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Inhibition of transforming growth factor beta1-induced hepatoma cell apoptosis by liver tumor promoters: characterization of primary signaling events and effects on CPP32-like caspase activity

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Abstract

The effects of the liver tumor promoters phenobarbital, clofibrate, dieldrin, and DDT on transforming growth factor-β1 (TGFβ)-induced apoptosis were studied in FTO-2B hepatoma cells. Inhibition of apoptosis by these compounds was strongly correlated with a decrease in CPP32-like caspase activity. Similar effects were obtained with insulin and dexamethasone. CPP32-like activity may thus provide a useful tool for quantiation of apoptosis under various treatment conditions. Diverse effects on apoptosis-associated cellular signaling proteins were observed: insulin led to an activation of the MAP kinases ERK1/2, of PKB/Akt and of NF-κB, phenobarbital and clofibrate enhanced NF-κB activity solely, while dexamethasone slightly enhanced NF-κB activity and increased the expression of Bcl-x_L. Since inhibition of apoptosis was still detectable if the anti-apoptotic compounds were administered more than 10 h after TGFβ, the diverse primary signals appear to converge at a presumably late stage of apoptosis, but upstream of activation of CPP32 or related caspases.

Keywords: apoptosis; hepatoma cells; tumor promoters; CPP32 (caspase-3) activity; anti-apoptotic signaling

Abbreviations: ActD, actinomycin D; CPP32, cysteine protease P32 (caspase 3); DTT, dichlorodiphenyltrichloroethane; DEVD-AFC, N-acetyl-Asp-Glu-Val-Asp-aminotrifluoromethylcoumarin; EMSA, electrophoretic mobility shift assay; ERK1/2, extracellular signal-regulated kinases 1/2; MAP kinase, mitogen activated protein kinase; NF-κB, nuclear-factor-κB; PI(3) kinase, phosphoinositide-3′-OH kinase; PKB/Akt, protein kinase B; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TGFβ, transforming growth factor-β1; TNF, tumor necrosis factor α

Introduction

A variety of endogenous and exogenous compounds with diverse chemical structure act as tumor promoters in rat liver.[1–4] While some of these compounds (e.g. phenobarbital) require comparatively high doses to exert their promoting effects, others act at very low, hormone-like concentrations (e.g. dioxins, peroxisome proliferators, oestrogens), probably via specific high-affinity receptors.[4] Apart from stimulation of cell division, inhibition of active cell death, also referred to as apoptosis, has been recognized as an important mechanism of the carcinogenic action of non-genotoxic tumor promoters. A variety of different tumor promoters have been shown to inhibit apoptosis *in vivo*,[2,3,5] and a limited number of promoting agents tested so far was also active at inhibiting apoptosis induced by different stimuli *in vitro*.[6–10] Anti-apoptotic activity is also a hallmark of certain endogenous hormonally active agonists like insulin and insulin-like growth factor I, which act as survival factors in several cell systems.[7,11,12] Because of the diversity of primary effects elicited by different classes of tumor promoters, the molecular mechanisms underlying inhibition of apoptosis are only poorly understood. Regardless of their differences in primary signaling events, however, it seems likely that they ultimately converge into a common pathway.

Apoptosis can be subdivided into several phases, such as induction, signaling, and execution, which are regulated by positive and negative control factors.[13–16] Within the signaling phase, pro-apoptotic stimuli may be counteracted by anti-apoptotic signals which mediate cell protection and survival. Kinase-dependent signal transduction via the Ras/Raf MAP kinase pathway, the phosphoinositide-3′-OH (PI(3)) kinase/protein kinase B (PKB/Akt) pathway and the nuclear factor-κB (NF-κB) pathway have been demonstrated to protect cells from apoptosis under a variety of different experimental settings.[17–20] These signals may ultimately modify the activity of different pro- or anti-apoptotic members of the Bcl-2 family of proteins which are key regulators of apoptosis.[15,16,21] Once the decision has been made to enter execution of apoptosis, proteases of the ICE/CED3 (caspase) multi-gene family become activated, probably via a proteolytic cascade.[22,23] The observation that viral or synthetic caspase inhibitors efficiently block apoptosis under a variety of experimental conditions, including apoptosis in mouse liver *in vivo*,[24,25] underlines their central role in the apoptotic process. The activity of caspases can be quantitatively determined by use of specific synthetic substrates such as the tetrapeptide DEVD-AFC, which detects CPP32 (caspase-3) and related protease activities.[22,23] CPP32 is widely expressed in all tissues examined and appears to be one of the most

important mediators of apoptosis in mammalian cells.[22,23] Moreover, CPP32-like caspases have recently been shown to become activated during transforming growth factor-β1 (TGFβ)-induced apoptosis in primary hepatocyte cultures.[26]

Using FTO-2B cells, a subclone derived from the well differentiated H4IIEC3 rat hepatoma cell line,[27] we have now analyzed the effects of different liver tumor promoters (phenobarbital, clofibrate, dieldrin, DDT) and hormone-like agents (insulin, dexamethasone) on TGFβ-induced apoptosis, on activation of CPP32-like caspases, and on cell signaling pathways which are known to play a role in suppression of apoptosis.

Results

Characterization of TGFβ-induced apoptosis

Treatment of FTO-2B cells with 5 ng/ml TGFβ led to efficient induction of apoptosis as detected by the appearance of apoptotic (condensed and fragmented) nuclei in Hoechst 33258 stainings and extensive inter-nucleosomal DNA fragmentation, which is characteristic for apoptotic cells (for typical examples see Figures 1A and B). Gross visual inspection of TGFβ-treated cultures revealed that, starting around 12–16 h after TGFβ application, apoptotic cells progressively detached from the culture dishes, and at 24 h after treatment a high percentage of apoptotic cell was in the culture supernatants. Therefore, cells in the supernatants were collected and combined with adherent cells for analysis of DNA fragmentation and caspase activity. CPP32-like caspase activity started to rise around 10 h after TGFβ application, showing a $>$tenfold increase over untreated controls at 24 h after TGFβ treatment (Figure 1C). If YVAD-AFC, which detects ICE-like (caspase-1) activities, was used as a substrate, no increase in activity was seen (data not shown). Simultaneous treatment of cells with TGFβ and the anti-apoptotic agents tested, namely phenobarbital, clofibrate, dieldrin, DDT, insulin and dexamethasone, led to an inhibition of both, DNA fragmentation and CPP32-like caspase activity (for typical examples see Figures 1B and D). Qualitatively similar results were obtained when TGFβ was removed from the medium prior to addition of the inhibitors (data not shown), suggesting that the anti-apoptotic

