Antiapoptotic effect of EGF on mouse hepatocytes associated with downregulation of proapoptotic Bid protein

Chantal Éthier, Valérie-Ann Raymond, Lina Musallam, Robert Houle, and Marc Bilodeau

Laboratoire d’hépatologie cellulaire, Centre de recherche du Centre Hospitalier de l’Université de Montréal-Hôpital Saint-Luc, Montreal, Quebec, Canada H2X 1P1

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Address for reprint requests and other correspondence: M. Bilodeau, Centre de Recherche du CHUM-Hôpital Saint-Luc, 264, Boul. René-Lévesque Est, Montréal, QC, Canada, H2X 1P1 (E-mail: Marc.Bilodeau@umontreal.ca).

EGF is a powerful growth factor, particularly efficient in causing hepatocyte death in animals and cell culture models (18, 38). The Fas signaling pathway involves activation of the receptor by the agonist Fas ligand followed by receptor homooligomerization (1), recruitment of the adaptor FADD protein and of inactive caspase 8 proteins present close to the cell membrane (28). This causes aggregation of procaspase 8 proteins and subsequent activation (4, 36). Caspase 8 can directly activate downstream caspases such as caspase 3 (type I cells). Alternatively, caspase 8 can cleave a cytoplasmic protein called Bid leading to mitochondrial release of cytochrome c and the subsequent activation of caspase 9 followed by activation of caspase 3 (type II cells). Hepatocytes are thought to undergo apoptosis mainly through the second pathway and are thus considered type II cells (44).

The process of apoptosis can be inhibited. Antiapoptotic conditions are characterized by a decrease in the intensity of the apoptotic response to a given stimulus, a delay in the apoptotic response, or a switch to a different mode of cell death or injury. The mechanisms through which resistance to apoptosis is afforded are being intensively scrutinized as it can teach us on how cells normally defend themselves, and therefore, how we can eventually manipulate this response.

Cytokines, among which several growth factors, have frequently been identified as inhibitors of apoptosis. Three pathways are thought to be central to the survival signals afforded by growth factors: 1) phosphatidylinositol 3-kinase (PI3-kinase), 2) Ras/MAPK, and 3) Jak/signal transducers and activators of transcription pathways (48). Other ways to counteract apoptosis is through modulation of the cell matrix (6), decrease in glutathione levels (35), or by directly interfering with cell death machinery (i.e., caspases and Bcl-2 family proteins). It is particularly interesting to note that almost every cell type possesses regulatory proteins to inhibit the apoptotic pathway; these inhibitors of apoptosis proteins have been the subject of intense research and their protective role against Fas hepatotoxicity has been recently described (9).
EGF and hepatocyte growth factor are the growth factors studied mostly for their capacity to induce hepatocyte resistance toward apoptosis (17, 30, 46). Increased expression of Bcl-xl (30, 34), involvement of the EGF receptor (EGFR) autophosphorylation (34) as well as PI3-kinase (52), and, to a lesser degree, MAPK pathways (42) have been identified as possible mechanisms of the antiapoptotic effect of these growth factors in hepatocytes. Phosphorylation and inactivation of the proapoptotic Bad has also been demonstrated in other cell systems (10, 12, 57). c-Jun NH2-terminal kinase has been rather implicated in the antiapoptotic response to TNF-α, a cytokine that has the capacity to convey pro- and antiapoptotic messages (32).

In this paper, we report studies showing that the mechanisms of EGF-induced hepatocyte resistance toward apoptosis also involve a decrease in the level of expression of the Bid protein.

MATERIALS AND METHODS

Collagenase D, avian myeloblastosis virus (AMV)-reverse transcriptase, and Taq DNA polymerase were purchased from Roche Diagnostics (Laval, QC, Canada). HEPES, newborn calf serum, TRIZol, and cell culture media were from Gibco-BRL (Burlington, ON). Purified anti-mouse Fas antibody (Jo2 clone) was from Research Diagnostics (Flanders, NJ). EGF, actinomycin D (ActD) and Hoechst 33258 from Sigma-Aldrich (Oakville, ON, Canada), and PD-168393 and LY-294002 were from Calbiochem (San Diego, CA). Goat anti-human/mouse Bid antibody was from R&D Systems (Minneapolis, MN). Peroxidase-conjugated anti-goat IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies actin kit was from Oncogene Research Products (Cambridge, MA). Ac-IETD-7-amino-4-methylcoumarin (Ac-IETD-AMC) and Ac-DEVD-AMC were from Bionse International (Camarillo, CA). Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Hepatocyte isolation and culture. The care and use of animals for these studies were reviewed and approved by the Comité Institutionnel de Protection des Animaux du Centre Hospitalier de l’Université de Montréal-Hôpital Saint-Luc. All animals received humane care according to the guidelines of the Canadian Council on Animal Care.

