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Cytokine regulation of pro- and anti-apoptotic genes in rat hepatocytes: NF-κB-regulated inhibitor of apoptosis protein 2 (cIAP2) prevents apoptosis☆

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***Background/Aims*: In acute liver failure, hepatocytes are exposed to various cytokines that activate both cell survival and apoptotic pathways. NF-κB is a central transcription factor in these responses. Recent studies indicate that blocking NF-κB causes apoptosis, indicating the existence of NF-κB-regulated anti-apoptotic genes. In the present study the relationship between NF-κB activation and apoptosis has been investigated in hepatocytes.**

***Methods*: Primary rat hepatocytes were exposed to a cytokine mixture of tumor necrosis factor α, interleukin-1β, interferon-γ and lipopolysaccharide. Modulation of signalling pathways was performed by using dominant negative adenoviral constructs. Apoptosis and NF-κB activation were determined by caspase-3 activity, Hoechst staining and electrophoretic mobility shift assay, respectively. Furthermore, expression and regulation of apoptosis-related genes were investigated.**

***Results*: (1) Inhibition of NF-κB activation results in apoptosis. (2) Inhibitor of apoptosis protein (IAP) family members, inhibitor of apoptosis protein1 (cIAP1), and X-chromosome-linked IAP, are expressed in rat hepatocytes. cIAP2 is induced by cytokines in an NF-κB-dependent manner and overexpression of cIAP2 inhibits apoptosis. (3) The anti-apoptotic Bcl-2 family member A1/Bfl-1 and the pro-apoptotic members Bak and Bid are induced by cytokines and NF-κB-dependent. (4) Nitric oxide inhibits caspase-3 activity in hepatocytes.**

***Conclusions*: In inflammatory conditions, hepatocyte survival is dependent on NF-κB activation and cIAP2 contributes significantly to this protection.**

***Keywords*: Inflammation; Apoptosis; NF-κB; Adenovirus; Inhibitor of apoptosis protein family; Bcl-2 family; Nitric oxide**

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Abbreviations: TNF-α, tumor necrosis factor α; TNFR-1, TNF-α type I receptor; FADD, Fas-associated protein with death domain; TRADD, TNFR-associated death domain protein; TRAF2, TNFR-associated factor 2; RIP, receptor interacting protein; IAP, inhibitor of apoptosis protein; HIAP, human IAP; XIAP, X-chromosome-linked IAP; NO, nitric oxide; iNOS, inducible NO synthase; CM, cytokine mixture; IL-1β, interleukin-1β; IFN-γ, interferon-γ; ActD, Actinomycin-D; V-PYRRO/NO, O^2-vinyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate; L-NAME, N^G-nitro-L-arginine methyl ester; Ad5IκBAA, adenoviral dominant-negative IκB-α; Ad5LacZ, adenoviral β-galactosidase; Ad5dnFADD, adenoviral dominant-negative FADD; AdHIAP1, adenoviral human IAP1; EMSA, electrophoretic mobility shift assay.

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1. Introduction

In acute liver failure or acute viral hepatitis, hepatocytes are exposed to high levels of a variety of cytokines. These cytokines, in particular tumor necrosis factor α (TNF-α), activate both cell survival and apoptotic pathways in hepatocytes [1,2]. Trimerization of the TNF-α type I receptor (TNFR-1) by TNF-α results in docking of adaptor proteins such as Fas-associated protein with death domain (FADD), TNFR-associated death domain protein (TRADD), TNFR-associated factor 2 (TRAF2) and the serine/threonine kinase receptor interacting protein (RIP) [3]. Recruitment of FADD to TNFR-1-bound TRADD results in the activation of caspase-8 followed by the activation of effector caspases, in particular caspase-3, resulting in apoptosis [4].

TRADD is also a docking protein for TRAF2 and RIP. These proteins are involved in the induction of the NF-κB and Jun Kinase (JNK) survival pathways. The NF-κB survival pathway is initiated by the activation of NF-κB-inducing kinase (NIK). This protein activates an IκB kinase (IKK) complex, which results in the specific phosphorylation and proteasomal degradation of the inhibitor of NF-κB, IκBα. The release of NF-κB is followed by its migration to the nucleus, where it activates the transcription of NF-κB-responsive genes [5].

Blocking of the NF-κB pathway in TNF-α-stimulated hepatocytes results in a shift towards apoptosis. This implies the existence of NF-κB-regulated anti-apoptotic genes [6–8]. Recent data suggest that members of the inhibitor of apoptosis protein (IAP) family may represent these genes. In different cell types, but not yet in hepatocytes, it has been demonstrated that the expression of members of the IAP family is regulated by NF-κB [9–11]. The IAP family was originally discovered in baculovirus and subsequently identified in human cells. This family includes cIAP2 (also known as human IAP1: HIAP1), cIAP1 (also known as human IAP2: HIAP2), X chromosome-linked IAP (XIAP), Survivin [11] and Livin [12]. These proteins have been reported to directly bind and inhibit the activation of caspase-3, -7 and -9. Signalling initiated from caspase-8 is therefore blocked at the effector caspase level [13]. Another family which is also critical in regulating cell death is the Bcl-2 protein family [14–16]. This family consists of pro- (e.g. Bak, Bid and Bax) and anti-apoptotic (e.g. Bcl-2, Bcl-XL and A1/Bfl-1) members, which together regulate the integrity of the mitochondrial membrane [17,18]. The regulation of the Bcl-2 family by cytokines has been investigated in different cell types, but little is known about its expression and regulation in hepatocytes.

Finally, inducible nitric oxide synthase (iNOS) may act as an anti-apoptotic gene. Protection against TNF-α-induced apoptosis has been achieved by nitric oxide (NO) [19–21]. NO is a product of iNOS, which has been found to be an NF-κB-regulated gene [22]. Although the inhibitory effect of exogenous nitric oxide on caspase activity has been demonstrated before [20], it is not clear whether inhibition of iNOS activity would result in apoptosis in cytokine-exposed hepatocytes.

