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Betulin, betulinic acid and butein are inhibitors of acetaldehyde-induced activation of liver stellate cells

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Abstract:
Liver fibrosis has been reported to be inhibited *in vivo* by oleanolic and ursolic acids; however, the activity of other triterpenes like betulin and betulinic acid has not been examined. Butein has also been reported to prevent and partly reverse liver fibrosis *in vivo*, although its mechanism of action is poorly understood. Therefore, the aim of this study was to determine the antifibrotic potential of butein, betulin, and betulinic acid and examine their mechanisms of action *in vitro*. This study was conducted in rat stellate cells (HSCs) that were treated with acetaldehyde, which is the most reactive product of ethanol metabolism.
Butein, betulin, and betulinic acid were preincubated with rat HSCs at non-toxic concentrations. Treatment effects were measured in regard to acetaldehyde-induced toxicity and cell migration, and several markers of HSC activation were evaluated, including smooth muscle α-actin (α-SMA) and procollagen I expression. In addition, changes in the release of reactive oxygen species (ROS) and cytokines such as tumor necrosis factor-α (TNF-α) and tumor growth factor-β1 (TGF-β1) and changes in the production of metalloproteinase-2 (MMP-2) and tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) were determined.
In vitro, HSCs were protected against acetaldehyde-induced toxicity by betulin but not by betulinic acid and butein. However, butein, betulin, and betulinic acid inhibited the production of ROS by HSCs treated with acetaldehyde and inhibited their migration. Butein also inhibited acetaldehyde-induced TGF-β1 production. Butein, betulin, and betulinic acid down-regulated acetaldehyde-induced production of TIMP-1 and TIMP-2. Betulin decreased the acetaldehyde-induced activity of MMP-2, but butein and betulinic acid did not.
The results indicated that butein, betulin, and betulinic acid inhibited the acetaldehyde-induced activation of HSCs. Each drug functioned in a different manner, whereby some were acting as either antioxidants or inhibitors of TIMPs expression and butein additionally acted as an inhibitor of TGF-β production.

Key words:
liver stellate cells, acetaldehyde, butein, betulin, betulinic acid, cytokines, MMP-2, TIMPs

Abbreviations: ADH – alcohol dehydrogenase, AP-1 – activator protein-1, CYP2E1 – cytochrome P450 2E1, ERK – extracellular signal-regulated kinases, NF-κB – nuclear factor kappa B, PMA – phorbol 12-myristate 13-acetate

Introduction

Liver fibrosis is caused by a variety of etiologic agents, including chronic viral hepatitis, alcohol toxicity, autoimmune disease, and inherited metabolic disorders. For all of these diseases, there is a common pathologic mechanism leading to fibrosis that includes the generation and proliferation of smooth muscle α-actin (α-SMA)-positive myofibroblasts of periportal and perisinusoidal origin. These cells are produced as a consequence of the activation of hepatic stellate cells (HSCs). HSCs exist in the normal liver as quiescent retinoid-storing cells that activate in response to injury; once activated, these cells become

proliferative and profibrogenic [1, 29]. The activated HSCs are a rich source of type I and III fibrillar collagen and also secrete high levels of tissue inhibitors of metalloproteinase (TIMPs) [31]. The activation of HSCs is mediated by several soluble factors, including growth factors, cytokines, chemokines, and oxidative stress products. Activation of HSCs is associated with sequential expression of several key cytokines and their surface receptors, which include transforming growth factor β (TGF-β) and its receptors [5]. Endogenous expression of TGF-β in the liver induces liver fibrosis, and blockade of TGF-β signalling by multiple methods has been shown to prevent the progression of liver fibrosis in experimental animals [40].

The development of liver fibrosis in alcoholics has been linked to the oxidation reaction of ethanol to the highly reactive compound – acetaldehyde. At concentrations that have been detected in hepatic venous blood during alcohol consumption, acetaldehyde has been shown to stimulate type I collagen synthesis and gene transcription in cultured rat and human HSCs through protein kinase C (PKC) activation [34]. Acetaldehyde has also been shown to increase levels of NF-κB (p65) and its binding to the α_2(I) collagen promoter [23], which has been shown to be enhanced by the accumulation of H_2O_2 [6, 24, 33]. CYP2E1 is an important source of reactive oxygen species (ROS) in alcohol-induced injury and fibrosis by generating superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Furthermore, inhibition of CYP2E1 activity by diallyl sulfide (DAS) has been shown to prevent the induction of collagen I gene expression in rat stellate cells overexpressing CYP2E1 [21]. Oxidative stress also activates the c-Jun NH_2-terminal kinase (JNK), which is a protein that regulates the secretion of proinflammatory cytokines by cultured HSCs [20, 22].

Matrix metalloproteinases (MMPs) are a family of zinc metallo-endopeptidases that are rapidly expressed by HSCs in response to a diverse number of hepatic toxins [18]. Numerous *in vitro* experiments have examined the role of MMPs in the activation of HSCs. In addition, the proliferation of HSCs has been shown to be promoted by pericellular collagen I proteolysis acting *via* αvβ3 integrins [43]. Conversely, MMPs can also contribute to the regression of liver fibrosis through the cleavage of the fibrillar extracellular matrix (ECM) and have been shown to promote the apoptosis of activated HSCs. Thus, MMPs appear to play a dual role in liver fibrosis, depending on the activation state of HSCs [7].

To prevent the progression of hepatic fibrosis, various types of compounds that interfere with HSC proliferation and activation have been developed as antifibrogenic agents. Among these agents, butein (3,4,2',4'-tetrahydroxychalcone) is a polyphenolic compound extracted from the stem bark of cashews and *Rhus verniciflua* Stokes that has been shown to suppress liver fibrosis induced by carbon tetrachloride [11]. In addition, butein has been shown to inhibit the myofibroblastic differentiation of rat HSCs [38]. Furthermore, butein derivatives with an improved bioavailability have been shown to have a potent antiproliferative effect. This effect is mediated by the activation of ERK, which leads to the transcriptional activation of AP-1 and consequently to heme oxygenase 1 expression in hepatic stellate cells [12]. However, butein also exhibits anti-inflammatory and antitumor effects through activation of other pathways, including ERK 1/2 and NF-κB signaling [12, 25, 42].

Natural triterpenoids such as ursolic and oleanolic acids have been investigated for their hepatoprotective effects. The mechanisms underlying these protective effect are complex and include the suppression of enzymes that play a role in liver damage such as cytochrome P450, cytochrome b5, CYP1A, and CYP2A. These protective effects are also mediated by an increase in antioxidant substances like glutathione, metallothioneins, and glutathione-S-transferase that simultaneously provide protective effects for liver mitochondria. Oleanolic acid protects the mouse liver from hepatotoxic tetrachloromethane, acetaminophen, phalloidin, and cadmium, whereas it has no effect on the toxicity of α-amanitine and allyl alcohol [13–17, 28, 37]. The protective effect of betulin, betulinic acid, and oleanolic acid against ethanol-induced cytotoxicity in HepG2 cells has also been noted [35]. Ursolic acid has been observed to have a similar protective effect in *in vitro* models of ethanol-induced hepatic damage in rats [28]. However, the antifibrotic activity of betulin and betulinic acid and the mechanisms underlying their antifibrotic effects have not been examined.