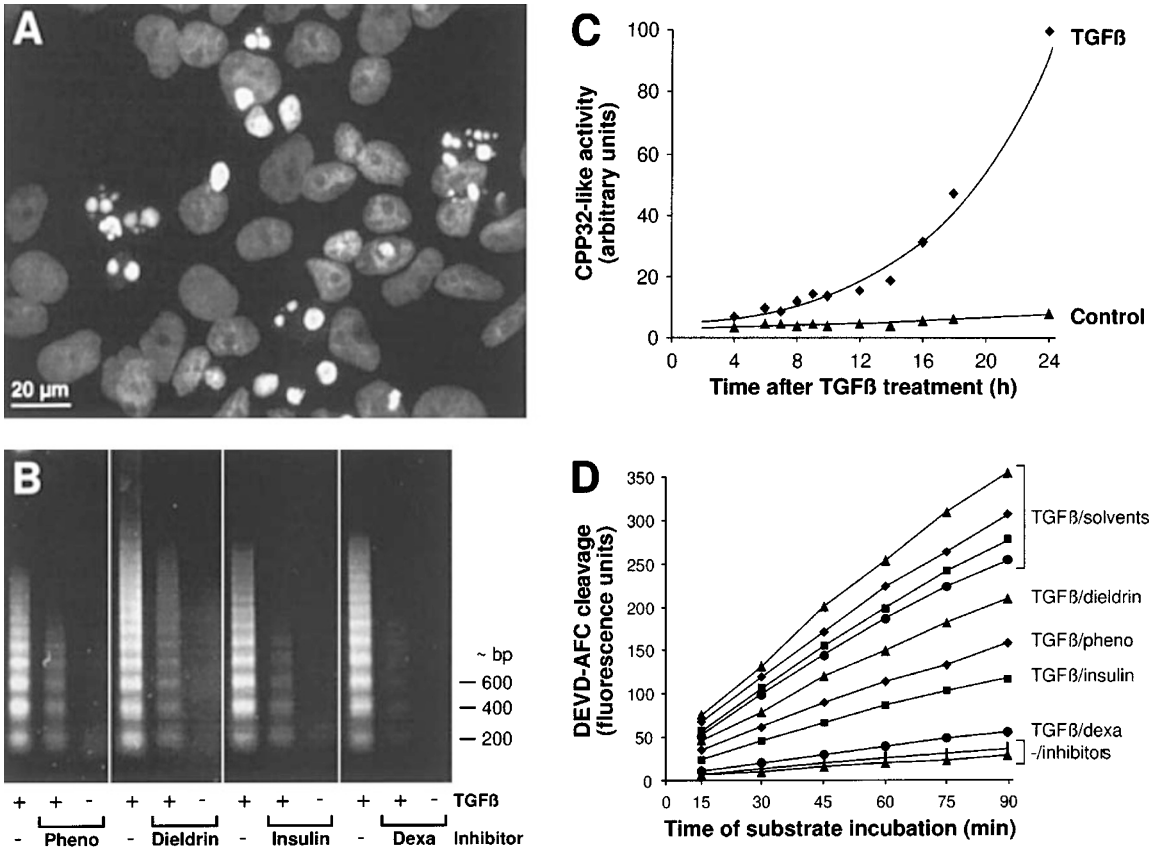

Figure 1 Characteristics of TGFβ-induced apoptosis in FTO-2B hepatoma cells. (**A**) Hoechst 33258 staining of normal nuclei (large, faintly stained) and apoptotic nuclei (brightly stained, condensed and fragmented) at 16 h after treatment with 5 ng/ml TGFβ. (**B**) TGFβ-induced DNA fragmentation and inhibition by simultaneous treatment of cells with anti-apoptotic agents for 24 h (exemplified for phenobarbital (pheno), dieldrin, insulin and dexamethasone (dexa)). Cells not treated with TGFβ received the solvent (4 mM HCl/1% BSA) instead. (**C**) Time-dependent increase in CPP32-like caspase activity after treatment of cells with 5 ng/ml TGFβ (means of 3–4 independent experiments). Starting at 10 h after TGFβ application, values were significantly different from solvent controls ($P<0.05$). (**D**) Inhibition of CPP32-like activity by simultaneous treatment of cells with 5 ng/ml TGFβ and the anti-apoptotic agents shown in (**B**). Representative examples of the caspase assay (DEVD-AFC cleavage) 16 h after treatment are shown

effects are not due to physicochemical interaction with TGFβ in the medium.

Correlation between inhibition of DNA fragmentation and CPP32-like caspase activity

For quantitative assessment of DNA fragmentation and CPP32-like caspase activity, results obtained with the various treatments were expressed as a percentage of TGFβ/solvent controls to compensate for variations in apoptotic response due to solvent effects (on average TGFβ/solvent controls were $\pm$25% compared to TGFβ alone). As shown in Figure 2, TGFβ-induced apoptosis, as estimated by DNA fragmentation analysis, was inhibited by $\sim$50% by treatment of cells with either clofibrate, dieldrin and phenobarbital, while only $\sim$25% inhibition was observed with DDT. The strongest anti-apoptotic effects were seen with insulin and dexamethasone (60–80% inhibition). Similar results were obtained by quantitation of apoptotic nuclei (data not shown). Control cultures treated with the various anti-apoptotic agents or their solvents alone showed DNA fragmentation values that were less than 10% of TGFβ-treated cells, indicating a low level of spontaneous apoptosis. In parallel to the results on DNA fragmentation, CPP32-like caspase activity was inhibited by 40–60% by clofibrate, dieldrin and phenobarbital, 30% by DTT, and $>$60% by dexamethasone and insulin (Figure 2). The inhibitory effects were highly significant for all compounds analyzed. When data from all treatment groups were combined and subjected to Pearson's correlation analysis, a highly significant correlation between DNA fragmentation and CPP32-like caspase activity was obtained (r^2=0.87; $P<0.0001$). To test, if the various anti-apoptotic agents are direct inhibitors of CPP32-like caspase activity, extracts from apoptotic cells were incubated with the different agents at concentrations used for treatment of cells in culture and assayed for CPP32-like activity. Under these conditions, no inhibition of CPP32-like caspase activity was seen (data not shown), demonstrating that the various compounds do not interfere with the protease activity itself.

Persistence of anti-apoptotic activity

Since the apoptosis inhibitory effects of the various compounds observed at 16–24 h after treatment (Figure 2) could also be attributed to a delay in apoptotic response, the duration of suppression of TGFβ-mediated apoptosis was tested next. For this purpose, cells were incubated for 48 h with TGFβ and a selected panel of four characteristic inhibitors. As shown in Figure 3, the inhibitory activity of dexamethasone, dieldrin and phenobarbital on CPP32-like caspase activity and DNA laddering was not significantly different between 24 and 48 h of treatment. In contrast, insulin showed not only a complete loss of anti-apoptotic activity after 48 h of incubation but rather appeared to actively trigger TGFβ-stimulated cells into apoptosis when present for prolonged periods of time.