In this study we want to investigate the relationship between NF-κB activation and apoptosis in hepatocytes. To mimick acute liver inflammation, in this study a mixture of cytokines is used containing, TNF-α, interleukin-1β (IL-1β), interferon-γ (IFN-γ) and lipopolysaccharide. The results of our study demonstrate that in inflammatory conditions hepatocyte survival is dependent on activation of NF-κB. The NF-κB-regulated gene cIAP2 inhibits caspase-3 activity and prevents apoptosis.

2. Materials and methods

2.1. Animals

Specified pathogen-free male Wistar rats (220–250 g) were purchased from Harlan, Zeist, The Netherlands. They were housed under standard laboratory conditions with free access to standard laboratory chow and water. The study as presented was approved by the local Committee for Care and Use of Laboratory Animals.

2.2. Hepatocyte isolation and experimental design

Hepatocytes were isolated as described previously [23]. Cell viability was consistently more than 90% as determined by trypan blue exclusion. Isolated hepatocytes were plated at a density of 125 000 cells per cm^2 in William's medium E (Life Technologies Ltd., Breda, The Netherlands) supplemented with 50 μg/ml gentamycin, 2 μg/ml fungizone (Biowhittaker, Verviers, Belgium) and 20 mU/ml insulin (Novo Nordisk, Bagsvaerd, Denmark). During the attachment period (4 h) 50 nmol/l dexamethasone (Sigma, St. Louis, MO) and 5% FCS (Life Technologies Ltd.) were added to the medium. Cells were cultured in a humidified incubator at 37°C/5% CO_2. Experiments were started 24 h after isolation. Hepatocytes were exposed to a cytokine mixture (CM) composed of 20 ng/ml recombinant mouse TNF-α (R&D Systems, Abingdon, UK), 10 ng/ml recombinant human IL-1β (R&D Systems), 100 U/ml recombinant rat IFNγ (Life Technologies Ltd.) and 10 μg/ml LPS (*Escherichia coli*, serotype 0127:B8, Sigma, St. Louis, MO). Fifteen hours prior to CM exposure, cells receiving adenoviral constructs were infected with a Multiplicity of Infection of 10 (as determined by plaque assay). In the case of double virus infections, cells received each virus at an MOI of 10 simultaneously. Thirty minutes prior to addition of cytokines, some cultures were exposed to 200 ng/ml of the transcriptional inhibitor Actinomycin-D (ActD) or 250 μmol/l NO-donor V-PYRRO/NO [24], or 2.5 mmol/l of the aspecific NOS-inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, Sigma). Each experimental condition was performed in triplicate wells. Two hours after addition of CM, cells were harvested for preparation of nuclear extracts. Ten hours after the addition of CM or at the indicated time-points in the time course study, cells were harvested and rinsed three times with ice-cold phosphate buffered saline prior to the addition of Trizol reagent (RNA isolation) (Life Technologies Ltd.) or hypotonic cell lysis buffer (protein analysis). This buffer consisted of 25 mmol/l HEPES (Sigma), 5 mmol/l $MgCl_2$ (MERCK, Darmstadt, Germany), 5 mmol/l EDTA (Sigma), 2 mmol/l PMSF (Sigma), 10 μg/ml Pepstatin A (Roche Biochemicals, Almere, The Netherlands) and 10 μg/ml Leupeptin (Roche Biochemicals), pH 7.5. Cells were stored at −80 (RNA) or −20°C (protein). Each experiment was performed three times, using hepatocytes from different isolations.

2.3. Adenoviral constructs

Recombinant, replication-deficient adenovirus Ad5IκBAA was used to inhibit NF-κB activation as described previously [25]. This adenovirus

Table 1
Oligonucleotide primers used for the analysis of pro- and anti apoptotic genes by RT-PCR

Primers (species)	Sense and antisense	PCR product (bp)	# cycles	Annealing temperature (°C)
cIAP2 (rat)	5′-ACATTTCCCCAGCTGCCCATTC-3′ 5′-CTCCTGCTCCGTCTGCTCCTCT-3′	622	30	60
cIAP1 (rat)	5′-CCAGCCTGCCCTCAAACCCTCT-3′ 5′-GGGTCATCTCCGGGTTCCCAAC-3′	502	30	61
XIAP (rat)	5′-CGCGAGCGGGGTTTCTCTACAC-3′ 5′-ACCAGGCACGGTCACAGGGTTC-3′	510	28	61
iNOS (rat)	5′-CGAGGAGGCTGCCTGCAGACTGG-3′ 5′-CTGGGAGGAGCTGATGGAGTAGTA-3′	1383	26	60
Bcl-2 (rat)	5′-GCTACGAGTGGGATACTGGAGA-3′ 5′-AGTCATCCACAGAGCGATGTT-3′	446	30	58
Bcl-XL (rat)	5′-GCATATCAGAGCTTTGAACAGGT-3′ 5′-CTTTCACAGAAGCGTGGTAGATT-3′	534	30	56
Bak (mouse)	5′-TCTCCACCACGACCTGAAAAAT-3′ 5′-GATATCAGCCAAAAAGCAGGTC-3′	494	30	56
Bid (mouse)	5′-AGTCAGGAAGAAATCATCCACAA-3′ 5′-CTCCTCAGTCCATCTCGTTTCTA-3′	361	30	58
Bax (rat)	5′-AGGATGATTGCTGATGTGGATAC-3′ 5′-CACAAAGATGGTCACTGTCTGC-3′	300	30	56
A1/Bfl-1 (rat)	5′-ATCCACTCCCTGGCTGAGAACT-3′ 5′-ACATCCAGGCCAATCTGCTCTT-3′	311	30	56
GAPDH (rat)	5′-CCATCACCATCTTCCAGGAG-3′ 5′-CCTGCTCACCACCTTCTTG-3′	576	22	58

contains a construct driven by the cytomegalovirus promoter-enhancer in which IκBα has been mutated at serines 32 and 36. Therefore, mutant IκBα cannot be phosphorylated and binds NF-κB irreversibly, preventing its activation. As a control virus Ad5LacZ was used, which contains the *E. coli* β-galactosidase gene. The Ad5dnFADD expresses a FADD mutant lacking the death effector domain. It is therefore unable to bind caspase-8 [26]. The AdHIAP1 virus contains human IAP1 which is the human homologue of rat cIAP2 [27].