Hepatocytes were isolated from fed adult male Balb/c mice, C57BL/6 mice (Bid+/+) and C57BL/6 Bid-deficient mice (Bid−/−) by using the two-step collagenase perfusion method previously described (34). Cells were seeded onto plastic petri dishes in William's E medium supplemented with 10% FBS at a density of 26,000 cells/cm². After 2 h, medium was replaced by fresh serum-free medium for the indicated times. Cultures were kept with the different conditions in 5% CO₂ atmosphere at 37°C.

Apoptotic nuclei measurement. Hepatocytes isolated from Balb/c, C57BL/6 (Bid+/+), and (Bid−/−) mice were incubated in William’s E medium in the presence of Fas alone (250 ng/ml), Fas + ActD (12.5 ng/ml) with or without EGF (50 ng/ml). At the end of the culture period, medium was gently removed and the cells were fixed with 5% formaldehyde solution at room temperature for 20 min. Cells were stained with Hoechst 33258 (0.25 μg/ml) for 15 min. Apoptosis was quantified as previously described (34). Four hundred cells were counted, and apoptotic nuclei were expressed as a percentage of the total number of fluorescent nuclei.

Biochemical determination of cell death. To determine cell death, we measured alanine aminotransferase (ALT) levels released in the medium from the cells cultured in the same conditions as described above at the indicated time as detailed elsewhere (34). This represents secondary necrosis after apoptosis in culture. ALT levels for each sample were calculated as the ratio of ALT present in the medium vs. the sum of ALT present in the medium and in adherent cells after sonication. Results are presented as the percentage of ALT release at each time point or compared with the control.

Semiquantitative RT-PCR mRNA determination. Total cellular RNA was extracted with TRIzol according to the manufacturer’s recommendations. Briefly, 750 μl of TRIzol was added for every 2.5 E⁶ cells isolated from Balb/c mice and cultured in the presence of Fas with or without EGF for 4 h. RNA was extracted with 1:5 volume of chloroform and centrifuged at 12,000 g at 4°C. The aqueous phase was incubated for 10 min at room temperature with 1:3 volume of isopropanol and centrifuged at 12,000 g at 4°C. The resultant RNA pellet was washed in 75% ethanol and resuspended in diethyl pyrocarbamate-treated water.

PCR primers were made against mouse Bid and GAPDH (as internal control) sequences with oligonucleotide primers designed by using the Primer program (Genetic Computer Group, Madison, WI) and synthesized by the Sheldon Center of McGill University (Montréal, QC, Canada). The forward and reverse primers for Bid were 5'-CGATGAGATGGACCA-CAAC-3' and 5'-AAGACATCACGAGGAAAG-3', and for GAPDH were 5'-GAGGGGCCATCCACAGTTCTG-3' and 5'-CCCTCTATGCACCTCACTA-CATGGT-3', respectively. Two hundred fifty nanograms of RNA were mixed with 10 mM Tris, pH 8.3, 1 mM MgCl₂, 50 mM KCl, 100 μg/ml BSA, 100 μM dNTPs, 1 μM primers, 200 U/ml RNAse inhibitor, 125 U/ml AMV reverse transcriptase, 20 U/ml Taq DNA polymerase, and 20 μCi/ml α-[32P]dCTP for a total reaction volume of 50 μl. The reaction was initially carried on at 50°C for 15 min followed by PCR at 95°C for 30 s, 59°C for 1 min, and 72°C for 90 s by using a PTC-100 programmable thermal controller (MJ Research, Waltham, MA).