Time-dependence of anti-apoptotic effects

In the experiments described so far, TGFβ and inhibitors of apoptosis were administered at the same time and were continuously present throughout the entire cultivation period. In subsequent experiments, different inhibitory agents were administered at various time intervals after start of TGFβ treatment in order to narrow the time window required for anti-apoptotic effectiveness. Since we observed some variability in the onset of apoptotic response between individual experiments, additional cultures were always analyzed in parallel to determine the kinetics of TGFβ-induced apoptosis in the absence of the inhibitors. Data obtained by determination of CPP32-like caspase activity are shown in Figure 4; DNA fragmentation analyses revealed qualitatively similar results (data not shown). Dexamethasone and insulin could be added as late as 12 and 16 h, respectively, after TGFβ without

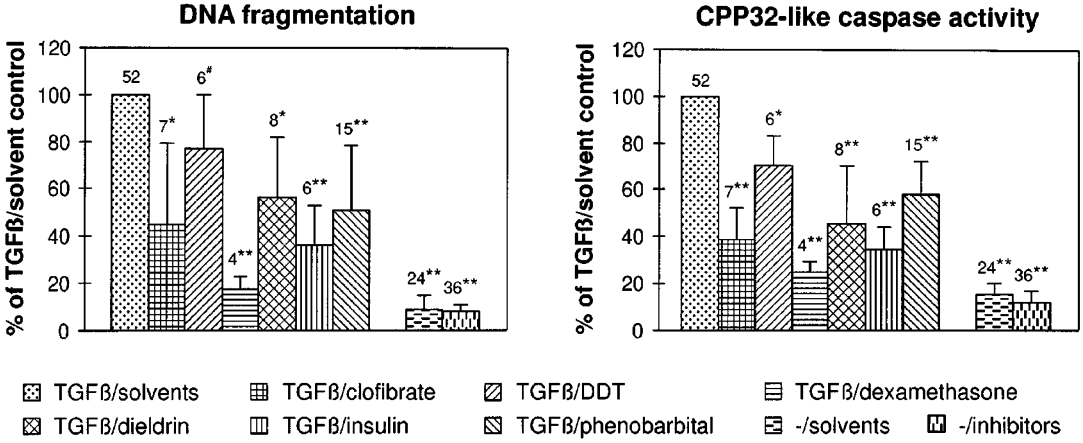

Figure 2 Effects of apoptosis inhibitory agents on TGFβ-induced DNA fragmentation and CPP32-like caspase activity. FTO-2B cells were incubated with 5 ng/ml TGFβ either alone or simultaneously with the various anti-apoptotic agents indicated. DNA fragmentation was analyzed 24 h and CPP32-like caspase activity 16 h after treatment. Values for each treatment group are expressed as a percentage of the respective TGFβ/solvent controls. Data on non-TGFβ-treated cells (4 mM HCl/1% BSA instead of TGFβ) were combined for the various inhibitors and their solvents, respectively. Bars represent means plus standard deviation; numbers above bars indicate the number of experiments analyzed and asterisks mark significant differences from TGFβ/solvent controls (*$P<0.01$, **$P<0.001$, #P=0.056; Student's *t*-test)

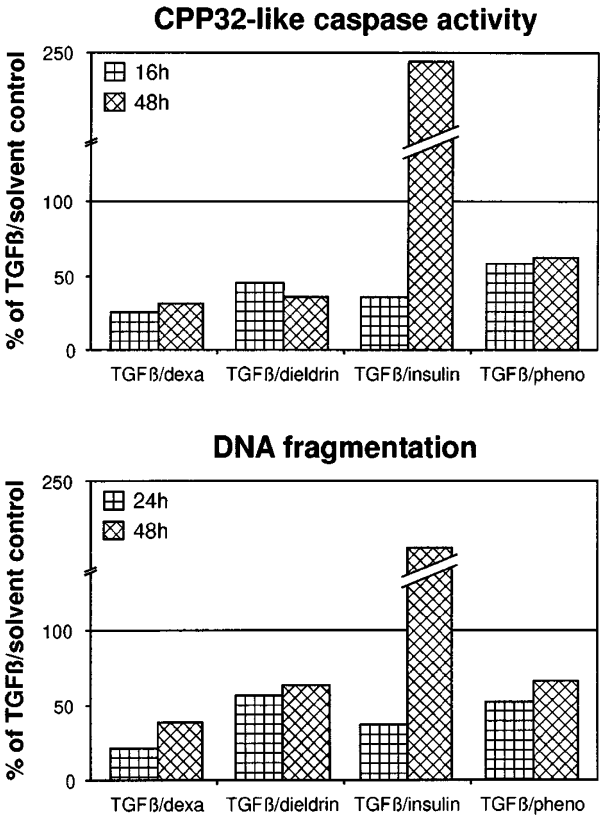

Figure 3 Persistence of anti-apoptotic activity. FTO-2B cells were incubated with 5 ng/ml TGFβ either alone or simultaneously with dexamethasone (dexa), dieldrin, insulin, or phenobarbital (pheno) and DNA fragmentation and CPP32-like caspase activity were analyzed 48 h thereafter. Cultures were supplemented with fresh inhibitors 24 h after the first treatment. Results are representative for two independent experiments. Values from Figure 2 obtained after 16 h (CPP32-like caspase activity) and 24 h (DNA fragmentation) of treatment are shown for comparison

significant loss in inhibitory effectiveness on CPP32-like caspase activity, when compared to experiments where TGFβ and the inhibitors were given simultaneously (Figure 4). Similar effects have previously been reported for the anti-apoptotic activity of insulin in Hep3B human hepatoma cells.[7] The effective time window was somewhat narrower in the case of phenobarbital and dieldrin, which showed a gradual loss of inhibitory activity when added later than about 10 h after TGFβ. One has to consider, however, that the fraction of *viable* cells decreases with time after TGFβ as is reflected by the steady increase in CPP32-like activity prior to the addition of inhibitors (see dashed lines in Figure 4) – which necessarily leads to a time-dependent decrease in the number of cells which could be potentially protected by the various anti-apoptotic agents. Therefore, the loss of inhibitory effectiveness at the later times of inhibitor application is actually somewhat less than implicated by the data shown in Figure 4.

Role of transcription for anti-apoptotic activity

To address the question, whether or not transcriptional activity is required for inhibition of apoptosis, cells were treated simultaneously with actinomycin D (ActD) and either dexamethasone, insulin, dieldrin or phenobarbital. Since ActD itself proved to be an efficient inducer of apoptosis in FTO-2B cells, treatment with TGFβ could be omitted in these experiments. As shown in Figure 5, ActD-induced DNA fragmentation was not significantly affected by the various agents. Similarly, there was no alteration in CPP32-like activity in cultures treated with ActD and either insulin, dieldrin or phenobarbital when compared to ActD/solvent controls; only dexamethasone led to a slight reduction of CPP32-like caspase activity. The reason for the differential behavior of the glucocorticoid is not clear, it is noteworthy, however, that inhibition of CPP32-like activity by simultaneous ActD/dexamethasone treatment was much less pronounced than after TGFβ/dexamethasone treatment (see Figure 2).