Therefore, the aim of this study was to examine the protective effects of butein, betulin, and betulinic acid (Fig. 1) on acetaldehyde-induced cytotoxicity in rat liver stellate cells (HSC). Several markers of HSC activation were examined, including α-SMA, procollagen I, TNF-α, TGF-β1, ROS, MMP-2, TIMP-1, and TIMP-2.

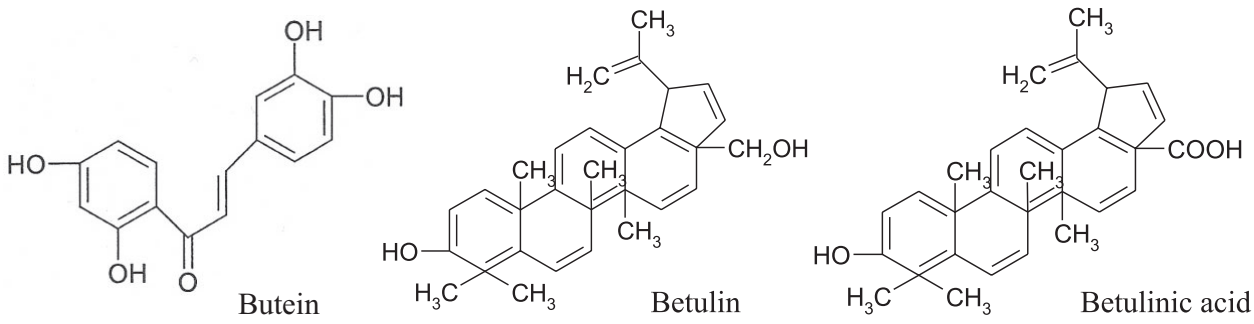

Fig. 1. Chemical structures of butein, betulin, and betulinic acid

Materials and Methods

Cell cultures

A rat liver stellate cell line, CFSC-2G, was kindly provided by Dr. Marcos Rojkind (Department of Clinical Investigation, Walter Reed Army Medical Center, Washington, D.C., USA). CFSC-2G cells were cultured in Eagle's Medium (MEM) supplemented with 5% heat-inactivated fetal calf serum (FCS), 1% nonessential amino acids (NEAA), and 1% antibiotic and antimycotic, pH 7.4. The cells were seeded in tissue culture plates (Falcon, Bedford, MA, USA) and incubated at 37°C in a humidified atmosphere of 5% CO_2. CFSC-2G cells were subcultured twice a week by trypsinization in a 0.25% trypsin-EDTA solution after washing with Ca-Mg-free saline. The culture media, antibiotic, antimycotic, 0.25% trypsin-EDTA, FCS, and NEAA were obtained from Sigma-Aldrich (Steinheim, Germany). In some experiments, Hanks' Balanced Salt Solution (HBSS) (Sigma-Aldrich) was used.

The influence of butein, betulin and betulinic acid on the viability of CFSC-2G cells treated with acetaldehyde

CFSC-2G cells were grown in 96-well plastic plates (Nunc, Roskilde, Denmark) at a high density of 2×10^4 cells/well in Eagle's Medium (MEM) supplemented with 5% FCS. After a 24-h incubation, the medium was replaced with fresh media containing 0.1% FCS and either 10 μM butein, 10 μM betulin or 1 μM betulinic acid (Sigma-Aldrich). All concentrations of substances examined were chosen from preliminary experiments and were not toxic towards HSCs as determined by an MTT assay. After another 24 h of incubation, different acetaldehyde concentrations (0.075–3 mM) were added. Acetaldehyde was purchased from Merck (Darmstadt, Germany) and maintained as a 1 M stock solution. The cells treated with acetaldehyde were maintained in closed-lid containers in a humidified CO_2-incubator at 37°C for 24 h. The toxicity of these treatments was determined by an MTT assay. Data are presented as % of control cell viability. For all subsequent experiments, 175 μM of acetaldehyde was used.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

CFSC-2G cells were grown in 96-well plastic plates (Nunc, Roskilde, Denmark) at a low density of 5×10^3 cells/well in Eagle's Medium (MEM) supplemented with 5% FCS for 24 h. Then, the medium was replaced with fresh media contained the appropriate treatment. After a 96-h incubation at 37°C in a humidified atmosphere of 5% CO_2, the proliferation rate of cells was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The CFSC-2G cells were incubated for 3 h with the MTT solution (5 mg/ml). Formazan crystals were solubilized overnight in SDS buffer (10% SDS in 0.01 M HCl, Sigma-Aldrich), and the reaction product was quantified spectrophotometrically by measuring absorbance at a wavelength of 570 nm using an E-max Microplate Reader (Molecular Devices Corporation, Menlo Park, CA, USA). These data are presented as % of control cell proliferation (optical density values).

Migration assay

Cell migration was assessed using an *in vitro* wound closure assay.

CFSC-2G cells were plated at a density of 6×10^5 on 4 cm culture dishes (Nunc) in 5% FCS-MEM for 24 h. Then, one linear wound was generated by scraping each well with a sterile pipette tip (P300). The wounded monolayers were rinsed twice with culture medium to remove all cellular debris. Afterwards, the medium was replaced with fresh media containing 0.1% FCS-MEM and, in some cultures, either 10 μM butein, 10 μM betulin or 1 μM betulinic acid. After a 24-h incubation, 175 μM acetaldehyde was added to the wells with or without the test substances present. Control cells were cultured in 0.1% FCS-MEM. The number of cells that had migrated into the wounded area after 24 h was estimated in control wells and in the cultures treated with acetaldehyde that had or had not been pretreated with the examined substances. Plates were stained according to the May-Grünwald-Giemsa method. Cell imaging was performed under an Olympus BX51 System Microscope (Olympus Optical, Tokyo, Japan), and micrographs were prepared using analySIS software (Soft Imaging System GmbH, Münster, Germany). Cells that migrated to the wounded areas were counted, and the results were expressed as the mean number of cells that had migrated to 100 randomly selected wounded areas taken from three micrographs.

Measurement of superoxide anion (O_2^-) production by cytochrome c reduction assay

CFSC-2G cells were grown in 96-well plastic plates (2×10^4 cells/well) for 24 h at 37°C in a humidified atmosphere of 5% CO_2. Then, the cultures were washed twice with HBSS, and the culture medium was replaced with fresh 0.1% FCS-MEM with or without either 10 μM butein, 10 μM betulin or 1 μM betulinic acid. The next day, an assay for superoxide anion production was performed [19]. Briefly, HBSS (207.5 μl), 12.5 μl of cytochrome c solution in HBSS (final concentration of 75 μM), 5 μl of either SOD solution (final concentration of 60 U/ml) or 5 μl HBSS, and 25 μl acetaldehyde solution in HBSS (final concentration of 175 μM) was added into each well in a 96-well plate. Also, control wells contained cells that were incubated without acetaldehyde. The microplate was incubated at 37°C for 60 min and transferred to a microplate reader. The absorbance values at 550 nm (the differences in OD between samples with and without SOD) were converted to nanomoles of O_2^- based on the following extinction coefficient of cytochrome c: $\Delta E_{550} = 21 \times 10^3\ M^{-1}cm^{-1}$. The results are expressed as nanomoles of O_2^- per 1×10^6 cells per 60 min. All chemicals were purchased from Sigma-Aldrich.