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared using a final concentration of 0.25% Nonidet P-40 as described previously [28]. EMSA for NF-κB was performed as described previously [28].

2.5. Caspase-3 enzyme activity assay

Caspase-3 enzyme activity was assayed in cells using a caspase-3 activity kit with fluorimetric detection (Promega) according to the manufacturer's instructions. 20 μg of protein was used.

2.6. Microscopic determination of apoptosis

Hoechst 33342 (Sigma-Aldrich Chemie, Schnelldorf, Germany) was used to detect apoptotic nuclei in hepatocytes. Hepatocytes were seeded on glass coverslides and exposed to CM with or without ActD or Ad5Iκ BAA. Fifteen hours after addition of CM, cells were incubated with 4.7 μg/ml Hoechst 33342 for 5 min at 37°C/5% CO_2. Glass coverslides were rinsed twice in HBSS (Life Technologies Ltd.) and placed upside down on microscope slides. Fluorographs were visualized and monitored using a Leitz fluorescence microscope.

2.7. RNA isolation and reverse-transcriptase polymerase chain reaction (RT-PCR)

RNA was isolated using the Trizol method (Life Technology Ltd.) according to the manufacturer's instructions. Reverse transcription was performed on 5 μg of total RNA using random primers in a final volume of 75 μl (Reverse Transcription System, Promega, Madison, WI). Each PCR was performed as described previously [29]. PCR primers are listed in Table 1. For every PCR, GAPDH was used as internal control. Each PCR product was loaded on a 2% agarose gel and stained with ethidium bromide.

2.8. Western blot analysis

Hepatocytes were scraped and cell lysates were obtained by three cycles of freezing (−80°C) and thawing (37°C) followed by centrifugation for 5 min at 13 000 rpm. Western blot analysis for iNOS was performed as described before [30]. Blots were incubated with a dilution of 1:1000 of rabbit anti-rat iNOS antibody [30].

2.9. Statistical analysis

Results are presented as the mean ± standard deviation. One-way analysis of variance and Student-Newman–Keuls test were used to determine the significance of differences between experimental groups. A P value of less than 0.05 ($P < 0.05$) was considered to be statistically significant.

3. Results

3.1. Specific inhibition of the NF-κB pathway results in apoptosis

To investigate the relationship between NF-κB activation and caspase-3 activity, hepatocytes were incubated with a cytokine mixture (CM) for 10 h with or without adenoviral constructs that selectively inhibit various signalling pathways. Functionality of Ad5IκBAA and Ad5dnFADD was demonstrated by EMSA and caspase-3 assay, respectively. In the presence of CM alone, NF-κB was clearly activated as determined by EMSA (Fig. 1). The activation of NF-κB

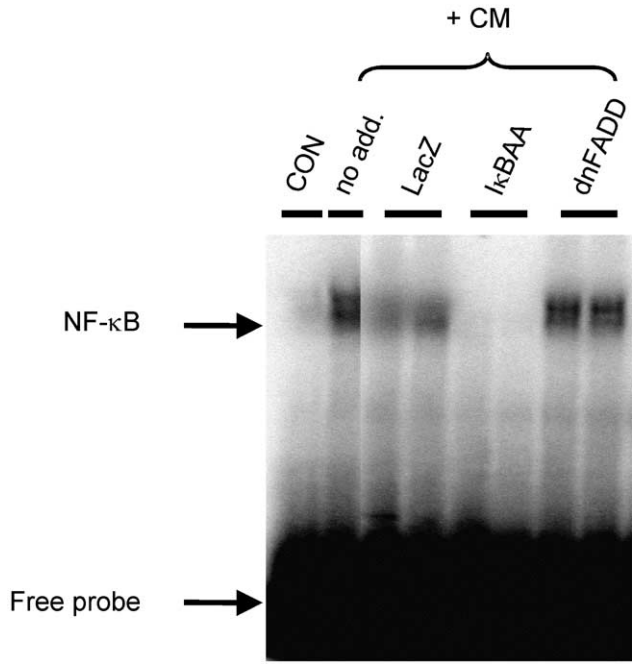

Fig. 1. NF-κB activation is FADD-independent and inhibited by Ad5IκBAA. Cultured rat hepatocytes were treated as indicated in the figure. NF-κB activation was measured by EMSA. One or two representative samples per condition are shown. (Con = control hepatocytes; no add. = no additives; CM = cytokine mixture; Ad5dnFADD and Ad5IκBAA express mutated FADD, and IκB-α, respectively; Ad5LacZ = control virus).

was inhibited by the dominant negative IκB-construct, whereas control (LacZ) virus had no effect.

Next, the corresponding effect on caspase-3 activity was determined. A positive control experiment was performed with CM in the presence of ActD, which induces apoptosis in many cell types, including hepatocytes. The induction of caspase-3 activity was FADD-dependent, as shown in Fig. 2A. ActD alone or CM alone had no effect on caspase-3 activity compared to control hepatocytes (data not shown). Specific inhibition of NF-κB activation by Ad5IκBAA resulted in FADD-dependent induction of caspase-3 activity. Control (LacZ) virus had no effect on caspase-3 activity. Caspase-3 activity started to rise at least 3 h after cytokine addition in hepatocytes with a blocked NF-κB pathway (Fig. 2B). Caspase-3 activity peaked at 6 h and returned to normal 24 h after cytokine addition.

