Biochemical Pharmacology, Vol. 58, pp. 1891–1898, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00268-3

Prevention of Cycloheximide-Induced Apoptosis in Hepatocytes by Adenosine and by Caspase Inhibitors

*W. Marty Blom, Hans J. G. M. de Bont, Irma Meijerman, Gerard J. Mulder and J. Fred Nagelkerke**

Division of Toxicology, Leiden Amsterdam Centre for Drug Research, Leiden University, Leiden, The Netherlands

ABSTRACT. The mechanism by which cycloheximide induces apoptosis in isolated rat hepatocytes was studied. Cycloheximide (1–300 μM) induced apoptosis within 3–4 hr in the hepatocytes. Specific apoptotic characteristics such as blebbing, phosphatidyl serine (PS) exposure, chromatin condensation, and nuclear fragmentation were induced. Cycloheximide (CHX) dose dependently activated the caspase-3-like proteases, but not the caspase-1-like proteases. Pretreatment of the hepatocytes with 100 μM of the caspase inhibitors z-Val-Ala-DL-Asp-fluoromethylketone or Ac-Asp-Glu-Val-Asp-aldehyde completely abrogated the caspase activation and the apoptosis. Addition of adenosine (100 μM) reduced phosphatidyl serine exposure and other morphological characteristics of apoptosis by 50%; however, it did not prevent the activation of the caspases, suggesting that adenosine inhibited downstream of caspase activation. The adenosine receptor antagonist 8-[4-[[[[(2-aminoethyl)amino]-carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine abolished the capacity of adenosine to prevent apoptosis, indicating that prevention was receptor-mediated. During apoptosis, the mitochondrial membrane potential in apoptotic cells (cells with PS exposition) was decreased to 50–60% of the control value; in the population viable cells, however, the mitochondrial membrane potential remained stable. Prevention of apoptosis by the caspase inhibitor z-Val-Ala-DL-Asp-fluoromethylketone or adenosine prevented the decrease in mitochondrial membrane potential. In conclusion, CHX rapidly induces apoptosis in isolated rat hepatocytes, which is inhibited by adenosine at a relatively late step. BIOCHEM PHARMACOL **58**;12:1891–1898, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. cycloheximide; apoptosis; liver; adenosine; mitochondria; caspases

The protein synthesis inhibitor CHX† has been used in the past to prevent apoptosis. However, it was shown recently that CHX could also induce apoptosis in selected cell types [1, 2]. *In vivo*, CHX induced apoptosis in the liver [3, 4], similar to other, endogenous compounds such as tumor necrosis factor-α and Fas ligand [5, 6], suggesting that apoptosis in the liver may be an important pathophysiological process. However, the development of apoptosis in liver cells has been characterised poorly; it has been only recently that the upregulation of *c-myc*, *c-fos*, and *p53* after CHX-treatment *in vivo* [4] and activation of caspases in hepatocytes after stimulation of the Fas pathway have been described [7]. In general, during apoptosis several characteristic morphological changes are induced in cells, both in the cytoskeleton (leading to bleb formation) and in the nucleus (chromatin condensation and nuclear fragmentation). Recently, it was shown that upon an apoptotic stimulus the $\Delta\Psi$ decreases and mitochondrial factors (cytochrome *c* and apoptotic-inducing factor (AIF) [8, 9] are released from the mitochondria. Also characteristic of apoptosis is the exposition of phosphatidyl serines on the extracellular side of the plasma membrane and the activation of caspases.