Measurement of hydrogen peroxide (H_2O_2) production

CFSC-2G cells were grown in 96-well plastic plates (2×10^4 cells/well) with or without either 10 μM butein, 1 μM betulin or 1 μM betulinic acid. After a 24-h incubation at 37°C, the cultures were washed twice with HBSS and the medium was replaced with HBSS (100 μl/well) with 175 μM acetaldehyde for 60 min at 37°C. In control wells, cells were incubated without the inducer. Next, all wells were washed twice with HBSS, and a measurement of intracellular hydrogen peroxide was performed. The assay was based on horseradish-dependent peroxidation (HRPO) of phenol red by H_2O_2, leading to the formation of a compound with an absorbance at 600 nm [27]. Briefly, the cells were covered with 100 μl/well of the assay solution, which was prepared on the day of the experiment and consisted of HBSS, phenol red (Sigma-Aldrich, at a final concentration 0.56 mM), and HRPO (Serva, Heidelberg, Germany) at a final concentration 20 U/ml. Additionally, 10 μl/well of 1 M NaOH was added. After 3 min of incubation, the plate was read at 600 nm in the microplate reader. The results are expressed as nanomoles H_2O_2 per 10^6 cells per 60 min based on the phenol red extinction coefficient ($E_{600} = 19.8 \times 10^3\ M^{-1}cm^{-1}$).

Treatment of CFSC-2G cells with acetaldehyde after preincubation with either 10 μM butein, 10 μM betulin or 1 μM betulinic acid

CFSC-2G cells were grown in 6-well plastic plates (4×10^5 cells/ml with 5 ml/well) in 5% FCS-MEM for 24 h in a humidified CO_2-incubator at 37°C. Then, the medium was replaced with fresh 0.1% FCS-MEM with or without either 10 μM butein, 10 μM betulin or 1 μM betulinic acid. Subsequently, the cells were incubated for another 24 h at 37°C. After that, the inducer, acetaldehyde (at a final concentration of 175 μM), was added to the medium. Plates were prepared in duplicate. After 24 h of incubation, the cultures were washed twice with PBS, and the cells were harvested for western blot analysis of α-SMA, procollagen I, TIMP-1, and TIMP-2.

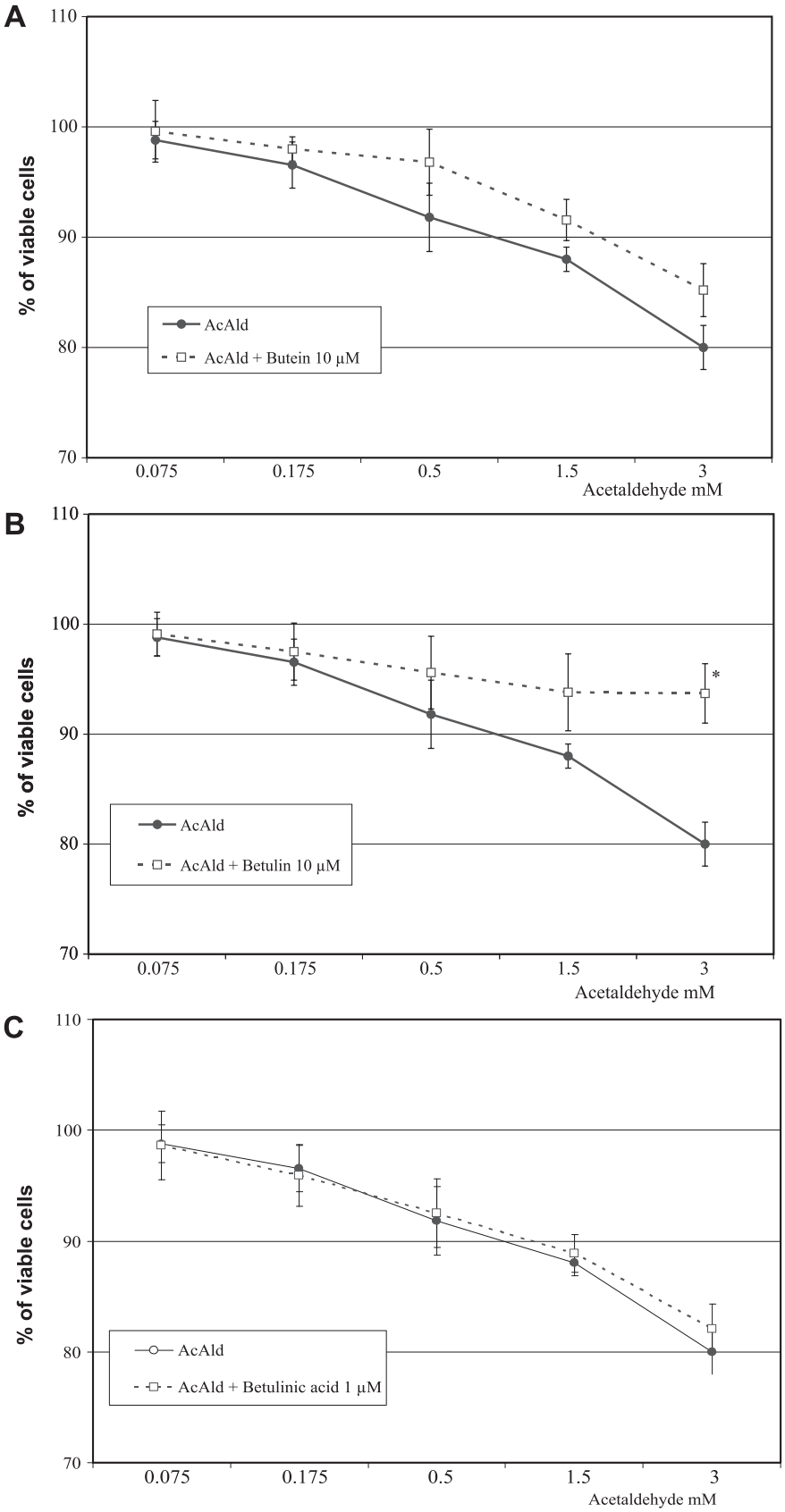

Fig. 2. The effect of butein (**A**), betulin (**B**), and betulinic acid (**C**) on acetaldehyde-induced toxicity in CFSC-2G cells. CFSC-2G cells were preincubated with these substances for 24 h. Later, acetaldehyde was added at the indicated concentration. After 24 h of incubation, the toxicity was determined by the MTT method. Values are the means ± SD of the results from five experiments. * Statistically significant at $p \leq 0.05$ compared to cells incubated with acetaldehyde alone (Wilcoxon test)