Amplification efficiency conditions were determined after a kinetic study to ensure all experiments were performed within the exponential phase of amplification where PCR products remain proportional to initial template concentration. After amplification, samples were electrophoresed onto 8% polyacrylamide gels, dried and exposed for 1 h at −80°C on Kodak X-ray film (Universal X-ray of Canada, Dorval, QC, Canada). Films were subjected to scanning densitometry (Scanjet Plus; Hewlett-Packard Canada, Mississauga, ON, Canada) with quantification of bands by using Collage version 4.0 software (Fotodyne, New Berlin, WI).

Western blot analysis. Cell lysate was prepared from Balb/c mouse hepatocytes cultured in the presence of Fas, ActD + Fas, PD-168393 (10 μM), and LY-294002 (40 μM) with or without EGF in PBS pH 7.4 containing 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, and 100 μM benzamidine. Protein was quantitated according to Bradford (8). Protein samples (100 μg) were separated by 15% SDS-PAGE and transferred overnight to a Hybond-ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Mississauga, ON, Canada). Blots were incubated with goat anti-human/mouse Bid polyclonal antibody (1:500) followed by horseradish peroxidase (HRP)-conjugated anti-goat antibody (1:10,000) and mouse monoclonal anti-actin antibody (1:5,000) followed by HRP-conjugated anti-mouse antibody (1:10,000) to assess equal protein load-
ing. Bound antibodies were detected by using Renaissance Western blot chemiluminescence Reagent Plus (New England Nuclear Life Science Products, Boston, MA).

Measurement of caspase protease activity. To measure the enzymatic activity of caspases, cells isolated from Bid+/+ and Bid−/− mice cultured in the presence of Fas with or without EGF were lysed in lysis buffer [10 mM HEPES (pH 7.4), 5 mM MgCl₂, 4 mM KCl, 0.1 mM EDTA, 0.1% 3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 10 μg/ml soybean trypsin inhibitor, and 100 mM benzamidine] for 15 min on ice and were then centrifuged at 13,000 g for 10 min. The proteolytic activity of caspase 3 and 8 was measured by the cleavage of caspase 3 (Ac-IETD-AMC) and caspase 8 (Ac-DEVD-AMC) fluorescent substrate (100 μM) in a reaction mixture containing 100 μg protein for caspase 3 and 200 μg protein for caspase 8 in assay buffer of 100 mM HEPES (pH 7.2), 200 mM NaCl, 2 mM EDTA, 20% sucrose, 0.2% CHAPSO, and 20 mM DTT over a period of 30 min at 37°C in the dark. Fluorescence was measured in a SPECTRAMax GEMINI microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) by using 380 and 460 nm as excitation and emission wavelengths, respectively. The maximal substrate cleavage rate (Vmax/s) was calculated by using SOFTmax Pro software (Molecular Devices, Sunnyvale, CA), and the activity of caspase was derived from a calibration curve relating Vmax/s to increasing units of human recombinant caspase 3 or 8 (Calbiochem, San Diego, CA).

Statistical analysis. All data represent the values of at least three experiments each from a different cell isolation. Data are expressed as means ± SE. Differences among groups were analyzed by using one- and two-way ANOVA for repeated measures or Student’s t-test. A P value below 0.05 was considered significant.

RESULTS

EGF significantly decreases Bid expression in primary mouse hepatocyte cultures. Exposure of primary cultures of mouse hepatocytes to increasing concentrations of EGF caused a dose-dependent decrease in the expression of the 23-kDa Bid protein (measured at 4 h after the beginning of exposure; Fig. 1A). Kinetic analysis of the effect of 50 ng/ml of EGF revealed that the decrease in Bid expression was first observed after 4 h of exposure and was maintained for ≥12 h (Fig. 1B). The maximal decrease in Bid expression was 54% compared with basal levels. No evidence of the truncated form of Bid was found in control and EGF-treated cells.

