regulate JNK1/2 pathway function, as well as to promote the activities of the ERK1/2 and AKT pathways, in primary hepatocytes. They also conclusively demonstrate that the FAS receptor plays an essential role in the activation of the JNK1/2 pathway by DCA in primary rodent hepatocytes.

Use of ASM −/− hepatocytes has previously revealed that bile acid-induced ceramide levels were ASM-dependent and that a lack of ceramide generation resulted in a failure of bile acids to promote activation of the FAS receptor and the JNK1/2 pathway. However, parallel data by ourselves and others have shown that insulin can stimulate JNK1/2 signaling in these cells, and that DCA activates the insulin receptor [7,8,11,12,36]. In agreement with prior findings, loss of ceramide generation in ASM −/− hepatocytes significantly reduced JNK1/2 activation by DCA: however, basal and DCA-induced activation of JNK1/2 signaling was restored to ~65% of those levels found in parental cells by NVP-ADW742 and by expression of dominant negative IGF-1 receptor, which *inhibit* IGF-1/insulin receptor function. These findings argue in primary hepatocytes that basal and DCA-induced signaling by the IGF-1/insulin receptors acts to impede DCA from promoting JNK1/2 activation via ASM/ceramide and the FAS receptor. Further studies outside the scope of the present manuscript will be required to define the signaling pathway(s) that are downstream of the IGF-1/insulin receptors, but not of ERBB1, that may be responsible for signaling pathway cross-talk in hepatocytes resulting in reduced bile acid-induced activation of JNK1/2.

In addition to the above findings, we also noted that unlike AG1478 or Iressa, expression of dominant negative ERBB1 abolished basal ERK1/2, AKT and JNK1/2 activity as well as bile acid-stimulation of these pathways. In contrast to data using dominant negative ERBB1, Iressa did not further enhance bile acid toxicity in hepatocytes expressing the molecular inhibitor and the ability of this agent to promote bile acid toxicity was lost in ERBB1−/− fibroblasts, indicating that Iressa is a relatively specific ERBB1 inhibitor. Thus surprisingly, our data support the concept that over-expressed dominant negative ERBB1 may be a pleiotropic inhibitor of multiple plasma membrane receptors in primary hepatocytes, e.g. FAS receptor, insulin/IGF-1 receptors and that in contrast to established scientific dogma, inhibition of ERBB1 function by small molecule inhibitors in primary hepatocytes appears to more closely correlate with a ''specific'' ERBB1-dependent modulation of cell signaling than use of a molecular ''dominant negative molecule'' approach [29,30].

Findings from this laboratory and other groups have linked bile acid-induced apoptosis to ligand-independent activation of the FAS receptor (e.g. [2,7,23,24]). The mechanism by which both NVP-ADW742 and expression of a dominant negative IGF-1 receptor enhanced bile acid toxicity was investigated: enhanced toxicity was dependent on caspase 8, but only partially dependent on signaling through the FAS receptor, FADD and BID. This would suggest inhibition of insulin/IGF-1 receptor function promotes DCA-induced cell death via both a FAS receptor – caspase 8 – BID-dependent pathway and by an additional pro-apoptotic pathway that requires caspase 8 activation but does not primarily utilize death receptor, FADD or BID function to promote cell killing. Protection of mitochondrial function by over-expression of BCL-XL abolished NVP-ADW742 and DCA-induced apoptosis. Thus, the most likely additional mechanism(s) for cell killing besides death receptor activation is that combined NVP-ADW742 and DCA exposure also promotes mitochondrial dysfunction and/or an apoptotic ER stress response leading to activation of caspase 9 and caspase 3, which in turn promotes caspase 8 activation, and further mitochondrial dysfunction, which in turn amplifies the apoptotic signal.

NVP-ADW742 more potently enhanced DCA toxicity than inhibition of ERBB1 by AG1478 or Iressa. Expression of constitutively activated AKT but not MEK1/2 partially protected hepatocytes from DCA and NVP-ADW742

exposure. As NVP-ADW742, but not AG1478/Iressa caused a strong promotion of DCA toxicity, our findings in this manuscript tend to argue that DCA-induced activation of the insulin receptor (and AKT) generates a more profound cytoprotective signal than DCA-induced activation of ERBB1 (and ERK1/2), in general agreement with the findings of Faubion et al. [2].

Enhanced signaling by the JNK1/2 pathway has been proposed by many groups to play a positive role in apoptosis (e.g. [31]). Previously, we discovered that DCA activated JNK1 and JNK2 to similar levels in primary rodent hepatocytes treated with DCA and MEK1/2 inhibitors, and that inhibition of DCA-induced total JNK1/2 or JNK2 signaling promoted cell death, whereas inhibition of DCA-induced JNK1 signaling was protective [22]. However, in the present studies we noted in wild type mouse hepatocytes treated with NVP-ADW742 and DCA that inhibition of JNK1/2 signaling blunted the apoptotic response. Thus the downstream "output" of JNK1/2 signaling towards cell survival after DCA exposure may depend upon whether signaling by ERBB1, MEK1/2, insulin/IGF-1 receptors and AKT has been inhibited in parallel. Contemporaneous studies have also suggested that JNK1 and JNK2 may differentially regulate the apoptotic response of cells exposed to various noxious stimuli [32–35]. In rat hepatocytes, Liedtke et al. have presented data arguing that JNK1/2 pathway signaling is protective versus TNFα-induced apoptosis [34]. This is similar to findings of Roulston et al. who discovered in fibroblasts that TNFα-induced apoptosis could be enhanced by inhibition of JNK1/2 and p38, but not ERK1/2/5, signaling [35,36].

Previous studies by this laboratory have shown that modulation of transcription factor function plays a partial role in protecting against bile acid toxicity, with expression of dominant negative forms of C/EBPβ, CREB and c-Jun enhancing DCA lethality [22]. Bile acids are also known to directly modulate the function of nuclear orphan steroid binding receptors such as PXR and FXR, whose transcriptional functions can also be facilitated by MAPK pathways, and it is possible that these orphan steroid binding receptors may represent another target for the potentiation of bile acid apoptotic effects by NVP-ADW742 [37]. Furthermore, although not examined in the present studies, in the cholestatic liver prolonged exposure to high levels of bile acids can result in changed expression of multiple detoxification genes, e.g. cytochrome P450 enzymes due to the prolonged activation of orphan steroid receptors, as well as the regulation of other orphan nuclear receptors such as CAR by xenobiotics, e.g. potentially NVP-ADW742 [38–40]. Thus, in vivo, the modulation of NVP-ADW742 toxicity by bile acids is likely to be significantly more complicated than the model in vitro system used in the present studies.

In conclusion, our findings in primary rat hepatocytes and MEFs argue that bile acid-induced activation of the insulin receptor and of ERBB1 generate a cytoprotective signal versus the toxic effects of DCA exposure. Inhibition of ERBB1 function tended to promote DCA-induced cell killing through a mechanism, which was dependent solely on FAS receptor mediated activation of the apoptotic cascade. Inhibition of insulin/IGF-1 receptor function promoted DCA toxicity through death receptor-dependent and mitochondrial dysfunction-dependent pathways. Additional in vivo studies will be required to determine whether IGF-1 receptor inhibitors cause liver toxicity in animals with normal or obstructed bile flow.

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