Effects of anti-apoptotic agents on cell signaling

The subsequent experiments were aimed to investigate the mechanisms by which the inhibitory agents interfere with TGFβ-mediated apoptosis of FTO-2B cells. We first studied the effects of insulin, dexamethasone and phenobarbital on the activity of different kinases reported to mediate anti-apoptotic signals in other cell systems. The use of phosphoprotein-specific antibodies enabled activity measurements by Western analysis. As shown in Figure 6, treatment of cells with insulin resulted in very rapid but only transient increases in the phosphorylation of the extracellular signal-regulated kinases ERK1/2 (reflecting MAPK/ERK kinase activity), c-Jun (reflecting c-Jun N-terminal kinase activity) and PKB/Akt (reflecting PI(3) kinase activity). The effects were most pronounced for phospho-PKB/Akt which was increased from almost undetectable levels in untreated cells by a factor of $\sim$30-fold at 1 h after addition of the hormone. By contrast, phenobarbital and dexamethasone failed to induce the activation of either of these proteins. We subsequently studied by Western analysis the expression of Bax and Bcl-x_L, which are known to be key regulators of apoptosis. While neither Bax nor Bcl-x_L expression levels were affected by insulin or phenobarbital treatment of cells, dexamethasone led to a time-dependent increase in Bcl-x_L levels which reached a maximum (threefold increase) at 4 h after addition to the cultures and remained elevated throughout the entire 24 h observation period (Figure 6).

Activation of NF-κB by anti-apoptotic agents

The activity of the transcription factor NF-κB which mediates anti-apoptotic signals in different cell systems was analyzed by electrophoretic mobility shift assay (EMSA). For this purpose, a ^{32}P-labeled double-stranded oligonucleotide probe containing the NF-κB consensus sequence was incubated with total cell extracts and the extent of sequence-specific DNA binding was monitored. As a positive control we used tumor necrosis factor α (TNF) which has been shown to activate NF-κB in other cells. As expected, the DNA binding activity of the transcription factor was markedly enhanced following incubation of cells with TNF for 45 min (Figure 7). Increases in DNA binding were also seen in cells

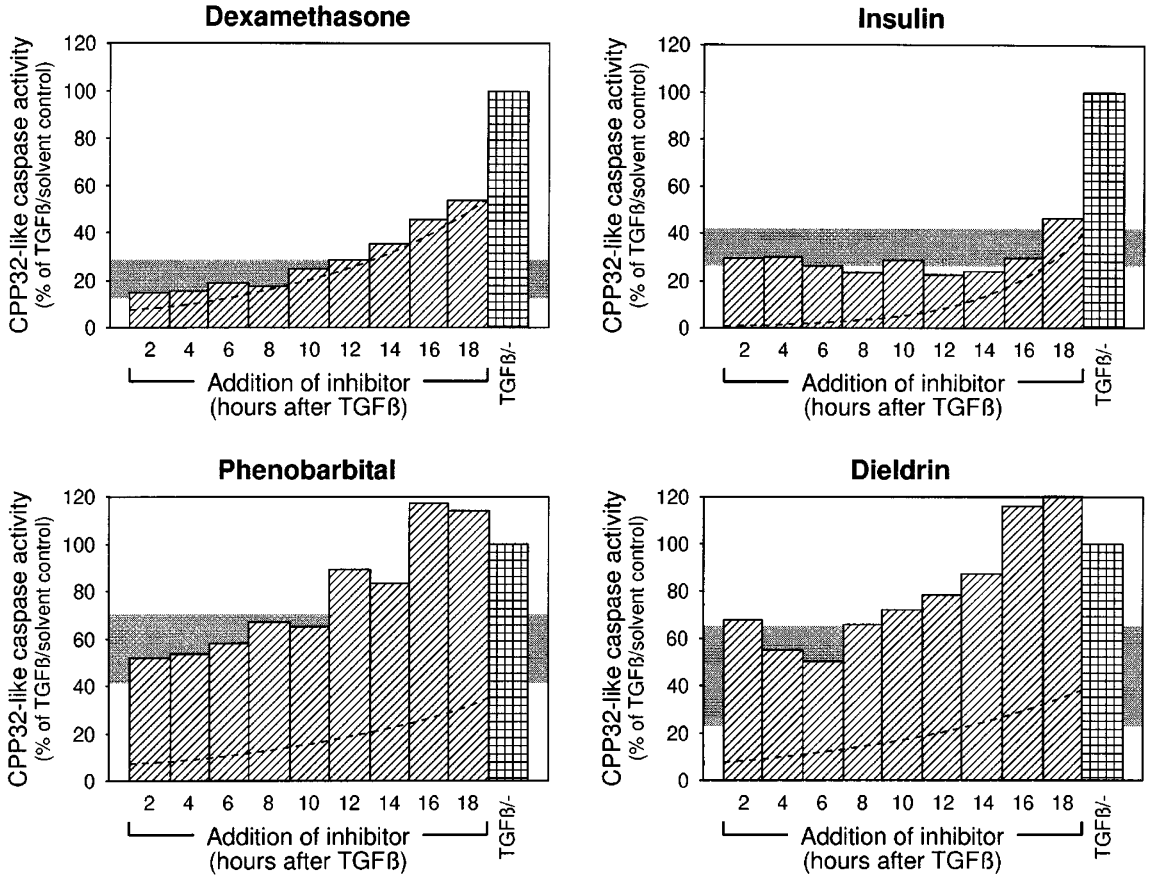

Figure 4 Time window of anti-apoptotic activity. FTO-2B cells were incubated with 5 ng/ml TGFβ and the anti-apoptotic agents indicated were added at various time intervals (2–18 h) thereafter. CPP32-like caspase activity (bars) was analyzed 24 h after TGFβ treatment and expressed as a percentage of TGFβ/solvent controls (TGFβ/−). Results are representative for two independent experiments. Grey areas indicate the range of inhibition of apoptosis observed in experiments where TGFβ and the anti-apoptotic agents were given simultaneously. Dashed lines show the kinetics of CPP32-like caspase activity determined at the times of inhibitor application in parallel cultures treated with TGFβ alone

treated with insulin, phenobarbital, clofibrate and dexamethasone. The extent and time course of changes, however, were clearly different for the various compounds analyzed (Figure 7). Insulin mediated a comparatively slight increase in NF-κB binding, which persisted for only about 90 min. Similarly, phenobarbital led to a rapid increase in transcription factor binding which persisted, however, throughout the entire observation period of 12 h. Most pronounced effects were seen in clofibrate treated cells, where a progressive enhancement of oligonucleotide binding was observed with length of incubation. Finally, dexamethasone slightly increased NF-κB DNA-binding, but only at the later incubation times of 6 and 12 h.