To confirm that activation of caspase-3 activity results in apoptosis, staining with Hoechst 33342 was performed to detect apoptotic nuclei (Fig. 3). Nuclear fragmentation and condensation of chromatin were observed in many hepatocytes exposed to CM in the presence of actinomycin-D or Ad5IκBAA, but not in control hepatocytes or hepatocytes exposed to CM alone.

These data indicate the importance of NF-κB-regulated transcription of anti-apoptotic genes that inhibit caspase-3 activity. Therefore, we investigated their expression and their effect on caspase-3 activity. In order to approximate the in vivo situation, experiments were performed in the presence of a mixture of cytokines (CM).

3.2. cIAP-2 is an NF-κB-regulated gene and prevents apoptosis in hepatocytes

IAP protein family members are potent inhibitors of caspases and may play an important role in maintaining the pro-/anti-apoptotic balance after NF-κB activation. Both cIAP1 and XIAP, but not cIAP2 are clearly expressed in freshly isolated rat hepatocytes corresponding to hepatocytes in vivo (Fig. 4A). However, cIAP2 expression is strongly increased 3 h after cytokine addition (Fig. 4B) preceding the 6-h-peak of caspase-3 activity. Elevated cIAP2 expression persists at least 12 h after cytokine exposure. cIAP1 and XIAP expression are not increased by cytokines.

Experiments on hepatocytes exposed to CM alone or in the presence of Ad5IκBAA reveal that cIAP1 and XIAP are not regulated by NF-κB (Fig. 4C), whereas the cytokine-induced expression of cIAP2 is NF-κB dependent. To demonstrate that cIAP2 is able to inhibit caspase-3, hepatocytes were incubated with CM alone or in the presence of Ad5IκBAA and/or AdHIAP1 (Fig. 5). The increase in

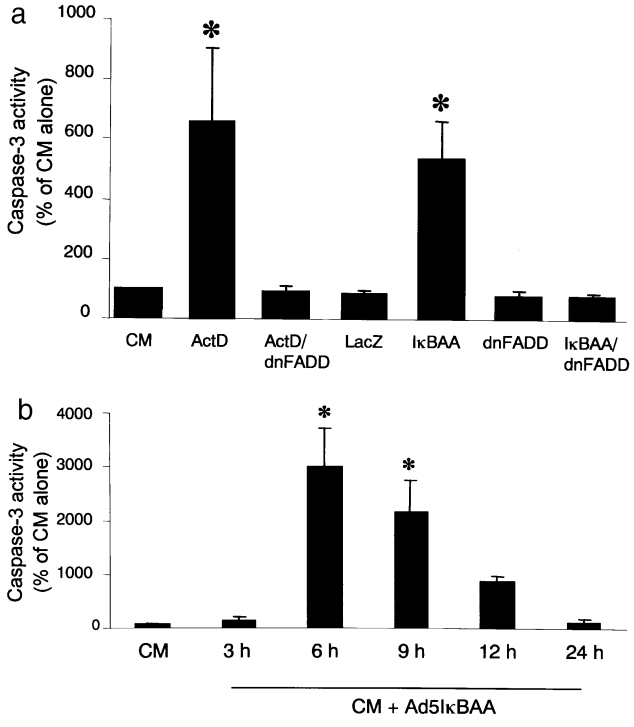

Fig. 2. Specific inhibition of NF-κB results in a FADD-dependent increase of caspase-3 activity, peaking at 6 h after CM exposure. (A) Cultured rat hepatocytes were treated as indicated in the figure. All conditions were performed in the presence of CM. (B) Time course study on hepatocytes exposed to CM for 3, 6, 9, and 12 h and the adenoviral construct Ad5IκBAA. Caspase-3 enzyme activity is shown as percentage of CM alone. The data represent mean of three independent experiments ± SD. *$P < 0.05$ (compared with other groups). (CM = cytokine mixture; ActD = Actinomycin-D; adenoviral constructs (Ad5) expressing dnFADD, and IκBAA; LacZ = control virus).