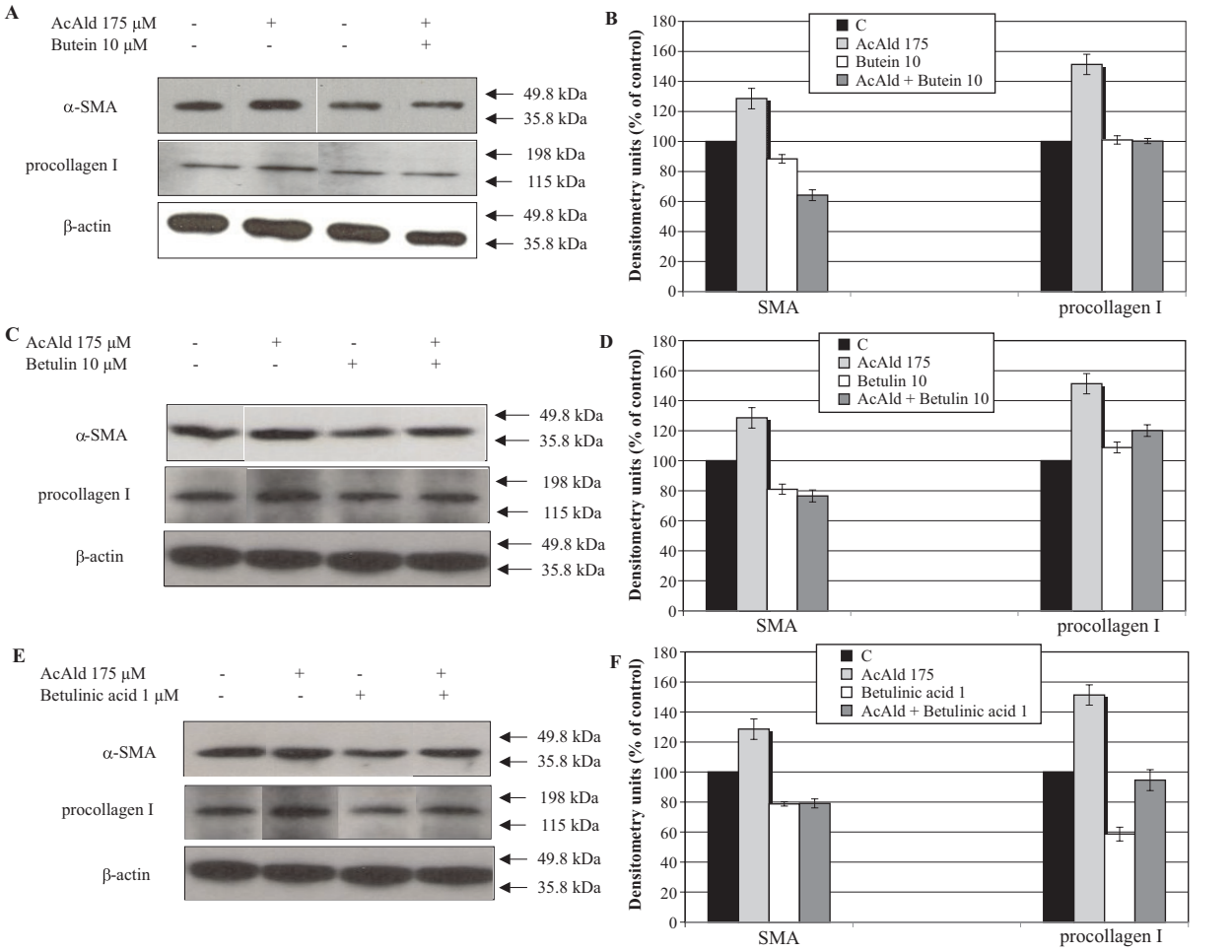

Fig. 3. Preincubation of CFSC-2G cells for 24 h with either butein (**A**, **B**), betulin (**C**, **D**) or betulinic acid (**E**, **F**) induces quiescence among cells activated by a 24 h incubation with acetaldehyde (AcAld). The markers of HSC activation such as α-SMA and procollagen I were measured by western blotting. β-Actin expression served as a loading control. On the right, the arrows indicate the position of the molecular weight markers used in the experiments. Representative blots are shown. Experiments were done in triplicate, and the bar represents the mean ± SD. * Significantly different from respective control (cells incubated without acetaldehyde), $p \leq 0.05$. # Statistically significant at $p \leq 0.05$ compared to cells treated with acetaldehyde alone. The examined substances significantly changed the acetaldehyde effect, $p \leq 0.01$ (two-way ANOVA). (C – control CFSC-2G cells without acetaldehyde, triterpenes or chalcone)

TGF-β, TNF-α, MMP-2, and TIMP-1 assay

CFSC-2G cells were cultured in duplicate in 24-well plastic plates (Nunc) and treated as described above for the 24 h time point. Cell culture supernatants were collected, centrifuged, and frozen immediately at –80°C for further analysis *via* cytokine, MMP-2, and TIMP-1 assays. The culture supernatants were stored for no longer than 3 weeks. Rat TGF-β, TNF-α, MMP-2, and TIMP-1 present in the supernatants from the CFSC-2G cells were measured by an ELISA method using kits from Bender MedSystems Diagnostics (TNF-α) and R&D Systems (TGF-β, MMP-2, and TIMP-1). These kits contain a specific monoclonal antibody immobilized on a 96-well microtiter plate that binds TGF-β, TNF-α, MMP-2, or TIMP-1, which is detected by a second enzyme-conjugated specific polyclonal antibody. Following several washings to remove unbound substances and antibodies, a substrate solution was added to the wells. Color development was stopped by sulfuric acid, and the color intensity was measured using a microtiter plate reader (E-max, Molecular Devices Co, Menlo Park, CA) at 450 nm (correction at 550 nm or 620 nm). The detection limits were the following for each antigen: TGF-β > 4.61 pg/ml, TNF-α > 11.2 pg/ml, MMP-2 > 0.16 ng/ml, and TIMP-1 > 3.5 pg/ml. Intra-assay variations were less than 10%.