Discussion

Liver tumor promoters inhibit TGFβ-induced apoptosis and CPP32-like caspase activity

In our study we analyzed the effects of a variety of liver tumor promoters and hormone-like agents on TGFβ-induced apoptosis, on activation of CPP32 and related caspases which play a central role in the apoptotic process, and on different signal transduction pathways which are known to be involved in anti-apoptotic signaling. Inhibition of apoptosis by liver tumor promoters such as phenobarbital, nafenopin or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been shown to occur both *in vivo* and *in vitro*.[2,3,5,6,8–10] In addition, insulin and insulin-like growth factor I, which have also been implicated to play a role in liver tumor development, efficiently suppress apoptosis in different cell lines including hepatoma cells.[7,11,12] In extention of these findings, our results demonstrate that the tumor promoters clofibrate, dieldrin, and to a lesser extent DDT, are also effective inhibitiors of TGFβ-induced apoptosis as shown by analysis of internucleosomal DNA fragmentation. Moreover, dexamethasone, commonly known as an inducer of apoptosis in lymphocytes and other cells of the immune system, has been found in the present study to be an almost complete inhibitor of apoptosis in FTO-2B hepatoma cells. Similar protective effects on apoptosis of other cell types have been described recently.[28–30] Except for insulin, the anti-apoptotic activity of the various compounds persisted for at least 48 h, suggesting that the observed inhibition of apoptosis is not due to a shift in the time response to the apoptotic stimulus. The reason for the differential behavior of insulin is not clear; it may, however,

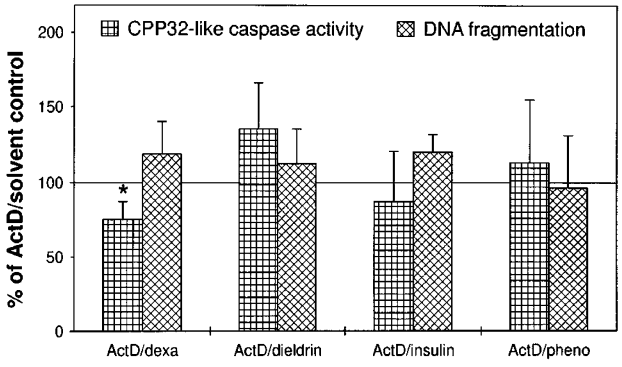

Figure 5 Effects of anti-apoptotic agents on ActD-induced DNA fragmentation and CPP32-like caspase activity. FTO-2B cells were incubated with 100 ng/ml ActD either alone or simultaneously with the anti-apoptotic agents indicated. CPP32-like caspase activity was analyzed 16 h and DNA fragmentation 24 h after treatment. Values for each treatment group are expressed as a percentage of the respective ActD/solvent controls. Bars represent means plus standard deviation from 3–4 experiments and asterisks mark significant differences from ActD/solvent controls (*$P<0.05$; Student's *t*-test)

be related to a downregulation of the insulin receptor or a negative feedback control of the insulin receptor pathway. This might also explain the apparently paradoxical effect of sensitization of TGFβ-treated hepatoma cells seen after prolonged insulin treatment, if one would assume that insulin signaling is essential for liver cell survival.

The apoptosis inhibitory compounds used in our study produce different initial subcellular effects: Insulin and dexamethasone signal via their specific hormone receptors and clofibrate is assumed to act via the peroxisome proliferator activated receptor. In contrast, no intracellular receptors have been identified so far for phenobarbital and the pesticides dieldrin and DDT. Due to their differences in primary subcellular interactions, the concentrations required for inhibition of apoptosis ranged from 2 mM (phenobarbital) to 10^{-8} M (dexamethasone), with the other agents lying in between. Unexpectedly, the clofibrate concentration of 1–2 mM required for inhibition of apoptosis was very high, suggesting that the anti-apoptotic activity of clofibrate in FTO-2B cells may not be mediated via activation of the peroxisome proliferator activated receptor, although this receptor has recently been shown to be involved in suppression of apoptosis of primary hepatocytes.[10]

The protective effects of liver tumor promoters may depend on the apoptotic stimulus and cell type used. For example, nafenopin has been shown to suppress TGFβ-mediated apoptosis of FaO rat hepatoma cells but failed to inhibit apoptosis induced by DNA-damaging agents,[6] while the opposite has been reported with respect to the anti-apoptotic action of TCDD and phenobarbital in primary rat hepatocytes.[9] Under certain conditions, the barbiturate may even induce apoptosis of immortalized mouse hepatocytes that overexpress a c-*myc* oncogene.[31] The reason for these discrepancies is unclear. While phenobarbital was active as an inhibitor of TGFβ-induced apoptosis of FTO-2B cells in the present study, the potent tumor promoter TCDD failed to exert anti-apoptotic activity in this cell line (data not shown), although this dioxin strongly inhibits apoptosis of rat hepatocytes *in vivo*[5] and in primary cultures.[9] The lack of anti-apoptotic activity in FTO-2B cells, however, is not unexpected, since these cells show a defect in the Ah receptor system, as indicated by a lack of TCDD-mediated induction of dioxin-responsive cytochromes P450 (own unpublished observation).

To further characterize the anti-apoptotic activity of the various compounds studied, we analyzed their effects on TGFβ-induced activation of CPP32-like caspases which play a central role in the 'execution' phase of apoptosis and become activated prior to the occurrence of internucleosomal DNA fragmentation and gross morphological alterations of apoptotic cells.[13,14,22,23] In contrast to ICE-like caspases, CPP32-like caspases are activated during TGFβ-induced apoptosis both in primary hepatocytes[26] and in hepatoma cells (own results). Under all experimental conditions tested, inhibition of TGFβ-induced apoptosis was associated with a decrease in CPP32-like caspase activity, an effect which was highly correlated with the extent of DNA fragmentation. Notably, in the experiments with ActD, where no inhibition of apoptosis was observed, there were also no or only minor alterations in CPP32-like activity. Since the various anti-apoptotic agents themselves did not interfere with the caspase activity when added directly to the assay, our results demonstrate that their anti-apoptotic signals converge upstream of activation of CPP32 or related caspases. In light of the simplicity of the assay, determination of CPP32-like caspase activity may therefore be used as a simple and sensitive parameter for quantitation of apoptosis under different treatment conditions. The sensitivity of this assay is further underlined by the fact that only a small proportion of cells showed apoptotic morphology when analyzed 16 h after TGFβ treatment, where a strong increase in CPP32-like activity was observed. At this time, apoptotic nuclei were present in $<10\%$ of TGFβ-treated cells *versus* $<1\%$ in untreated controls.

Which primary signals may be relevant for anti-apoptotic action in hepatoma cells?