Expression of Bid was then evaluated under apoptotic and antiapoptotic conditions. Hepatocytes exposed to Fas antibodies undergo apoptosis in cell culture (37), and EGF has been shown to significantly decrease this response (34). Figure 2A demonstrates that EGF treatment caused a 46% decrease in full-length Bid (p23Bid) expression compared with controls at 4 h (0.527 ± 0.064 units in EGF vs. 0.969 ± 0.164 units in control cells; P < 0.05). Treatment with Fas alone also caused a decrease in p23Bid although not significant (0.683 ± 0.066 units; P > 0.05). Exposure to both Fas and EGF caused a 52% significant decline in the expression of p23Bid compared with Fas alone (0.333 ± 0.072 units in Fas + EGF vs. 0.683 ± 0.067 units in Fas cells; P < 0.05). These results demonstrate that EGF conserved its capacity to decrease Bid independently of the presence of Fas. The effect of EGF on Bid mRNA was analyzed then (Fig. 2B). EGF alone did not significantly alter Bid mRNA compared with controls (0.828 ± 0.08 units in EGF vs. 0.880 ± 0.05 units in control cells; P > 0.05). Fas alone increased Bid mRNA steady state but not up to a statistically significant level (1.22 ± 0.14 units in Fas vs. 0.880 ± 0.05 units in control cells; P > 0.05). When EGF was added to Fas, the level of Bid mRNA was significantly smaller, compared with Fas alone (0.817 ± 0.05 units in Fas + EGF vs. 1.22 ± 0.14 units in Fas cells; P < 0.05).

Decrease in Bid expression is dependent on the tyrosine kinase activity of the EGFR and is partly mediated through PI3-kinase signaling. To assess whether the effect of EGF on the level of Bid protein was specific to EGF, we used a specific inhibitor of the tyrosine kinase activity of this receptor (PD-168393) at a concentration previously shown to inhibit the phosphorylation of the EGFR as well as the activation of MAPK and PI3-kinase pathways (34). Figure 3A shows that PD-168393 alone had no effect on the level of expression of p23Bid at 4 h (0.458 ± 0.03 units in PD-168393 vs. 0.329 ± 0.07 units in control cells; P > 0.05),
whereas the combined use of PD-168393 with EGF totally reversed the decrease in p23Bid expression observed when EGF was used alone (0.444 ± 0.04 units in EGF vs. 0.095 ± 0.03 units in EGF cells; P < 0.001). We (34) have previously shown that PD-168393 significantly reduced the EGF protective activity toward Fas-mediated apoptosis in mouse hepatocytes.

The mechanism of Bid decrease by EGF was further studied by using a blocker of the PI3-kinase pathway (LY-294002). Figure 3B shows that LY-294002 alone had no effect on the level of Bid expression at 4 h (0.343 ± 0.07 units in LY-294002 vs. 0.338 ± 0.04 units in control cells; P > 0.05), whereas the combined use of LY-294002 with EGF partly reversed the decrease in p23 expression observed when EGF was used alone (0.225 ± 0.05 in EGF + LY-294002 vs. 0.178 ± 0.04 in EGF cells; P < 0.05). Increasing the LY-294002 concentration did not lead to a more complete abolition of the effect of EGF on hepatocytes. To prove that LY-294002 was effective in blocking PI3-kinase pathway, we assessed the level of AKT phosphorylation in EGF-treated cells in the presence or absence of LY-294002 (data not shown). Results show that at the concentration of LY-294002 used, complete loss of AKT phosphorylation was obtained. We finally evaluated the effect of LY-294002 on the apoptotic response (not shown). When cells were incubated in the presence of LY-294002 and Fas stimulation, the level of apoptosis was not different from cells incubated with Fas alone (28.76 ± 7.42% in Fas cells vs. 25.18 ± 5.60% in Fas + LY-294002 cells, P > 0.05) On the other hand, LY-294002 significantly reduced the protective effect of EGF on Fas-mediated apoptosis (10.84 ± 1.86% in Fas + LY-294002 + EGF cells vs. 6.56 ± 1.97% in

Fig. 2. Bid expression in the presence of Fas with or without EGF. A: full Bid protein using actin as a control for protein loading. B mRNA expression of Bid using GAPDH as a control gene in mouse hepatocytes incubated for 4 h in controlled, EGF-treated (50 ng/ml), Fas-treated (250 ng/ml), and Fas + EGF-treated conditions. Each point is the mean ± SE of 6 independent experiments. Data were analyzed by one-way ANOVA for repeated measures. Individual contrasts were analyzed by using Tukey's test: *P < 0.05. Representative blots for p23 and p15 protein and mRNA levels are depicted.