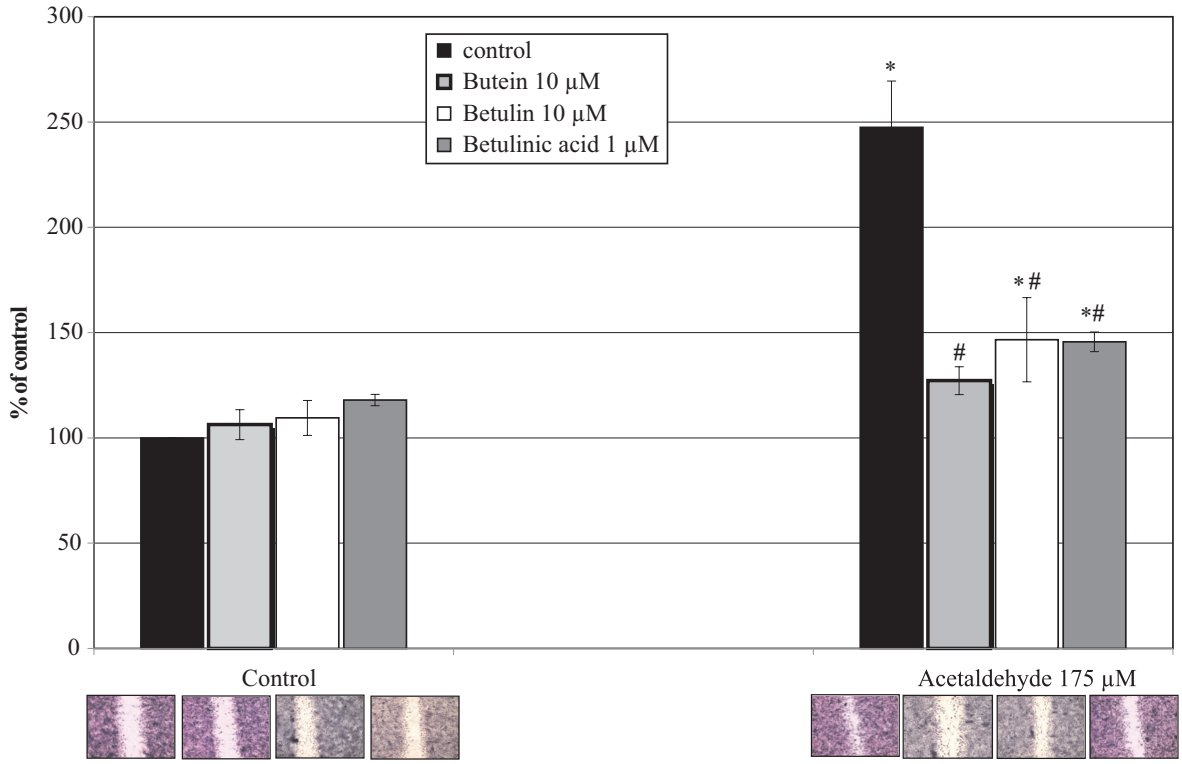

Fig. 4. Preincubation of CFSC-2G cells with butein, betulin, and betulinic acid inhibits cell migration. A wound healing assay was performed on CFSC-2G cells grown to a confluent cell layer. A wound was generated by scraping the culture dish in order to remove a linear area of cells. Cultures were treated with either butein (10 μM) betulin (10 μM) or betulinic acid (1 μM) for 24 h. Then, acetaldehyde at the indicated concentration was added and the cells were allowed 24 h to migrate. Representative images of the different conditions are shown. * Statistically significant at $p \leq 0.05$ compared to respective control (cells not treated or treated only with substances). # Statistically significant at $p \leq 0.05$ compared to cells treated with acetaldehyde alone (Wilcoxon test)

Statistical analysis

Values are expressed as the mean ± SD. The significance of differences was determined with the use of an analysis of variance (Statistica computer package). In addition, a two-way ANOVA test with a *post-hoc* Tukey's test and a Wilcoxon's paired test for comparisons inside groups were used; p values < 0.05 were considered to be significant.

Results

Acetaldehyde-induced HSC cytotoxicity is attenuated by betulin but not butein and betulinic acid

When HSCs were cultured on plastic plates and inhibited by a low (0.1%) level of FCS in the medium, they were very resistant to acetaldehyde toxicity. Acetaldehyde at concentrations between 75–175 μM exhibited a low toxicity towards HSCs *in vitro*, as measured by the MTT method. Therefore, we tested higher concentrations of acetaldehyde in these experiments (up to 3 mM). As can be seen from Figure 2, preincubation of HSCs with 10 μM betulin (a non-toxic concentration) inhibited the toxicity of acetaldehyde. Butein (10 μM) or betulinic acid (1 μM) did not protect HSC against acetaldehyde cytotoxicity. For further experiments, we used 175 μM acetaldehyde.

The influence of butein, betulin, and betulinic acid supplementation on acetaldehyde-induced α-SMA and procollagen I production

HSCs were starved with 0.1% FCS and subsequently incubated with acetaldehyde. The intracellular levels of α-SMA and procollagen type I were estimated by western blot. Acetaldehyde induced the production of both α-SMA and procollagen type I (Fig. 3). Preincu-

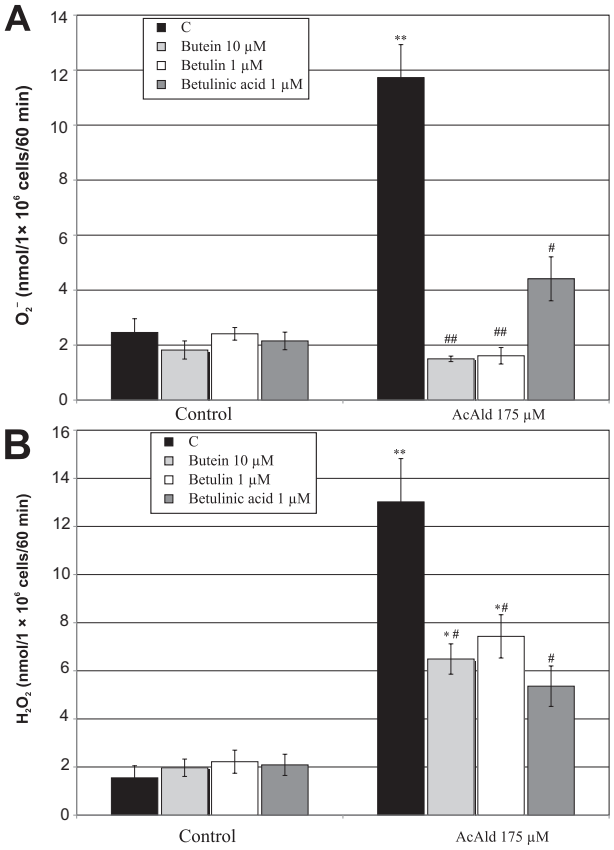

Fig. 5. Preincubation of CFSC-2G cells for 24 h with either butein (10 μM) betulin (1 μM) or betulinic acid (1 μM) inhibits acetaldehyde-induced superoxide anion (**A**) and H_2O_2 (**B**) production. Results are expressed as the mean ± SD of four independent experiments. * Significantly different from respective control (cells incubated without acetaldehyde and a substance examined or treated only with a substance), * $p \le 0.05$, ** $p \le 0.001$. # Statistically significant compared to cells treated with acetaldehyde alone, # $p \le 0.01$, ## $p \le 0.001$. The triterpenes and chalcone significantly changed the acetaldehyde effect, $p \le 0.01$ (two-way ANOVA)

bation with either 10 μM butein, 10 μM betulin or 1 μM betulinic acid significantly inhibited the expression of these markers of HSC activation.

The influence of butein, betulin, and betulinic acid on acetaldehyde-induced HSC motility

When the migration of HSCs was examined by the wound closure assay (Fig. 4), the addition of either 10 μM butein, 10 μM betulin or 1 μM betulinic acid to the incubation medium significantly inhibited the migration of cells in the area of the linear wound scrape through the monolayer of HSCs. Moreover, when HSCs were preincubated with either one of these triterpenes, the number of migrated cells was still higher compared to control cells.

The influence of butein, betulin, and betulinic acid on ROS production in acetaldehyde-treated HSCs

Ethanol and its metabolite acetaldehyde are known to be strong inducers of ROS in HSCs. Stellate cells were preincubated for 24 h with either 10 μM butein, 10 μM betulin or 1 μM betulinic acid, and then acetaldehyde was used as the inducer of the "oxidative burst". Notably, the examined triterpenes and chalcone significantly inhibited superoxide anion production (Fig. 5A). Production of H_2O_2 was also inhibited by these compounds; however, betulinic acid was slightly more effective than butein and betulin (Fig. 5B). It should be stressed that none of the substances examined alone induced O_2^- production.

The influence of butein, betulin, and betulinic acid on TGF-β1, TNF-α, TIMP-1, and TIMP-2 production in acetaldehyde-activated HSCs

Preincubation of HSCs with 10 μM betulin and 1 μM betulinic acid for 24 h did not change TGF-β1 and TNF-α production induced by acetaldehyde. In contrast, 10 μM butein inhibited the production of TGF-β1 and caused a non-significant decrease in TNF-α production (Fig. 6). However, butein seemed to have no effect on the concentration of MMP-2 present in the culture media (Fig. 7). Under similar experiment conditions, acetaldehyde-induced MMP-2 accumulation was significantly diminished by pretreatment with betulin but not by betulinic acid. TIMP-1 and TIMP-2 production was significantly inhibited by all three of the substances examined when measured by ELISA and western blot (Fig. 8).