In this study, we investigated these processes during development of apoptosis in hepatocytes that were exposed to CHX. To study the mechanisms involved, we used several compounds that may prevent the development of apoptosis and found that inhibitors of caspases blocked apoptosis. We also tested adenosine, which in general protects tissues [10–12], although it may also induce apoptosis in a variety of cell types such as neuronal and astroglial cells [13, 14], human thymocytes [15], T lymphocytes [16], the human leukaemia HL-60 cell line [17, 18], and endothelial cells [19]. We found that adenosine did not induce apoptosis, but rather seemed to have an anti-apoptotic effect. In the present study, we report the protec-

* Corresponding author: Dr J. F. Nagelkerke, Division of Toxicology, Leiden Amsterdam Centre for Drug Research, Sylvius Laboratories, PO Box 9503, 2300 RA Leiden, The Netherlands. Tel. +31 71 5276226; FAX +31 71 5276292; E-mail: nagelker@lacdr.Leidenuniv.nl

† *Abbreviations:* Ado, adenosine; AMDA, 5′-amino-5′-deoxyadenosine; ANV, annexin V conjugated with Alexa™488; CHX, cycloheximide; $\Delta\Psi$, mitochondrial membrane potential; DEVD-AMC, Ac-sp-Glu-Val-Asp 7-amino-4-methylcoumarin; DEVD-*cho*, Ac-Asp-Glu-Val-Asp-aldehyde; PI, propidium iodide; PS, phosphatidyl serine; TMRM, tetramethylrhodamine chloride; XAC, 8-[4-[[[[(2-aminoethyl)amino]-carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine; YVAD-AFC, Z-Tyr-Val-Ala-Asp(OMe)-7-amino-4-trifluoro-methylcoumarin; and zVAD-*fmk*, z-Val-Ala-DL-Asp-fluoromethylketone.

Received 31 August 1998; accepted 1 June 1999.

tive effect of adenosine against CHX-induced apoptosis in rat hepatocytes.

MATERIALS AND METHODS

Materials

Collagenase and HEPES were obtained from Boehringer. Hoechst 33258, PI, TMRM, and Alexa™488 were from Molecular Probes. Fluorescent annexin V was prepared by labelling human recombinant protein annexin V obtained from Bender Med Systems with Alexa™488 using the Alexa™488 protein labelling kit from Molecular Probes. The caspase inhibitors zVAD-*fmk* and DEVD-*cho* were obtained from BACHEM. Caspase substrates DEVD-AMC and YVAD-AFC were obtained from Calbiochem. Bovine serum albumin (type V), poly-L-lysine hydrobromide and all other chemicals were from Sigma. Male wistar rats (200–250 g) from Broekman were fed *ad lib*. and kept on a 12-hr day–light cycle for at least one week at the animal facilities of Sylvius Laboratories.

Isolation and Incubation of Rat Hepatocytes

Hepatocytes were isolated from liver by collagenase perfusion as described previously [20]. Viability of the freshly isolated cells was > 95% as determined by trypan blue exclusion. After isolation cells were kept on ice until use. Cells were preincubated for 45 min in Hanks'/HEPES buffer (pH 7.4, 37°) composed of 120 mM NaCl, 5 mM KCl, 4.2 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 25 mM HEPES supplemented with 10 mM glucose, and 1% (w/v) BSA, gassed for 30 min with 95% O_2/5% CO_2. Cells (3.0×10^5 cells/mL) were allowed to attach to circular glass coverslips coated with poly-L-lysine (1 mg/mL in water) [21]. The coverslips were placed in 6-well culture plates and 2 mL hepatocytes was added. The plates were incubated at 37° in a humidified atmosphere (5% CO_2/air) throughout the experiment. For experiments using cell suspension, 2 mL hepatocytes (3.0×10^5 cells/mL) was incubated in small polyethylene vials of 12 mL. The vials were incubated at 37° in a humidified atmosphere (5% CO_2/air) throughout the experiment, and were gently shaken every 30 min.

Morphological Determination of Apoptosis

After incubation the cells were carefully rinsed once with PBS (pH 7.4, 37°) and fixed for 20 min with 3.7% (w/v) paraformaldehyde in PBS at room temperature. Then, cells were washed twice with PBS prior to incubation with Hoechst 33258 (2 μg/mL) in PBS for 20 min. Cells were washed twice with PBS, and the glass coverslips were mounted with Polyaquamount. In normal hepatocytes, the nuclei had uncondensed chromatin, but in apoptotic hepatocytes the nuclear chromatin was condensed at the nuclear membrane or the nuclei were fragmented.