The mechanism of suppression of TGFβ-induced apoptosis by the various agents is not well understood. Anti-apoptotic activity may result from transcriptional activation of genes coding for apoptosis inhibitory proteins or from interference with biochemical cascades involved in activation of proteins or other factors necessary for survival of the cells. The observation that ActD, an inhibitor of transcription, efficiently triggers FTO-2B cells into apoptosis suggests that the survival of these cells depends on the presence of a constitutively expressed component with a short half-life. When this component is depleted by a transcriptional block, cells enter apoptosis within a few hours. In accordance with this assumption, labile protective proteins have been postulated on the basis of data on liver cell apoptosis induced by the protein synthesis inhibitor cycloheximide.[32] Our observation that none of the various agents used in this study was able to interfere with ActD-induced apoptosis may suggest that their anti-apoptotic activity is mediated via transcriptional induction

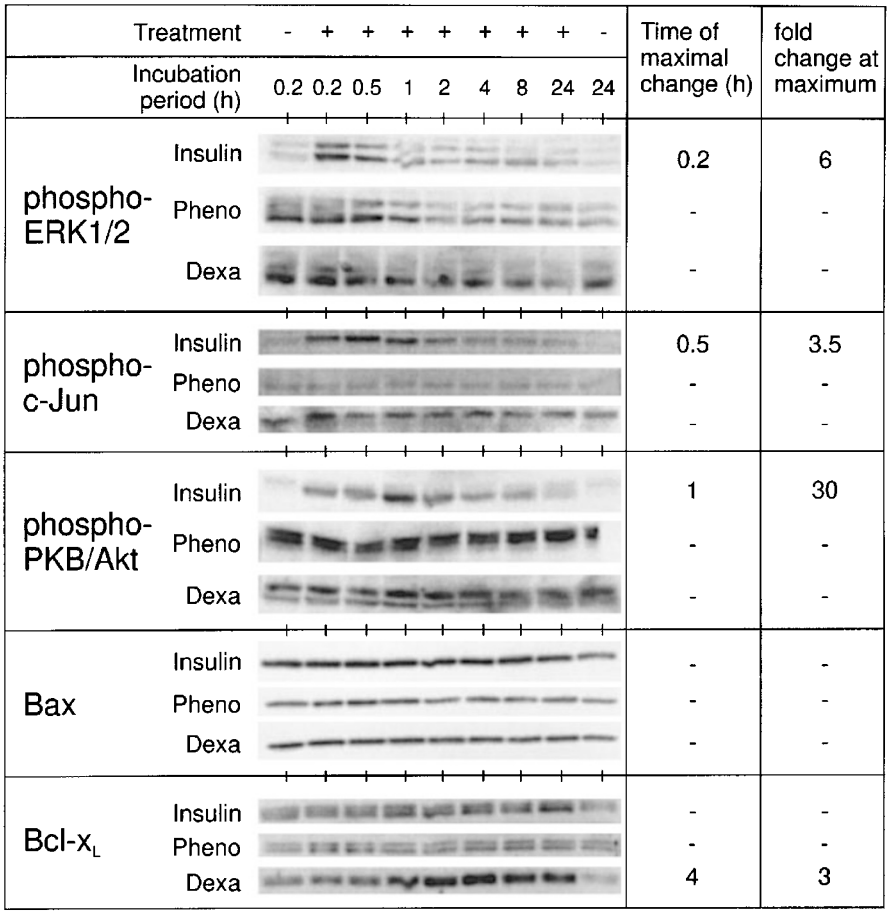

Figure 6 Subcellular responses mediated by anti-apoptotic agents. FTO-2B cells were incubated with 10^{-7} M insulin, 2 mM phenobarbital (Pheno), or 10^{-8} M dexamethasone (Dexa) for the indicated periods of time. Cellular extracts were separated by SDS–PAGE and proteins were detected by Western blotting using phosphospecific antibodies which allow the determination of the activity of kinases involved in the activation of ERK1/2, c-Jun and PKB/Akt, respectively. In addition, the expression of Bax and Bcl-x_L were analyzed. Signal intensities were quantified by image anlaysis and significant changes were expressed as fold change relative to values obtained with untreated control cells. Note that video camera exposure times were optimized for each individual experiment in order to allow for optimal detection of time-dependent changes within each treatment group; this advantage, however, impedes direct comparisons of signal intensities between the various treatments or proteins. In the case of PKB/Akt, the upper band seen after treatment of cells with phenobarbital and dexamethasone represents an unspecific signal, which is barely detectable in insulin-treated cells because of considerably shorter exposure times

of protective genes. Alternatively, it is tempting to speculate that their mode of action lies in the (presumably rapid) activation of a constitutively expressed survival/protection factor which is depleted in ActD-treated cells and is therefore not available for the anti-apoptotic action of the inhibitors. We have recently shown that ActD leads to a downregulation of Bcl-x_L protein levels in mouse hepatoma cells, while Bax levels remained unchanged, thus altering the ratio of anti- and pro-apoptotic members in favor of a pro-apoptotic state.[33] Since Bcl-2 family proteins may represent a possible target for the anti-apoptotic action of the different agents studied, we analyzed their effects on Bcl-x_L and Bax expression. While none of the apoptosis inhibitory compounds affected the expression of Bax, dexamethasone led to an about threefold increase in Bcl-x_L expression, which may contribute to the anti-apoptotic activity of the glucocorticoid. Although the other compounds did not interfere with the expression of either Bax or Bcl-x_L, they may modulate the activity of Bcl-2 family proteins, e.g. by activation of phosphatases or kinases that regulate interactions and functions of this class of proteins.[15,16,34–37]

TGFβ signals via transmembrane serine threonine receptor kinase-mediated activation of Smad proteins, which either directly or in complex with other proteins affect transcription of specific genes.[38] The TGFβ/Smad signal transduction pathway is likely to be inhibited by activation of ERK1/2 which are supposed to act at the level of the Smad proteins.[39] Activation of the insulin receptor by agonist binding leads to elevated ERK1/2 activity,[40] an effect also seen in this study with FTO-2B hepatoma cells. Direct interference with TGFβ-signaling by activation of MAP kinases may thus play some role in the case of insulin and could possibly explain the strong activity of the hormone to suppress TGFβ-induced apoptosis. The fact, however, that insulin – like all other agents tested – was still able to produce significant anti-apoptotic effects when added to the cell cultures more than 10 h after the TGFβ stimulus, i.e., long after primary signaling events and