Discussion

Our study explored the multiple effects of butein, betulin, and betulinic acid on acetaldehyde-activated rat stellate cells. Pretreatment of HSCs with the chalcone compound butein or the triterpenes betulin and betulinic acid influenced several of the cytotoxic effects of acetaldehyde-induced HSC activation, caused a decrease in α-SMA and procollagen type I production, and inhibited HSC motility. To assess the mechanisms by which butein, betulin and betulinic acid inhibited

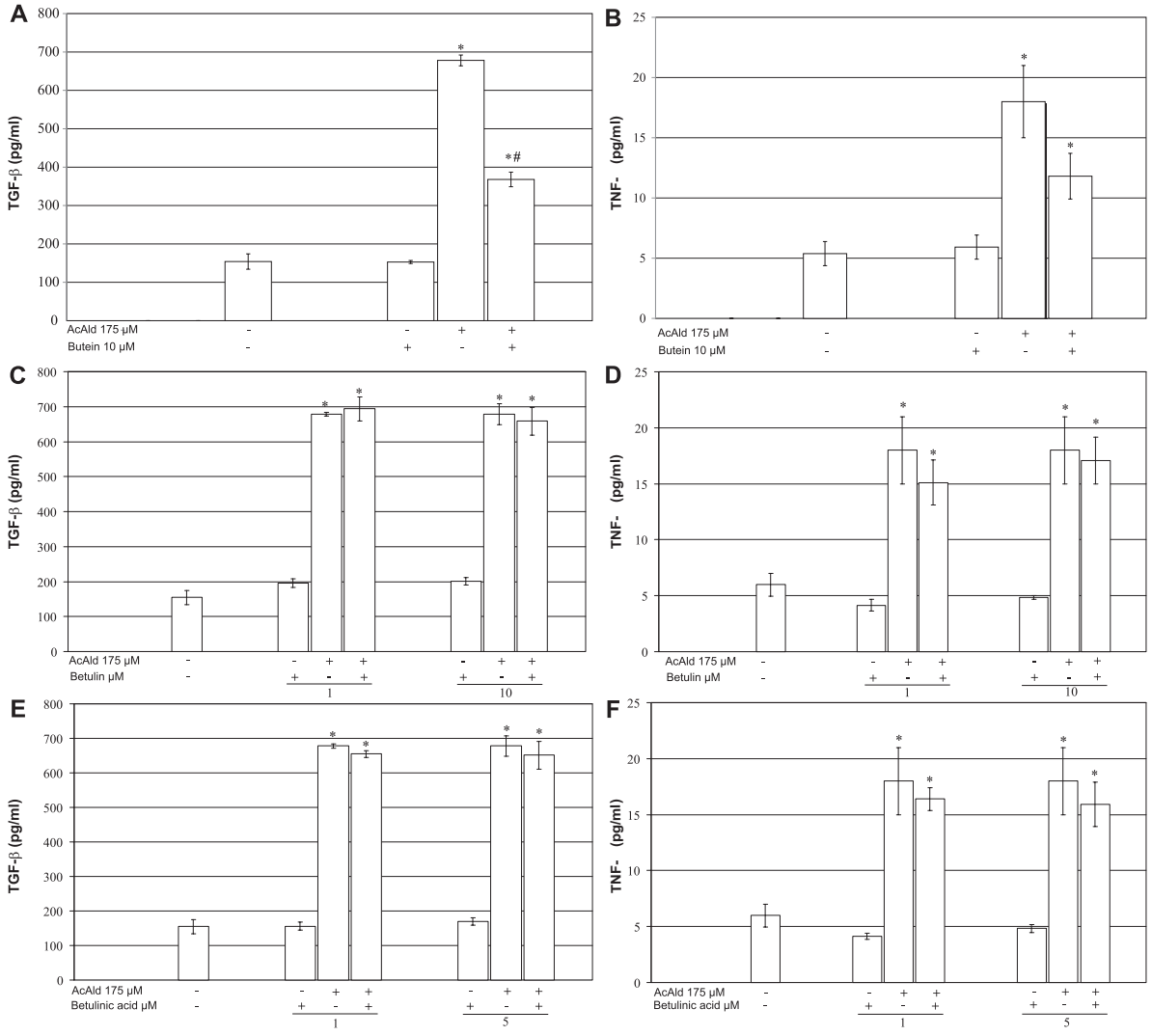

Fig. 6. Preincubation of CFSC-2G cells with butein (**A**, **B**) but not with betulin (**C**, **D**) or betulinic acid (**E**, **F**) inhibits acetaldehyde-induced TGF-β production. Production of TNF-α under these conditions was not statistically changed. The cells were preincubated with the triterpenes and chalcone at the indicated concentrations for 24 h. Afterwards, cells were induced to produce cytokines by the addition of acetaldehyde. The level of cytokines was measured by ELISA and is shown as the mean ± SD of three independent experiments. * Significantly different from respective control (cells not treated or treated only with a triterpene or chalcone), $p \leq 0.01$. # Statistically significant at $p \leq 0.05$ compared to cells treated with acetaldehyde alone. Only butein significantly changed the acetaldehyde-induced production of TGF-β, $p \leq 0.05$ (two-way ANOVA)

HSC activation, we examined their antioxidative properties.

In our experiments, butein inhibited acetaldehyde-induced production of superoxide anions in HSCs. Nevertheless, the antioxidative effects of butein have already been described by other authors. Butein has been shown to be a potent inhibitor of lipid peroxidation in rat liver microsomes and can substantially decrease the production of superoxide anion by macrophages isolated from rat peritoneal exudates [32]. Of particular interest is the interaction between TGF-β production and ROS formation in cultured HSCs. TGF-β was shown to increase the production of ROS [4, 32], which in turn induced the expression of α1(I) procollagen mRNA. ROS have also been identified as mediators of acetaldehyde-induced α1(I) procollagen gene expression [36]. The direct profibrogenic effect of ROS has also been observed in co-cultures of HSCs with HepG2 cells overexpressing CYP2E1 [6]. It should be stressed that butein inhibited both ROS generation and TGF-β production in our study.

In our experiments, betulin inhibited acetaldehyde-induced production of superoxide anions in HSCs by nearly 80%. In contrast, betulinic acid exhibited less

Tab. 1. Summary of potential antifibrotic activity of butein, betulin and betulinic acid

Parameters measured		Butein	Betulin	Betulinic acid
Acetaldehyde-induced toxicity		↓↑	↓	↓↑
HSC migration		↓	↓	↓
-SMA		↓	↓	↓
Procollagen type I		↓	↓	↓
ROS	O_2^-	↓	↓	↓
	H_2O_2	↓	↓	↓
TNF-α		*↓	↓↑	↓↑
TGF-β1		↓	↓↑	↓↑
MMP-2		↓↑	↓	↓↑
TIMP-1		↓	↓	↓
TIMP-2		↓	↓	↓

↓ – down-regulated, ↓↑ – not changed, * – statistically non-significant

inhibitory activity on superoxide production than betulin. These results demonstrate the differences in the activity of betulin and betulinic acid and parallel the findings of other authors who demonstrated that pretreatment of human neutrophils with lupeol and betulin, but not with betulinic acid, significantly inhibited fMLP-induced superoxide generation [39]. When superoxide production was induced by PMA, betulin, but not lupeol and betulinic acid, inhibited the superoxide burst and suppressed the phosphorylation of a 45 kDa protein in fMLP-induced neutrophils. It is interesting that the antioxidative activity of triterpenoids, as stated by those authors, was dependent on their structure. Betulin contains a -CH_2OH at its C17 position and suppresses fMLP and PMA-induced superoxide generation. On the other hand, betulinic acid contains a -COOH at the C17 position and has no effect on superoxide generation induced by fMLP and PMA. It should be noted that in our experiments, betulin inhibited superoxide anion generation from HSCs activated by acetaldehyde more effectively than betulinic acid, and it was also more effective in protecting of HSCs against the cytotoxicity of ethanol.