Fig. 3. Effect of a specific inhibitor of the activity of EGF receptor (EGFR) or phosphatidylinositol 3-kinase (PI3-kinase) on Bid protein expression. A: immunoblot analysis of full Bid protein expression using actin as a control for protein loading in mouse hepatocytes incubated for 4 h in controlled, EGF-treated (50 ng/ml), PD-168393-treated (10 μM), and EGF + PD-168393-treated conditions. Each point is the mean ± SE of 4 independent experiments. Data were analyzed by one-way ANOVA for repeated measures. Individual contrasts were analyzed by using Tukey's test. *P < 0.05, **P < 0.001. B: immunoblot analysis of full Bid protein expression using actin as a control for protein loading in mouse hepatocytes incubated for 4 h in controlled, EGF-treated (50 ng/ml), PD-168393-treated (40 μM), and EGF + PD-168393-treated conditions. Each point is the mean ± SE of 5 independent experiments. Data were analyzed by Student's paired t-test. *P < 0.05. Representative blots for p23 and p15 Bid protein levels are depicted.
Fas + EGF cells; \( P < 0.01 \) (not shown). However, the loss of protection was only partial.

**Decrease in Bid protein expression is not caused by an increase in the breakdown of Bid into its p15 fragment.** Bid is known to be cleaved into a smaller 15-kDa fragment on apoptotic conditions associated with activation of caspase 8. This fragment is considered to be the active form of Bid. One could expect that caspase activation should not occur after EGF stimulation, because exposure to EGF is not known to induce hepatocyte apoptosis. However, De Smet et al. (11) have described higher levels of the active form of caspase 8 in EGF-treated hepatocyte cultures after 48 h. It was therefore important to make sure that the decrease of the p23Bid protein observed with EGF was not due to a higher degree of breakdown into its p15 fragment. Careful analysis of the Western blots made with an antibody that recognizes both full-length and truncated Bid proteins did not reveal significant amounts of p15Bid under all of control, EGF, Fas, and Fas + EGF-treated conditions (Fig. 2A).

To assess the effect of EGF on the active fragment of Bid (p15Bid), higher levels of apoptosis were obtained by treating cells with Fas and ActD [an agent that has been shown to significantly potentiate the effect of Fas on hepatocytes (37)]. ActD was used at a concentration that did not induce apoptosis alone. In fact, there was no cell death observed even after 12 h as measured by Hoechst staining and DNA fragmentation (not shown). Figure 4B shows that p15Bid quickly appeared after the beginning of the culture with Fas and ActD and that its level remained stable for the next 8 h, whereas there was a 84% decrease in the level of p23Bid expression over time (\( P < 0.0006 \)). Analysis of Bid mRNA in these conditions revealed a severe decrease in its level of expression over time, this being consistent with the effect of ActD (not shown). Figure 4C represents the effect of EGF, in the presence of ActD and Fas over time; compared with the results obtained in Fig. 4B, EGF maintained its capacity to decrease Bid expression (\( P < 0.0004 \)) and this was not accompanied by any increase in the level of p15 fragment (\( P = 0.7034 \)). In fact, at 8 h, the levels of the p15 fragment of Bid were lower in EGF-treated conditions compared with controls (0.237 ± 0.07 units in ActD + Fas + EGF vs. 0.656 ± 0.09 units in ActD + Fas cells; \( P < 0.02 \)). Confirmation of this observation was obtained by calculating the ratio of p15/p23 Bid proteins and comparing the results obtained between ActD + Fas and

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**Fig. 4.** Kinetics of Bid protein expression. Immunoblot analysis of Bid protein expression (p23) and of its p15 fragment using actin as a control for protein loading in mouse hepatocytes incubated for ≥8 h in actinomycin D (ActD; 12.5 ng/ml) (A), ActD + Fas (250 ng/ml)-treated (B), and ActD + Fas + EGF (50 ng/ml)-treated conditions (C). D: measurement of the ratio of p15/p23 Bid protein expression in each condition. Each point is the mean ± SE of 5 independent experiments. Data were analyzed by one-way ANOVA for repeated measures. The decrease in time of the 23 kDa protein in ActD + Fas and ActD + Fas + EGF was statistically significant (\( P < 0.0006 \) and \( P < 0.0004 \) respectively). Representative blots for full and truncated forms of Bid protein levels are depicted.
ActD + Fas + EGF conditions (Fig. 4D): results show no increase in the p15/p23 ratio in between ActD + Fas and ActD + Fas + EGF conditions. Increase in the p15/p23 ratio over time in both conditions suggests that Bid cleavage does occur in both conditions and that EGF does not prevent the formation of truncated Bid.