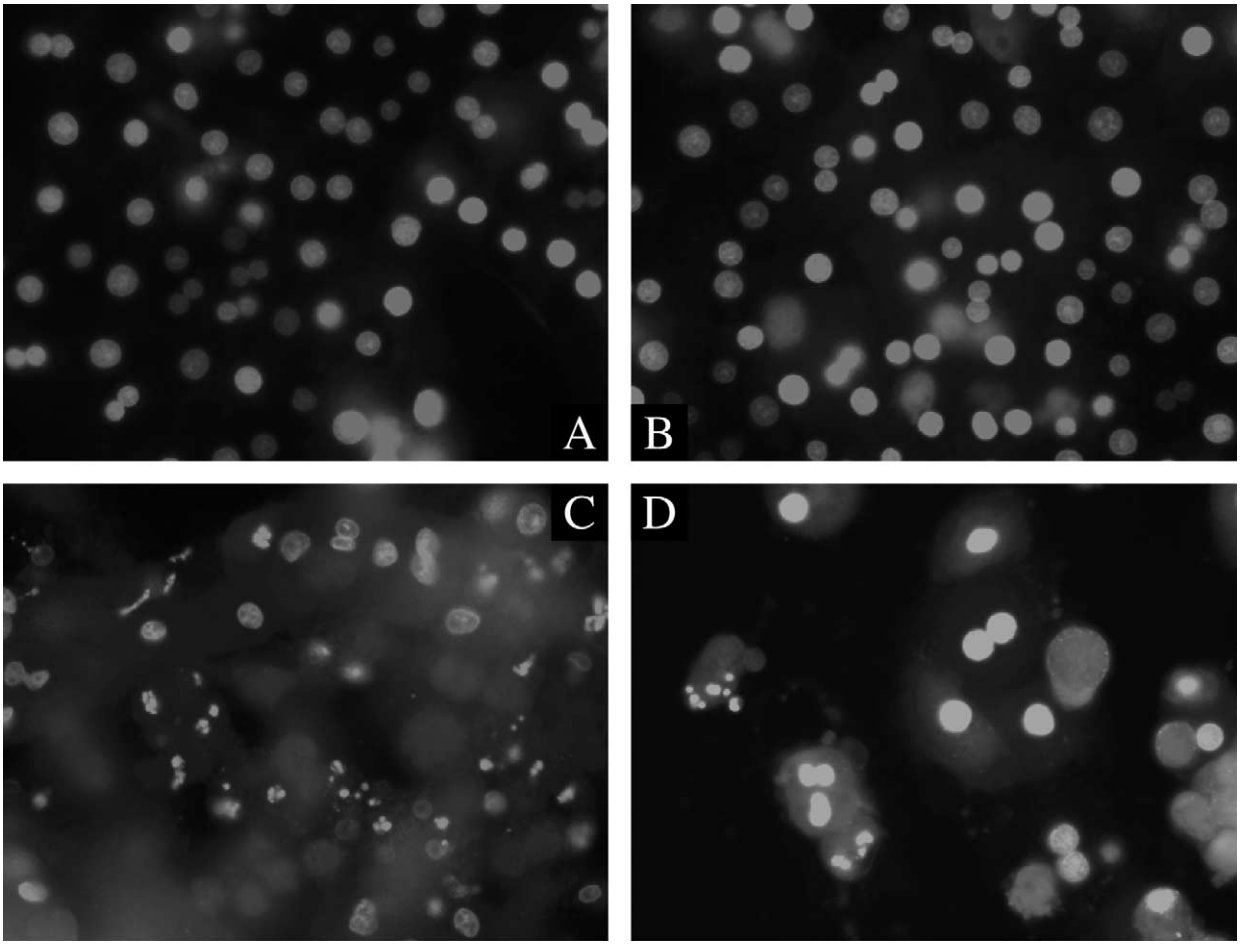

Fig. 3. Inhibition of NF-κB results in apoptotic nuclei. Control hepatocytes (A); hepatocytes exposed to CM (B); CM + ActD (C); and CM + Ad5IκBAA (D) were stained by Hoechst 33342 for determination of nuclear morphological alterations. The original magnification of all panels is 400 ×.

caspase-3 activity obtained with CM + Ad5IκBAA is completely prevented by AdHIAP1, demonstrating inhibition of caspase-3 by cIAP2 in hepatocytes.

3.3. Anti-apoptotic A1, and Pro-apoptotic Bak and Bid are NF-κB regulated genes

Since the Bcl-2 family plays an important role in regulating apoptosis, the regulation of Bcl-2 family members by cytokines in hepatocytes was investigated. With the exception of Bcl-2, messenger RNA (mRNA) levels of all investigated Bcl-2 family members were identical in freshly isolated hepatocytes, and primary cultured hepatocytes (data not shown). Bcl-2 expression was only shown in late cultured hepatocytes, but not in freshly isolated and early cultured hepatocytes. This allowed further investigation of these Bcl-2 family members, whereas Bcl-2 was not examined in detail. Exposure of hepatocytes to CM induced the expression of anti-apoptotic A1/Bfl-1 and pro-apoptotic Bak and Bid. This induction is abrogated in the presence of Ad5IκBAA, indicating that these genes are NF-κB-dependent. CM had no effect on anti-apoptotic Bcl-XL and pro-apoptotic Bax (Fig. 6A). mRNA expression of A1/Bfl is already increased 3 h after CM exposure (Fig. 6B) preceding the peak of caspase-3 activity and it is still elevated 12 h after CM exposure. Although Bid and Bak expression followed approximately the time-course of A1/Bfl, the induction of Bak expression was somewhat later.

3.4. Study on iNOS and its anti-apoptotic role in hepatocytes

Since exogenous NO is able to inhibit apoptosis in various cell types, in the present report the regulation of iNOS and its importance as an anti-apoptotic protein were investigated. Using Ad5IκBAA, we confirm that the cytokine-induced expression of iNOS in hepatocytes is NF-κB-dependent both at the mRNA and protein level (Fig. 7A). Control (LacZ) virus has no effect on iNOS expression. mRNA expression of iNOS is clearly induced after 3 h of CM exposure and remains unchanged after 12 h of CM addition (Fig. 7B).

Addition of V-PYRRO/NO to hepatocytes exposed to CM and ActD resulted in a 50% decrease of caspase-3 activity (Fig. 8). This confirms previous reports [20,21] and demonstrates that NO inhibits caspase-3 activity. Inhibition of iNOS