The antifibrotic activity of betulin and betulinic acid has not been investigated by other authors; however, oleanolic acid and ursolic acid, which are very similar in structure, have been shown to significantly reduce liver injury induced by CCl_4 in rats. These compounds reduced α-SMA expression and the activity of MMPs. *In vitro* experiments with stellate cells (HSC-T6) have revealed that either oleanolic acid (20 μM) or ursolic acid (10 μM) can reduce α-SMA and MMP-2 expression induced by PMA [30]. In addition, our experiments demonstrated that the expression of typical markers of stellate cell activation such as α-SMA and type I procollagen was significantly inhibited by both betulin and betulinic acid, as was acetaldehyde-induced MMP-2 production. The mechanism underlying the antifibrotic effects of ursolic and oleanolic acid is very complex. It includes the suppression of enzymes that play a role in liver damage such as cytochrome P450, cytochrome b5, CYP1A, and CYP2A. This protective mechanism also includes an increase in antioxidant substances such as glutathione, metallothioneins, and glutathione-S-transferase with simultaneous protective effects on liver mitochondria [12–16, 27, 36]. These findings suggest that the inhibition of ROS production in HSC by betulin or betulinic acid can be also caused by inhibition of CYP2E1; however, this potential effect needs further experimental support.

The production of TNF-α is one of the earliest events in many types of liver injury. The role of TNF-α as a critical inflammatory cytokine in the progression of Alcoholic Liver Disease (ALD) is also well documented [10]. In this study, we demonstrate that butein inhibits TNF-α production, which should benefit the resolution of liver fibrosis.

Our study shows that HSCs can be a rich source of several MMPs, especially MMP-2. Acetaldehyde sig-

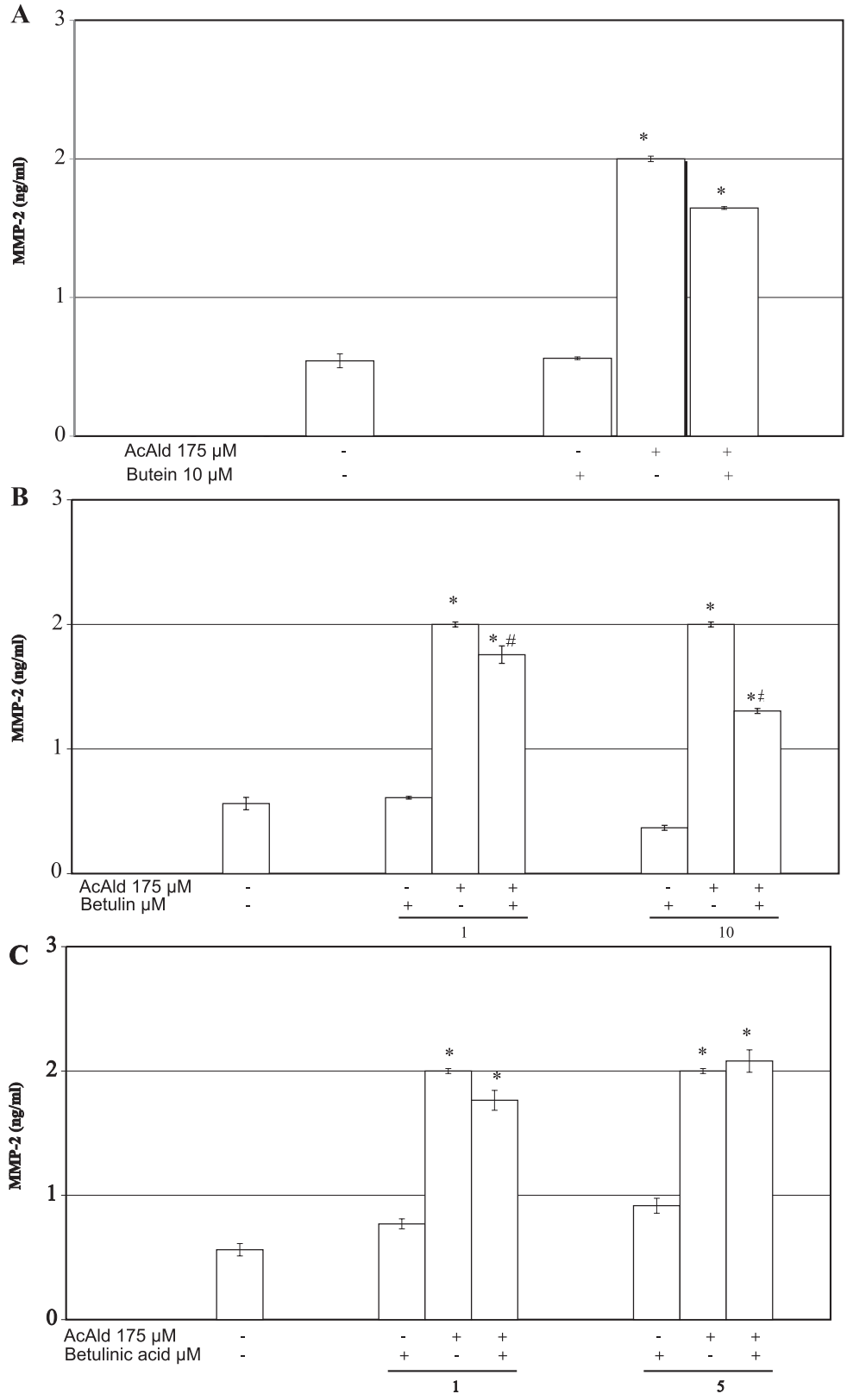

Fig. 7. Preincubation of CFSC-2G cells with betulin (**B**) but not with butein (**A**) and betulinic acid (**C**) decreases production of MMP-2 induced by acetaldehyde. The MMP-2 level was estimated by ELISA. * Significantly different from respective control (cells not treated or treated only with the triterpenes or chalcone), $p \leq 0.01$. # Statistically significant compared to cells treated with acetaldehyde alone, $p \leq 0.05$. Betulin significantly changed the acetaldehyde effect in a concentration-dependent manner, $p \leq 0.05$ (two-way ANOVA)