Imaging Techniques

Fluorescence microscopy was used for the analysis of the cells. The system consisted of an IM35 inverted microscope with a 100 watt mercury arc lamp (Zeiss) and a Nikon 40×/1.3 NA CF Fluor objective. Hoechst staining was detected with a 510-nm longpass emission filter, a 365-nm bandpass filter, and a 475-nm dichroic mirror. Images were recorded using a CCD instrumentation camera, controlled by a CC200 camera controller (Photometrics).

Flow Cytometric Analysis of the Percentage Viable, Apoptotic, and Dead Hepatocytes

DETECTION OF PS EXPOSURE AND PLASMA MEMBRANE PERMEABILISATION. The percentage viable, apoptotic, and dead hepatocytes was determined by flow cytometric analysis (FACscan, Becton Dickenson). In apoptotic cells the inward orientation of the PS is lost, and PS are located at the extracellular site of the plasma membrane. These extracellular PS bind to annexin V, which is used to discriminate between apoptotic and non-apoptotic cells. PS were visualised using Alexa™488-labelled annexin V (approximately 1 μg/mL annexin V and Alexa™488 in a stoichiometric complex of 1:1). Viable, non-apoptotic cells do not bind ANV, while apoptotic and dead cells are positive for ANV. To further discriminate between apoptotic and dead hepatocytes PI (10 μM final concentration) was included; apoptotic hepatocytes have an intact plasma membrane and do not take up PI, while dead cells are permeable to PI [22]. Briefly, a sample of 85 μL of the hepatocyte suspension was taken and centrifuged at 50 *g* for 1 min. The pellet was resuspended in 85 μL ice-cold buffer consisting of 10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1.0 mM $MgSO_4$, 1.8 mM $CaCl_2$, pH 7.4, and supplemented with 0.2 μL ANV and 10 μM PI. The samples were incubated for 15 min on ice in the dark; then 200 μL extra buffer was added and the samples were analysed.

PS EXPOSURE AND MITOCHONDRIAL MEMBRANE POTENTIAL. ANV (0.2 μL) also containing 0.02 μM TMRM was added at the indicated times to 85 μL of the hepatocyte suspension. Cells were incubated for 15 min at 37° and thereafter were centrifuged at 50 *g* for 1 min. The pellet was resuspended in 250 μL Hanks'/HEPES buffer at 37°. The samples were analysed using the flow cytometer.

Measurement of Caspase Activity

The activities of caspase 3 (CPP32)-like and caspase-1 (YVAD)-like proteases were measured as described [7]. At the indicated times, 1 mL cell suspension (3×10^5 cells/mL) was taken, centrifuged, and the cells were washed twice with ice-cold PBS; the cells were resuspended in a lysis buffer consisting of 10 mM HEPES, pH 7.0, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, and 5 mM EGTA. After 10 min on ice, the cells were disrupted

by four cycles of freezing and thawing and stored at −80°. Protein concentration was determined using the method described by Bradford [23]. For the assay, samples containing 50 μg protein were incubated with 40 nmol of the enzyme substrates DEVD-AMC or YVAD-AFC in a 100-mM HEPES buffer, pH 7.25, containing 10% (w/v) sucrose, 0.1% (v/v) Nonidet-P40, and 10 mM dithiothreitol. After cleavage of the substrates fluorescent AMC or AFC is released; this was monitored at an excitation of 360 nm and emission of 460 nm using a Perkin Elmer plate reader. Calibration curves were constructed using free AMC or AFC.

Statistics

Values are expressed as means ± SD. The statistical evaluation was performed with an unpaired two-tailed Student's *t*-test.