Bid deficiency is associated with a loss in the protective effect of EGF on hepatocyte apoptosis. To assess the role of Bid decreased expression in the protective role of EGF on hepatocyte apoptosis, hepatocytes obtained from Bid knock-out mice (Bid −/−) were analyzed compared with normal mice (Bid +/+).

First, the apoptotic response of Bid +/+ mice in Fas and Fas + EGF conditions was measured by determining the ratio of apoptotic bodies over 6 h and the release of ALT in the medium over 24 h (ALT release being the result of secondary necrosis after apoptosis in culture). No evidence of apoptosis or ALT release was found in EGF-treated Bid +/+ or Bid −/− cells in the absence of Fas stimulation. Figure 5A shows that EGF significantly decreased the ratio of apoptosis (P < 0.006) in Fas-treated cultures. Figure 5B shows that ALT release compared with the control is decreased between Fas and Fas + EGF-treated conditions at 6 h (P < 0.05) and 24 h (P < 0.001). The effect was not only a delay but rather a resistance, albeit partial, to the cytotoxic effects of Fas. Hepatocytes from Bid −/− mice cultured in the same conditions for ≤24 h (Fig. 5C) were found to be resistant to the effects of Fas as assessed by measuring the ratio of apoptotic bodies. These Bid −/− cells had very small increases in ALT release when treated with Fas; no significant effect of EGF was observed (Fig. 5D). Together, these results point toward the known resistance of Bid −/− cells to Fas stimulation.

These results were to be expected considering the results already published (56). However, careful analysis of these data revealed that, similar to what is observed with normal hepatocytes, cycloheximide, a protein synthesis blocker, significantly increased the apoptotic response to Fas in hepatocytes isolated from Bid −/− animals. Therefore, we used ActD, which has similar effects to cycloheximide on the hepatocyte sensitivity toward apoptosis, in combination with Fas and/or EGF to assess the antiapoptotic effect of EGF on Bid +/+ and Bid −/− hepatocytes.

Treatment of Bid +/+ hepatocytes with ActD + Fas resulted in a high degree of apoptosis (29.08 ± 3.63%) and cell death (29.60 ± 1.6%) early in culture (4 h). EGF treatment caused a decrease in apoptosis (P < 0.0001) and in ALT release (P < 0.004) when added with ActD + Fas conditions (Fig. 6, A and B). Bid −/− hepatocytes were shown to undergo apoptosis in response to ActD + Fas albeit at a lower level than normal hepatocytes at 6 h (14.77 ± 4.75% in Bid −/− vs. 5.47 ± 0.87% in Bid +/+ cells; P < 0.001). Figure 6, C and D, also shows no significant difference in the sensitivity of Bid −/− hepatocytes toward ActD + Fas when EGF was added to the medium. This result suggests that EGF loses its capacity to block apoptosis when Bid is absent. Confirmation of the sensitivity of Bid −/− cells to EGF was obtained by assessment of

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

**Fig. 5.** Effect of EGF on Fas-induced apoptosis. Isolated hepatocytes from Bid +/+ and Bid −/− mice were incubated for ≤24 h in controlled, Fas (250 ng/ml)-treated and Fas + EGF (50 ng/ml)-treated conditions. A: level of apoptosis over 6 h measured by Hoechst staining in Bid +/+ mouse. B: release of alanine aminotransferase (ALT) in the medium in Bid +/+ mouse compared with control. C: level of apoptosis measured by Hoechst staining in Bid −/− mouse. D: release of ALT in the medium in Bid −/− mouse compared with control. Each point is the mean ± SE of 4 (Bid +/+ ) and 3 (Bid −/− ) independent experiments. Data were analyzed by two-way ANOVA for repeated measures. Individual contrasts were analyzed by using Tukey’s test. *P < 0.05, **P < 0.001. The difference between Fas and Fas + EGF conditions in Bid +/+ mouse was statistically significant for the level of apoptosis (P < 0.006).
the phosphorylation of Akt in the absence and in the presence of EGF. Results demonstrate a potent and early phosphorylation of Akt both in Bid+/+ and Bid−/− cells (not shown).