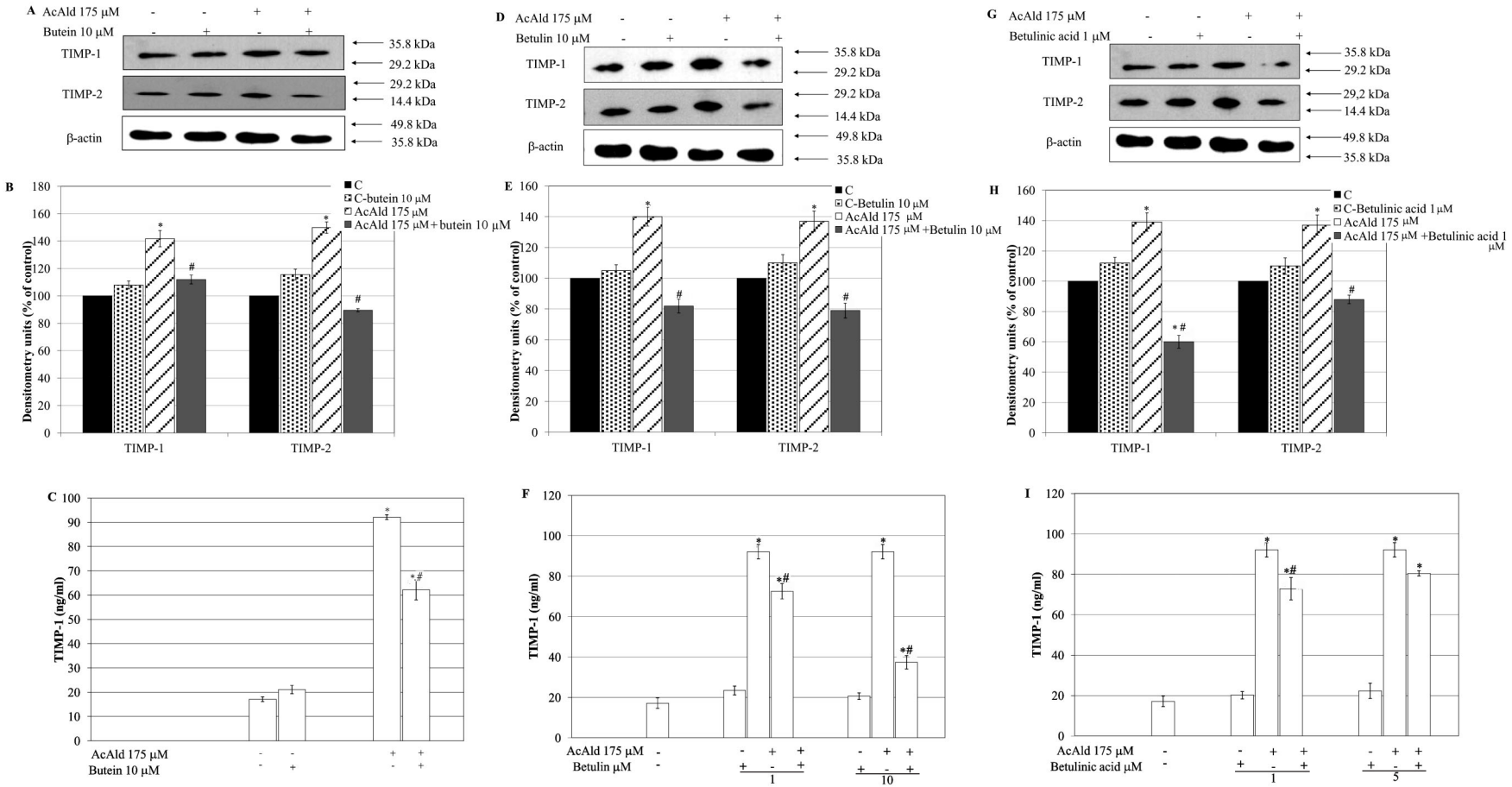

Fig. 8. The effect of preincubation of CFSC-2G cells with either butein (**A**–**C**), betulin (**D**–**F**) or betulinic acid (**G**–**I**) on the parameters related to extracellular matrix remodeling induced by acetaldehyde. Western blot analysis for TIMP-1 and TIMP-2 was performed on cell lysates derived from cells preincubated for 24 h with the triterpenes or chalcone, which were then treated for 24 h with acetaldehyde. The upper panel shows representative blots, the middle panel shows a densitometry analysis of bands, and the lower panel shows the TIMP-1 ELISA assay. * Significantly different from respective control (cells not treated or treated only with a triterpene or chalcone), $p \leq 0.01$. # Statistically significant compared to cells treated with acetaldehyde alone, $p \leq 0.05$. Betulin ($p \leq 0.001$), betulinic acid ($p \leq 0.01$) and butein ($p \leq 0.01$) significantly changed the acetaldehyde effect (two-way ANOVA)

nificantly increased the level and activity of MMP-2 as detected by western blotting and ELISA. MMP-2 is known to degrade basement membrane collagen; hence, its production during the early stages of cell activation may be profibrogenic. However, the delayed overexpression of MMP-2 may be important in the remodelling of the matrix during tissue repair processes [7]. Studies with HSCs have established that when activated, HSCs synthesize increased levels of ECM proteins, particularly fibrillar collagen, but shut down the expression of proteases such as MMP-13, which degrade fibrillar collagen [2, 3, 8]. Therefore, the reduction of the MMP-2 levels observed in our study reflects the antifibrogenic activity of betulin as opposed to butein and betulinic acid.

The regulation of ECM synthesis and its degradation by MMPs and their inhibitors (TIMPs) is a complex process. In general, TIMPs inhibit MMP activity by binding to the active sites of MMPs. Our study demonstrated that TIMP-1 and TIMP-2 secretion was induced in HSCs by acetaldehyde. This finding is similar to previous studies demonstrating significant TIMP expression after HSC activation [4, 9, 26]. In our study, butein, betulin, and betulinic acid significantly inhibited the production of both TIMP-1 and TIMP-2. Because TIMP-1 has been described [41] to have an antiapoptotic effect on activated HSCs, a decrease in its production may be a benefit towards the resolution of liver fibrosis. In addition, it should be noted that previous studies have demonstrated that butein at a concentration of 1 μg/ml reduced DNA synthesis without affecting cell viability and downregulated the expression of α-SMA, procollagen I expression, αI collagen, and TIMP-1 mRNA [37]. These findings suggest that butein is a potent inhibitor of stellate cell transformation, and our results confirm these observations.

Summing up, among the substances examined, betulin was the most potent in inhibiting HSC activation induced by acetaldehyde. Betulin protected HSCs against the toxicity of acetaldehyde, inhibited overproduction of α-SMA and procollagen I by activated HSC, and inhibited their acetaldehyde-induced motility and ROS production. Moreover, betulin inhibited the overproduction of MMP-2, TIMP-1 and TIMP-2, which are factors known to be involved in liver fibrosis. In contrast to these findings, betulinic acid inhibited α-SMA and procollagen I expression, HSC motility, and ROS and TIMP-1 and TIMP-2 production in acetaldehyde-induced HSCs. Butein inhibited superoxide anion and TGF-β production, as well as the release of TIMP-1 and TIMP-2 from acetaldehyde-activated HSCs. Our findings concerning the potential antifibrotic activity of butein, betulin, and betulinic acid based are presented in Table 1. It seems likely that among the substances examined, betulin is the most promising natural product for the prevention and treatment of liver fibrosis. All three substances examined in our study exhibited antifibrotic activity; however, no final conclusions concerning the structure and activity of these substances could be drawn.

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