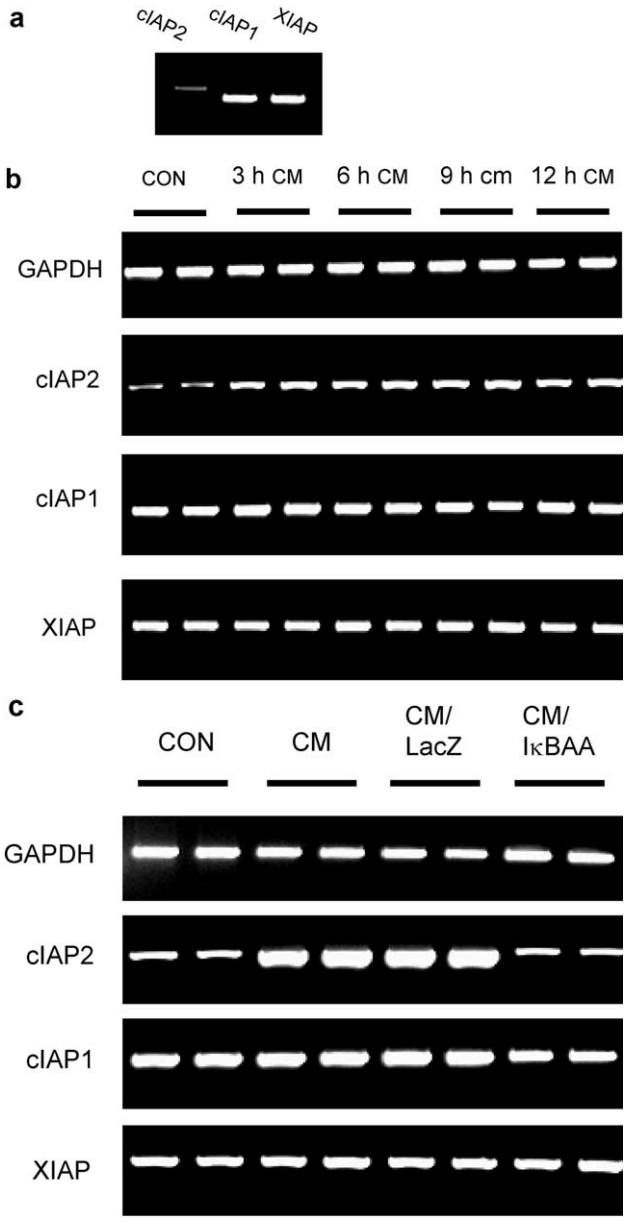

Fig. 4. cIAP2 is induced 3 h after cytokine exposure and NF-κB dependent. (A) cIAP2, cIAP1 and XIAP mRNA expression in freshly isolated rat hepatocytes. (B) mRNA expression of GAPDH, cIAP2, cIAP1 and XIAP in a time course study on cultured hepatocytes exposed to CM for 3, 6, 9, and 12 h. (C) mRNA expression of GAPDH, cIAP2, cIAP1 and XIAP in cultured rat hepatocytes treated with or without CM in the presence of adenoviral (Ad5) LacZ or IκBAA. Expression of mRNA was determined by RT-PCR. One of three representative experiments of $n = 2$ per condition is shown. (Con = control hepatocytes; CM = cytokine mixture; Ad5IκBAA express mutated IκB-α; Ad5LacZ = control virus).

activity in CM-exposed hepatocytes by L-NAME did not increase caspase-3 activity, as shown in Fig. 8.

3.5. Comparison of cytokine mixture- to TNF-α-induced gene expression

Since TNF-α alone is often used to study apoptosis in hepatocytes, we compared TNF-α and CM-induced expression of the NF-κB-regulated genes (Fig. 9). TNF-α and CM induced the expression of anti-apoptotic cIAP2 to the same extent. The expression of anti-apoptotic A1/Bfl is more strongly induced by CM compared to TNF-α alone, whereas iNOS and Bak are hardly induced by TNF-α.

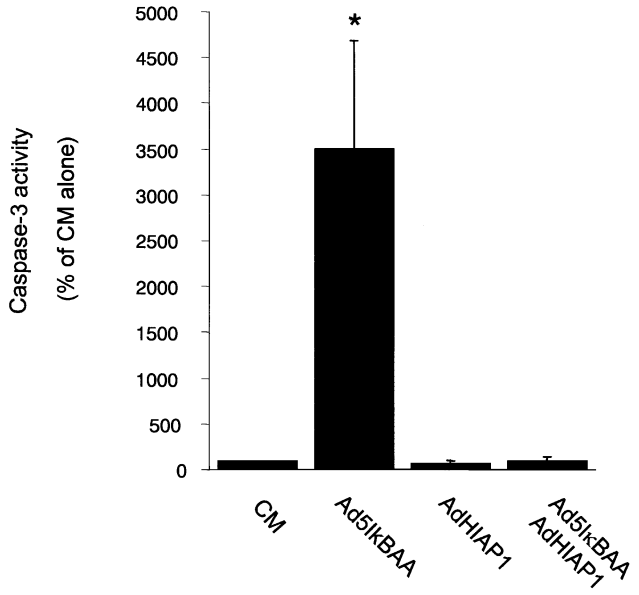

Fig. 5. cIAP2 prevents apoptosis in hepatocytes. Cultured rat hepatocytes were treated as indicated in the figure. All conditions were performed in the presence of CM. Caspase-3 activity is shown as percentage of CM alone. The data represent mean of three independent experiments ± SD. *$P < 0.05$ (compared with other groups). (CM = cytokine mixture; Ad5IκBAA express mutated IκB-α; AdHIAP1 express HIAP1; Ad5LacZ = control virus).

4. Discussion

In acute liver failure or acute viral hepatitis, hepatocytes are exposed to high levels of a variety of cytokines, such as TNF-α, IL-1β, IFN-γ and LPS. These cytokines simultaneously activate both survival and apoptotic pathways and it depends on the level of pro- and anti-apoptotic activities whether the balance will tip to one side or the other. Unlike Fas, cytokines like TNF-α are by themselves not sufficient to induce apoptosis [6]. Disturbance of NF-κB activation, either by ActD or an adenovirus expressing a mutated IκB-α, results in a shift towards apoptosis, as shown here by an increased FADD-dependent caspase-3 activity and nuclear fragmentation. Our study demonstrates that the anti-apoptotic effect of NF-κB is, to a large extent, due to the NF-κB-dependent transcription of the anti-apoptotic cIAP2 which inhibits caspase-3 activity.