RESULTS

CHX-Induced Apoptosis

CHX induced apoptosis in freshly isolated rat hepatocytes within 2–4 hr, as was assessed by several characteristics of apoptosis: formation of blebs, chromatin condensation (Fig. 1, A and B), exposition of phosphatidyl serines at the extracellular site of the plasma membrane (Fig. 2A), and shrinkage of cells (Fig. 2, B and C). In control hepatocytes the chromatin in the nucleus was uncondensed (Fig. 1A), while in apoptotic cells the chromatin was condensed at the nuclear membrane or the nucleus was fragmented (Fig. 1B). Apoptosis started approximately 2 hr after addition of CHX. In Fig. 2A, an experiment is shown in which approx. 4% of the hepatocytes were apoptotic at 30–300 μM CHX compared to 1% in non-exposed control cells after 2 hr. Apoptosis was maximal at 100 μM CHX and did not increase further at higher concentrations of CHX. At 100 μM CHX, apoptosis was induced in 6% and 10% of the hepatocytes at 3 and 4 hr, respectively (Fig. 2A), while in control hepatocytes 2% apoptosis was observed after 3 and 4 hr. We also determined the size of the hepatocytes by measuring the forward scatter in the flow cytometer (Fig. 2, B and C). Indeed, we found that the annexin-V-positive cells were smaller than the annexin-V-negative cells, confirming that apoptosis was induced in this group. Due to variation in the individual experiments with separate batches of hepatocytes, the time before the first apoptotic cells appeared and the extent of apoptosis differed, which resulted in a considerable variation (10–30% apoptosis after 4 hr). Therefore, the apoptosis caused by 100 μM CHX after a 4-hr incubation period in each individual experiment was set at 100% so as to make individual experiments comparable.

Apoptotic cells shrink; as an extra check for induction of apoptosis, the size of the hepatocytes in the different populations, obtained after labelling the cells with ANV and PI, was determined by measurement of the forward scatter (FSC) using flow cytometry. In the non-exposed cells, a very small population of apoptotic cells was present (Fig. 2B). Exposure to CHX resulted in shrinkage of the hepatocytes compared to the non-exposed cells. The size of the apoptotic cells was diminished compared to viable cells (Fig. 2, B and C). In addition, the population with small cells was increased after exposure to CHX compared to non-exposed control hepatocytes.

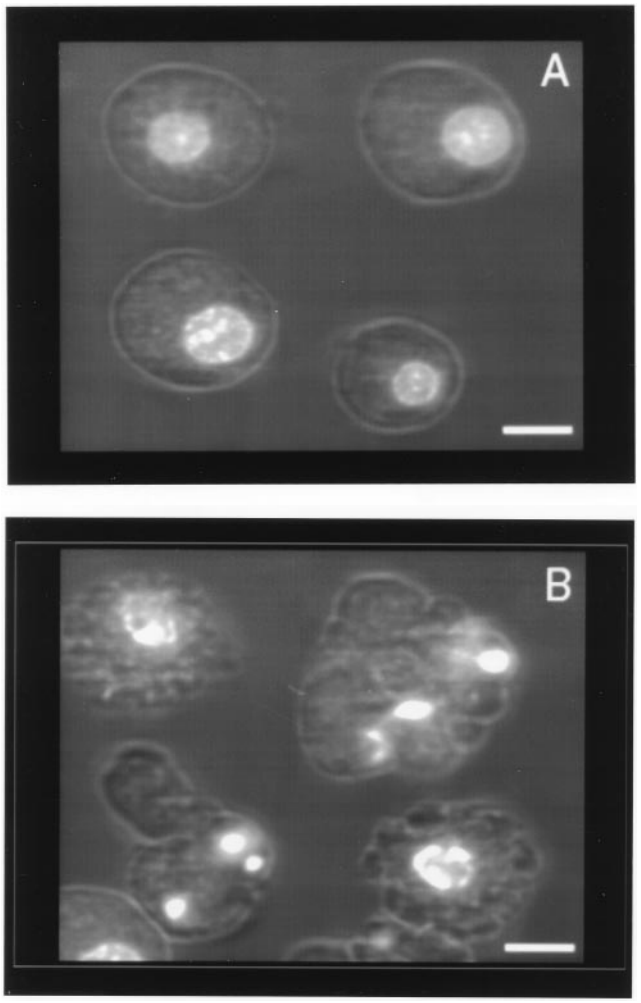

FIG. 1. CHX-induced morphological changes in apoptosis in rat hepatocytes. Freshly isolated rat hepatocytes were preincubated for 45 min at 37° followed by a 4-hr incubation in the absence or presence of CHX. (A) control cells and (B) cells exposed to 100 μM CHX. The cells were fixed with 3.7% paraformaldehyde and DNA was stained with Hoechst 33258. The cells were visualised using a Zeiss IM35 inverted microscope with a Nikon 40×\1.3 NA CF Fluor objective. The light microscopic image and the fluorescent image of the nuclei of the same cells are shown combined in one photograph. Bar is 10 μm.