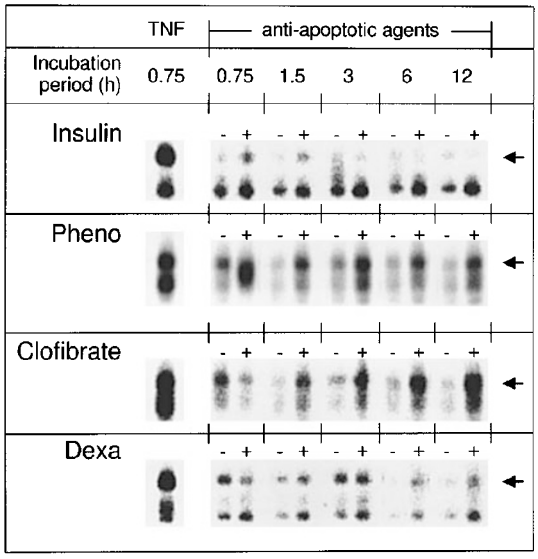

Figure 7 Activation of NF-κB by anti-apoptotic agents. The DNA binding activity of the transcription factor was analyzed by EMSA performed with extracts from untreated control cells or cells incubated for the indicated time periods with either of the anti-apoptotic compounds shown (for concentrations see legend to Figure 6; clofibrate, 1 mM). TNF-mediated response (10 ng TNF/ml) served as a positive control. Equal amounts of cellular extracts were incubated with ^{32}P-labeled oligonucleotides and the DNA-protein complexes were separated by polyacrylamide gel electrophoresis. The mobilities of the NF-κB-dependent DNA-protein complexes are indicated by arrows

transcriptional responses initiated by activation of the TGFβ receptor are assumed to be on the way, is not in favor of the assumption of an interference at an initial stage of TGFβ signaling. On the other hand, it should be noted that under the experimental conditions used cells may enter apoptosis assynchroneously, e.g. due to cell cycle dependence of the apoptotic response. Additional experiments using TGFβ-responsive reporter constructs or other parameters indicative of TGFβ-induced cell signaling will therefore be needed to further characterize possible interference with TGFβ signaling events. Studies addressing this issue are presently under way.

Opposing effects of ERK and c-Jun N-terminal MAP kinases on apoptosis have been described.[17] Treatment of FTO-2B cells with insulin led to an about sixfold increase in ERK1/2 activity, but only about threefold increase in c-*jun* phosphorylation, respectively, thus favoring the anti-apoptotic kinase pathway. Most pronounced effects of insulin (about 30-fold change) were seen with respect to PI(3) kinase activity, which is also involved in anti-apoptotic signaling.[18,19] This kinase acts through phosphorylation of the downstream serine/threonine kinase PKB/Akt, which has been demonstrated to provide a survival signal for cells, possibly by phosphorylation of Bad, a pro-apoptotic member of the Bcl-2 family.[36,37] Therefore, activation of diverse kinase cascades involved in mediating cell survival signals may be the prime mechanism of suppression of apoptosis of hepatoma cells by insulin. These pathways, however, do not appear to play a role for the anti-apoptotic action of the other compounds tested, namely dexamethasone and phenobarbital, which did not significantly affect the activity of the kinases determined. It should be noted that the different kinase pathways studied are also involved in regulation of cell replication. However, non of the anti-apoptotic agents used appeared to induce proliferation of FTO-2B cells, as estimated by total DNA and protein contents of the treated cultures (data not shown).

Another candidate mediating cell survival signals in several cell systems is NF-κB.[20] This transcription factor appears also relevant for regulation of liver cell apoptosis, since its ectopic expression ablates TGFβ-induced apoptosis of murine hepatocytes.[41] The activation of the transcription factor is initiated by phosphorylation of inhibitory IκB proteins which restrain NF-κB in the cytoplasm. IκB kinases have been shown to phosphorylate IκB proteins, but other kinases such as the mitogen-activated 90 kDa ribosomal S6 kinase also associate with and phosphorylate IκB.[42] This latter kinase can be activated via the Ras/Raf signal transduction pathway and is thus a downstream effector of the insulin receptor.[40] Activation of NF-κB was observed with all anti-apoptotic compounds tested in this study. While insulin and dexamethasone showed only moderate effects, both phenobarbital and clofibrate led to a significant and persistent activation of NF-κB in FTO-2B cells, which may, at least partly, contribute to their anti-apoptotic activity. Phenobarbital and ciprofibrate, a peroxisome proliferator closely related to clofibrate, have also been shown to activate the transcription factor in rat liver.[43,44] The mechanism of activation of NF-κB by phenobarbital and clofibrate is unclear; signaling via MAP kinases or PI(3) kinase does not appear to be involved since the barbiturate did not alter the activity of these kinases.

In summary, our results demonstrate that the primary signals that ultimately protect cells from apoptosis may be different for the various anti-apoptotic agents tested in this study. While insulin led to an activation of ERK1/2 kinases, PKB/Akt and NF-κB, dexamethasone showed no effect on kinase activities but slightly enhanced NF-κB DNA binding and increased the expression of Bcl-x_L. In contrast, phenobarbital and clofibrate stimulated the activity of the transcription factor NF-κB solely. Since all compounds produced anti-apoptotic activity even when given 10 h or later after TGFβ treatment, the present data suggest that, although the inhibitors used are diverse in structure and produce different initial cellular responses, their protective signals converge at a presumably late stage of apoptosis, but upstream of activation of CPP32 or related caspases.

Materials and Methods

Materials

Culture media were obtained from Biochrom, Berlin, Germany, fetal calf serum from GIBCO, Eggenstein, Germany, and culture dishes from Becton Dickinson, Heidelberg, Germany. Human transforming growth factor-β1 (TGFβ) and mouse tumor necrosis factor α (TNF) were from R&D Systems, Wiesbaden, Germany, phenobarbital from Geyer, Renningen, Germany, dichlorodiphenyltrichloroethane (DDT)

from EGA, Steinheim, Germany, and clofibrate, dieldrin, dexamethasone, insulin and actinomycin D (ActD) from Sigma, München, Germany.

Induction of apoptosis

FTO-2B cells (kindly provided by Dr. K. Kaestner) were maintained in a 1 : 1 mixture of Ham's F12 and modified Dulbecco's Eagle's medium containing 10% fetal calf serum in a humidified incubator (5% CO_2; 37°C). For all experiments, $4-5\times10^5$ cells were seeded in 3.5 cm culture dishes. After 24 h, cells were washed with PBS and incubated with fresh medium. For induction of apoptosis, 5 ng/ml TGFβ (1 μg/ml stock in 4 mM HCl/1% BSA) or 100 ng/ml ActD (100 μg/ml stock in PBS/4% ethanol) were added either alone or together with one of the following anti-apoptotic agents (final concentrations are given in parentheses): phenobarbital (2 mM), clofibrate (1–2 mM), dieldrin (25 μM), DDT (10^{-5} M), dexamethasone (10^{-8} M) or insulin (10^{-7} M). Clofibrate, dieldrin and DDT were dissolved in DMSO, phenobarbital in PBS, insulin in PBS/1% acetic acid and dexamethasone in PBS/10% ethanol. To keep solvent concentrations in the cultures as low as possible (0.1%), compounds were prepared as 1000× stocks, except for phenobarbital, where a 100× stock was used. Each experiment contained the following control incubations: (1) cells were treated with TGFβ or ActD and the solvents of the various anti-apoptotic agents (TGFβ/solvent or ActD/solvent controls); (2) cells were treated with the inhibitors or their solvents alone. Parallel incubations were used for analysis of DNA fragmentation and CPP32-like caspase activity.

In some experiments, cells were treated with TGFβ (same dose as above) and the various anti-apoptotic agents were added at different time intervals thereafter. Cells were analyzed 24 h after start of treatment with TGFβ. Parallel cultures, which were treated with TGFβ only, were used for determination of CPP32-like caspase activity present at the time of inhibitor application.