The NF-κB-dependence of rat cIAP2 in hepatocytes correlates with findings in other cell types [9,31,32]. Overexpression of the human homologue of rat cIAP2 (HIAP1) prevents caspase-3 activation in hepatocytes in which NF-

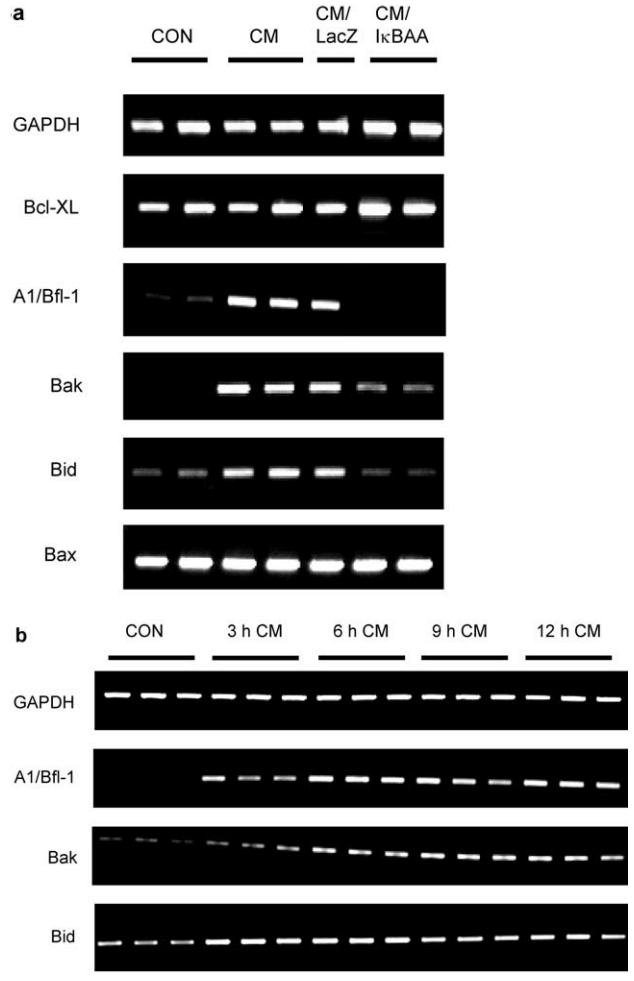

Fig. 6. Anti-apoptotic A1/Bfl, and Pro-apoptotic Bak and Bid are induced by cytokines in an NF-κB-dependent manner. (A) Cultured rat hepatocytes were treated with or without CM in the presence of adenoviral (Ad5) LacZ or IκBAA. mRNA expression of GAPDH, anti-apoptotic Bcl-XL and A1/Bfl-1, and pro-apoptotic Bak, Bid and Bax was determined by RT-PCR. One of three representative experiments of $n = 2$ per experimental condition is shown. (B) A time course study on hepatocytes exposed to CM for 3, 6, 9, and 12 h. mRNA expression of A1/Bfl, Bak and Bid and GAPDH was determined. One of three representative experiments of $n = 3$ per experimental condition is shown. (Con = control hepatocytes; CM = cytokine mixture; Ad5IκBAA express mutated IκB-α; Ad5LacZ = control virus).

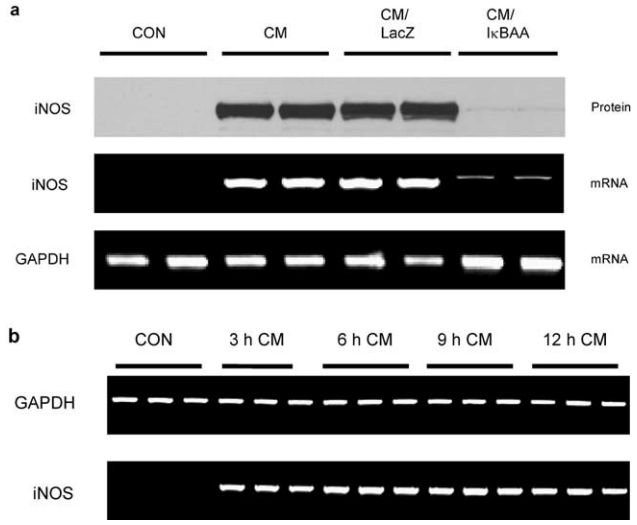

Fig. 7. iNOS is induced 3 h after cytokine addition and NF-κB-dependent. (A) iNOS protein and mRNA (iNOS and GAPDH) expression in cultured rat hepatocytes treated with or without CM in the presence of adenoviral (Ad5) LacZ or IκBAA. One of three representative experiments of $n = 2$ per condition is shown. (B) mRNA expression of iNOS and GAPDH in a time course study on hepatocytes exposed to CM for 3, 6, 9, and 12 h. One of three representative experiments of $n = 3$ per condition is shown. (Con = control hepatocytes; CM = cytokine mixture; Ad5IκBAA express mutated IκB-α; Ad5LacZ = control virus).

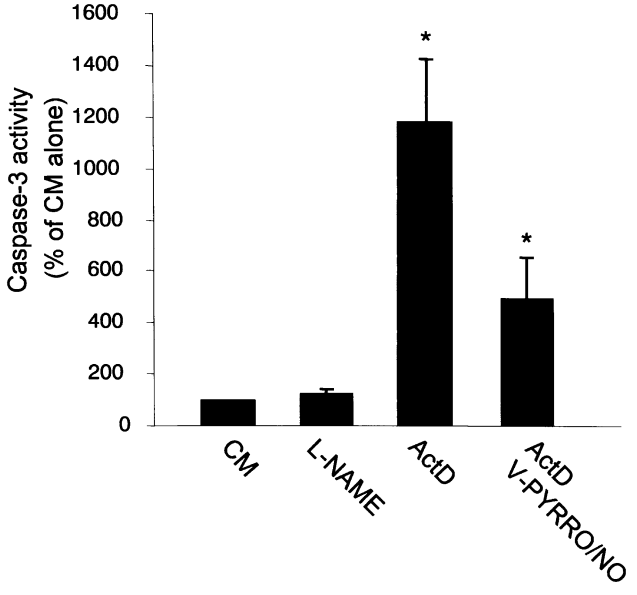

Fig. 8. Inhibition of iNOS does not affect caspase-3 activity, whereas exogenous NO is anti-apoptotic. Hepatocytes were treated as indicated in the figure. All conditions were performed in the presence of CM. Caspase-3 activity is shown as percentage of CM. The data represent mean of three independent experiments ± SD. *$P < 0.05$ (compared with other groups). (Con = control hepatocytes; CM = cytokine mixture; ActD = Actinomycin-D; L-NAME = inhibitor of iNOS; V-PYRRO/NO = NO-donor).