To confirm the loss of sensitivity of Bid−/− hepatocytes toward the antiapoptotic effect of EGF, a time course of caspase 8 and 3 activities was performed in Bid+/+ and Bid−/− hepatocyte cultures exposed to Fas with and without EGF. Figure 7A demonstrates a strong induction of caspase 3 activity in Bid+/+ hepatocytes starting after 4 h of Fas stimulation; the induction was significantly less in the presence of EGF (99.94 ± 3.91 units in Fas vs. 22.34 ± 1.70 units in Fas + EGF cells; P < 0.0001). In comparison, the induction of caspase 3 activity was more than three times less potent in Bid−/− cells (99.94 ± 3.91 units in Bid+/+ vs. 21.12 ± 5.46 units in Bid−/− cells; P < 0.0001), and there was no significant effect of EGF at 6 h (41.17 ± 20.96 units in Fas vs. 34.01 ± 8.13 units in Fas + EGF cells; P = 0.4). Similar results were obtained for caspase 8 activity (Fig. 7B).

When cells were incubated with EGF alone, no evidence of caspase 3 or 8 activity could be found (data not shown).
DISCUSSION

Results presented in these studies demonstrate that EGF dose dependently reduces the level of expression of Bid protein in mouse hepatocytes. The decrease occurs quickly and in a time frame compatible with the acquisition of resistance to the apoptotic response elicited by Fas. This effect is EGF-specific and is mediated by the activation of the EGFR, because a specific blocker of the activation of the EGFR totally abolished the effect of EGF on Bid expression. We have previously shown that this blocker significantly reduced the protection afforded by EGF (34). Results also show that EGF does not cause significant breakdown of Bid into its smaller active fragment but that, instead, EGF decreases the level of p15Bid observed in strong apoptotic conditions. The decrease in p15Bid is due to the decrease in p23 full fragment, because EGF did not significantly modify the ratio between the two forms of Bid. This argues against a significant effect of EGF on death-inducing signaling complex formation. However, we cannot completely rule out an effect of EGF at this level. The observation that EGF decreases caspase 8 activation has to be interpreted in the context that most identifiable caspase 8 activation occurs late in hepatocytes exposed to Fas. This has been understood as being due to the secondary activation of caspase 8 as occurring in type II cells (2, 5, 43, 44). A recent study (27) showed that ActD induced rat hepatocyte apoptosis by increasing the levels of FADD expression that resulted in caspase 8 activation. We do not believe that the concentration of ActD that we used was high enough to significantly modify FADD expression. As we have stated, we could not find any evidence of apoptosis or decrease in cell viability when treated in cultures with ActD only. This being said, even if increased FADD expression and subsequent caspase 8 activity would have been observed, the decrease in Bid expression and the antiapoptotic effect of EGF were still apparent in ActD + Fas-treated cells. EGF maintained its effect on Bid despite the presence of ActD, a potent mRNA synthesis blocker.

These results suggest that the effect of EGF is directly targeted to the p23Bid protein. The possibility that EGF acts on Bid mRNA stability is less likely, because EGF only decreases Bid mRNA under Fas stimulation. Posttranslational modifications of proteins involved in the regulation of the apoptotic response are recognized as determining their active/inactive conformation (29). It is possible that EGF modifies the stability or the activity of the Bid protein. In that regard, there is recent evidence showing that Bid can be phosphorylated by cellular kinases and that this affects its sensitivity toward caspase 8 (13). Further studies (15) have revealed that Bcl-2 family members can be degraded by the ubiquitin pathway and that the phosphorylation status of these proteins regulate their degradation. In that regard, Akt, a cellular kinase known to be activated by growth factors and to be involved in their antiapoptotic potential (19, 40, 46) has been shown to prevent Bid cleavage (55). Akt has also been shown to inhibit Bax conformational changes and its translocation to mitochondria (54). Therefore, the possibility that EGF through Akt phosphorylates Bid, thus increasing its degradation and/or preventing its cleavage into p15Bid, deserves to be addressed. To try to answer this hypothesis, we performed experiments by using LY-294002, a known PI3-kinase blocker (52). Our results show that LY-294002 only partially reversed the downregulation of Bid caused by EGF. The effect on the protection afforded by EGF toward Fas-induced apoptosis was also partial. These results strengthen the observation that modulation of Bid expression is important in the apoptotic response. They are in accord with another observation demonstrating the importance of the PI3-kinase pathway in hepatocyte survival (52). They also strengthen the hypothesis that the effect of EGF on Bid is through changes in stability and/or activity of the Bid protein. However, because the effect was only partial, these results also suggest that other signaling pathway are involved.