Determination of apoptotic nuclei

Cells grown on cover slips were fixed in ice-cold paraformaldehyde (3% in PBS), stained with bisbenzimide (Hoechst 33258; 10 μg/ml in PBS) for 10 min and evaluated under a fluorescence microscope.

Analysis of DNA fragmentation

For demonstration of internucleosomal DNA fragmentation which is characteristic for apoptotic cells, cytoplasmic DNA was isolated as previously described.[33] In brief, culture media containing non-adherent cells were collected; adherent cells were trypsinized, resuspended in fresh medium and combined with the culture supernatants. An aliquot of the combined cell suspension was used for determination of the total DNA content of the culture.[33] Cells were pelleted by centrifugation, lysed in 0.5% Triton X-100, 5 mM Tris, 20 mM EDTA, pH 7.4, and cell debris and nuclei were removed by centrifugation. Cytoplasmic DNA was extracted from the supernatants by phenol/chloroform extraction, precipitated with ethanol, treated with DNAse-free RNAse A and separated on 1.2% agarose gels. After ethidium bromide staining of DNA, the UV fluorescence was registered by use of a CCD video camera system (Raytest, Straubenhardt, Germany) and the fluorescence of the three smallest DNA fragments (~200–600 bp) was quantitated from the camera images using the software package TINA (V2.09g; Raytest). The fluorescence values of each sample were normalized to the amount of total DNA present in the culture and expressed as a percentage of the respective TGFβ/solvent or ActD/solvent controls.

Determination of CPP32-like caspase activity

For determination of CPP32-like caspase activity, culture media containing non-adherent cells were collected, cells were pelleted by centrifugation and suspended in 1 ml of lysis buffer (1 : 1 mixture of PBS with 50 mM Tris-HCl, pH 8, 120 mM NaCl, 5 mM EDTA, 0.5% Nonidet-P40). This suspension was then transferred onto the adherent cells in the culture dish and cells were lysed at 37°C for 10 min. The cell lysate was collected and centrifuged in a benchtop centrifuge (Biofuge 13, Heraeus, Germany) at maximum speed for 5 min. An aliquot of the supernatant was used for protein determination by the BCA protein assay (Pierce, Rockford, IL, USA). For measurement of CPP32-like caspase activity, 100 μl of the supernatant were diluted with 1.4 ml lysis buffer containing 25 μM DEVD-AFC (Biomol, Hamburg, Germany), incubated at 37°C and the fluorescence liberated at 505 nm (exitation 400 nm) was monitored in 15 min intervals for a total of 90 min using the Perkin Elmer LS-5B luminescence spectrophotometer. Under these conditions, there was a linear increase in fluorescence (see Figure 1D). To calculate CPP32-like caspase activity, the increase in fluorescence per hour was normalized to the protein contents of each sample and expressed as a percentage of the respective TGFβ/solvent or ActD/solvent controls.

Western analyses

Western analyses were performed essentially as recently described.[33] Cells adhering to the Petri dish were washed twice with PBS and lysed in a lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 10 mM sodium pyrophosphate, 25 mM sodium glycerophosphate, 2 mM sodium orthovanadate, 1 mM EGTA, 2 mM sodium fluoride, 0.3 μM aprotinin, 10 μM leupeptine, 1 μM pepstatin, 1 mM PMSF, pH 7.2) on ice, followed by sonication (5 s) and centrifugation (10 000 × *g*, 4°C, 10 min). Following protein determination using BCA (Pierce), protein concentrations were adjusted to yield optimal signals for each antibody used and diluted with 1 Vol. 2× Lämmli-buffer. Proteins (1 μg/lane for Bax and Bcl-x; 20–40 μg/lane for phosphospecific antibodies) were subsequently separated by SDS–PAGE and transferred to PVDF membranes (Bio-Rad, München, Germany). These were incubated with rabbit polyclonal antibodies detecting phospho-ERK1/2, phospho-c-Jun, phospho-PKB/Akt (all from New England Biolabs, Schwalbach, Germany), Bax (Santa Cruz Biotechnology, Heidelberg, Germany) or Bcl-x (PharMingen, Hamburg, Germany). After removal of unbound primary antibody, membranes were incubated with alkaline phosphatase-coupled goat anti-rabbit IgG secondary antibody (Dianova, Hamburg, Germany) and CDP-Star substrate (Tropix, Perkin Elmer, Langen, Germany) and chemiluminescence signals were monitored by use of a CCD camera system and quantitated by image analysis.

Electrophoretic mobility shift assay (EMSA)

Cells were plated at an initial density of 10^6 cells/well on 6-well plates. After 12 h, cells were washed once with PBS and cultured for at least 6 additional hours in medium without supplemental fetal calf serum. Serum was omitted from the cell incubations because components present in serum were found to lead to high background NF-κB activity. Cells were then treated with TNF, clofibrate, dexamethasone, insulin or phenobarbital for the indicated times, harvested by centrifugation and washed once in ice-cold PBS. Total cell extracts were prepared by resuspending the cell pellets in 60 μl of a high salt detergent buffer containing 20 mM HEPES, pH 7.9, 350 mM NaCl, 20% (v/v) glycerol, 1% (v/v) NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 2 mM PMSF, 50 μg/ml aprotinin, and

10 μg/ml leupeptin. After 10 min of incubation on ice the lysates were cleared by centrifugation and stored at -80°C. EMSA was carried out essentially as described by Pahl *et al.*[45]. The 5′-OH blunt end oligonucleotide with the high affinity NF-κB binding sequence (Promega, Mannheim, Germany) was labeled using T4 polynucleotide kinase and γ-[^{32}P]ATP (4500 Ci/mmol; ICN, Eschwege, Germany) followed by removal of non-incorporated nucleotides using the QiaQuick nucleotide removal kit (Qiagen, Hilden, Germany). The binding reactions were performed by adding equal amounts of the cell extracts containing about 10 μg of crude protein to 4 μl of 5 $\times$ binding buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 1 mM DTT, 2.5 mM $MgCl_2$, 20% (w/v) Ficoll), 2 μg poly(dI-dC) as a scavenger for nonspecific DNA-binding proteins, 2 μg of BSA, and 40 000 – 50 000 c.p.m. (Cerenkov counting) of the ^{32}P-labeled NF-κB specific oligonucleotide in a final volume of 20 μl. The reaction mixture was incubated at room temperature for 20 min. The samples were then separated on a 4% non-denaturing polyacrylamide gel run in 0.5 $\times$ TBE buffer at pH 8.3 for about 2 h. The dried gel was exposed for several hours to a bioimaging plate which was subsequently analyzed on the Fuji BAS 1500 bioimaging system (Raytest).

Statistical analysis

Student's *t*-test and Pearson's correlation analysis were performed using the GB-STAT^{TM} (V5.0) software package (Dynamic Microsystems, Silver Spring, MD, USA).

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