κB activation is prevented and cIAP2 expression is absent. These results clearly demonstrate the importance of cIAP2 in inhibiting apoptosis: in cytokine-exposed hepatocytes in which NF-κB activation is blocked, cIAP1 and XIAP are normally expressed, whereas expression of cIAP2 is abolished. Furthermore, the time course of cIAP2 mRNA induction precedes the activation of caspase-3. Our findings correlate with observations in neurons where overexpression of cIAP2 delays cell death [27,33]. Although cIAP1 (HIAP2) and XIAP are not NF-κB-dependent in hepatocytes, XIAP and cIAP1 are NF-κB-regulated in human endothelial cells [11,34] and human skin epithelial cells [35] and in the human fibrosarcoma cell line HT1080 [10].

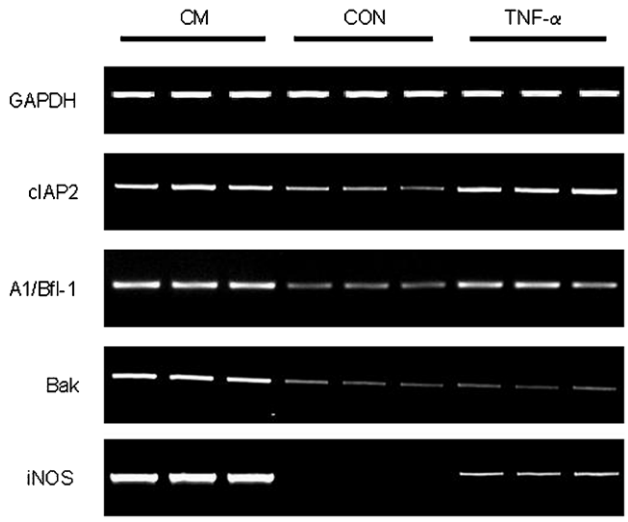

Fig. 9. Comparison of CM- to TNF-α-induced NF-κB-regulated gene expression. Hepatocytes were treated as indicated in the figure. mRNA expression of GAPDH, anti-apoptotic cIAP2 and A1/Bfl-1, and iNOS, and pro-apoptotic Bak, was determined by RT-PCR. One of three representative experiments of $n = 3$ per experimental condition is shown. (Con = control hepatocytes; CM = cytokine mixture; TNF-α = tumor necrosis factor α).

Little is known about the regulation of Bcl-2 family members in hepatocytes. The present report demonstrates that NF-κB is strongly involved in the cytokine-induced expression of pro-apoptotic Bak and Bid, and the anti-apoptotic A1/Bfl-1 in hepatocytes. Since inhibition of NF-κB tips the balance towards apoptosis, the upregulation of some pro-apoptotic genes appears surprising. On the other hand, the anti-apoptotic Bcl-2 family member A1/Bfl-1 is a potent inhibitor of apoptosis [14]. Furthermore, one may conclude from the observed effects of NF-κB inhibition, that the NF-κB-mediated activation of anti-apoptotic cIAP2 and A1/Bfl is dominant over the NF-κB activation of pro-apoptotic Bak and Bid. Since anti-apoptotic Bcl-2 is only expressed in late cultured hepatocytes, this expression is a culture artefact. Therefore, the role of Bcl-2 has not been investigated further.

Since TNF-α is frequently used to study apoptosis [6,26,36], we compared the effects of CM to TNF-α alone. This study demonstrates that some NF-κB-dependent genes are hardly induced by TNF-α alone. TNF-α induces similar induction of cIAP2 compared to cytokine mixture indicating that IL-1β and IFN-γ play a minor role in the induction of this gene. In contrast, TNF-α alone only partially induced Bak and iNOS expression, suggesting the presence of IL-1β and IFN-γ responsive elements in the Bak and iNOS promoter.

Although some studies reported NF-κB-regulated expression of anti-apoptotic Bcl-XL in various cell types [15,16], we do not observe NF-κB-regulated induction of Bcl-XL in hepatocytes. Also the mRNA expression of pro-apoptotic Bax is not regulated by NF-κB in hepatocytes.

The protective role of NO in hepatocytes has been established in different studies [20,21,37]. Our report confirms that NO is an inhibitor of caspase-3 activity. Furthermore, we confirmed that iNOS is regulated by NF-κB. However, the exact importance of iNOS-derived NO in the inhibition of caspase-3 remains to be clarified. Inhibition of NO synthesis using L-NAME in cytokine-exposed hepatocytes did not increase caspase-3 activity. An explanation of this observation is that cIAP2 is a very effective inhibitor of caspase-3. In cytokine-exposed hepatocytes in the presence of L-NAME, the NF-κB pathway is still intact (data not shown) and cIAP2 is induced and active. In this situation, the lack of NO is compensated by the presence of active cIAP2. In cytokine-exposed hepatocytes in which the NF-κB pathway is inhibited, we demonstrate that cIAP2 and iNOS are not expressed. In these hepatocytes, exogenous NO inhibits caspase-3 which is also shown by others [20]. From these observations we conclude that during inflammation various anti-apoptotic signals exist to protect hepatocytes against apoptosis. Exogenous NO could be useful as an anti-apoptotic agent in conditions in which NF-κB activation is compromised.

The results of the present report demonstrate that in inflammatory conditions hepatocyte survival is dependent on NF-κB activation. NF-κB activation results in the transcription of anti-apoptotic genes, in particular cIAP2, which in turn inhibits caspase-3 activity and apoptosis.

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