Our results suggested that the EGF-induced decrease in Bid protein is implicated in the antiapoptotic effect of EGF. To explore this hypothesis, we used hepatocytes isolated from the Bid −/− mouse model described by Yin et al. (56). This model was used, because no Bid transgenic mouse has been developed and hepatocyte transfection in vitro would have been very difficult to achieve. Our experiments confirmed that Bid −/− hepatocytes are resistant to Fas stimulation used alone but that they can be sensitized by ActD. This implies that hepatocytes can undergo Fas-induced apoptosis without Bid. Additional data are also available to demonstrate that cells can undergo apoptosis in the absence of Bid (5). Mechanisms through which apoptosis can occur in Bid −/− have not been described; it is possible that Bid analogs, such as the human Bik protein, are involved (7).

Experiments performed with EGF in hepatocytes isolated from Bid −/− mice revealed that the absence of Bid significantly decreases the protection afforded by EGF. Indeed, results showing no significant decrease in the rate of apoptosis and of cell death with EGF in Bid −/− cells and also no difference in the activity of both caspases 3 and 8 demonstrates that these cells respond to the antiapoptotic effects of EGF. They do however, respond to other EGF signals, because it was possible to observe a similar activation of Akt compared with Bid +/+ cells. Therefore, these results suggest that Bid is a likely target of the antiapoptotic effects of EGF.

Bid has first been identified through its capacities to interact with Bax and Bcl-2 (50) and was found to induce cytochrome c release from mitochondria (22, 33, 50). Bid is normally present in the cytoplasm in a pro-form and is cleaved by active caspase 8 (33) into a small p6.5 and a larger p15 fragment, the latter being considered the active fragment (21, 49). Truncated Bid induces a change in the conformation of the Bax-Bak proteins also present in the cytoplasm (16). Both truncated Bid and modified Bax-Bak then translocate to

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mitochondria and cause cytochrome c release (14). Bax and/or Bak have been shown to be essential for the proapoptotic effect of Bid (53). It is therefore conceivable that a decrease in Bid expression will be associated with a less strong apoptotic signal. Increased expression of Bcl-xL caused by EGF (34) is another mechanism of the resistance to apoptosis, because Bcl-xL has been shown to bind Bid (50) and to inhibit the effect of its active fragment (31). The ratio between anti- and proapoptotic members of the Bcl-2 family has been suggested to at least partly determine the susceptibility of cells to death signal (39). In hepatocytes, both the increase in Bcl-xL and the decrease in Bid observed with EGF will have a similar tilting effect on the apoptotic balance.

The importance of Bid in liver physiopathology has been demonstrated by experiments showing that Bid−/− mice are particularly resistant to the lethal hepatotoxicity of anti-Fas antibodies (56). This study also showed that hepatocytes isolated from these animals were resistant to the in vitro effects of Fas stimulation with a delay in the generation of the mitochondrial dysfunction and a decrease in the release of cytochrome c. Further studies have since implicated Bid in the pathophysiology of TNF-α hepatotoxicity (58) as well as in bile salt-induced liver injury (24, 47). In the latter study, Bid antisense has been used to decrease the expression of Bid, thereby decreasing the liver injury in vivo and hepatocyte toxicity in vitro. There is also evidence to show that granzyme B, a protein found in the cytotoxic granules of lymphocytes can directly cleave Bid, without caspase 8 activity, leading to the same apoptotic events already described (3, 45). This pathway could be important in immune-mediated liver injuries associated with intralobular lymphocytic infiltration as in the case in viral and autoimmune chronic hepatitis (55).

All these data underscore the key role of Bid in the transmission of the apoptotic message and in the genesis of liver injury. Our studies, by showing that EGF decreases the expression of Bid protein and that the effect of EGF on Bid is strongly associated with the antiapoptotic effect of EGF, confirm the importance of Bid in the apoptotic signal transmitted by Fas and describe a mechanistic pathway through which hepatocytes can escape from the apoptotic signal.

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DISCLOSURES

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