Involvement of Caspases in CHX-Induced Apoptosis

During apoptosis the caspases are activated. Figure 3 shows the effects of increasing concentrations of CHX (0.1–100 μM) on the activation of the caspase-3-like (DEVD-like) activity. CHX increased the DEVD-like activity during increasing incubation periods: it started to increase between

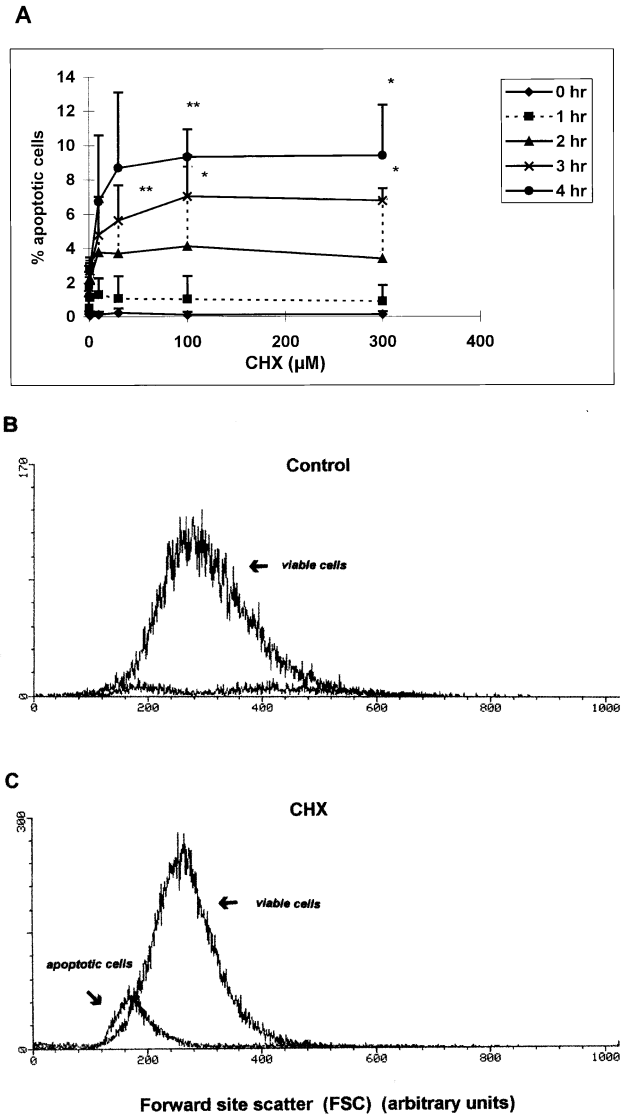

FIG. 2. Dose-dependent induction of apoptosis in rat hepatocytes by CHX. (A) Freshly isolated rat hepatocytes were preincubated for 45 min at 37° before CHX (3–300 μM) was added. The percentage of apoptosis was determined after 0 (◆), 1 (■), 2 (▲), 3 (✕), and 4 hr (●) by flow cytometric analysis of hepatocytes labelled with ANV and PI. Data are means ± SD of four independent experiments. *P ≤ 0.05 and **P ≤ 0.01 for a statistically significant increase in apoptosis compared to non-exposed control cells. The size of the hepatocytes was determined by measuring the forward scatter (FSC) of cells in the different populations obtained after labelling with ANV and PI. The different populations consisted of viable cells (ANV-negative and PI-negative) and apoptotic cells (ANV-positive and PI-negative). The frequency histogram of the FSC of viable cells and apoptotic cells is shown for (B) non-exposed hepatocytes (control) and (C) hepatocytes exposed to 100 μM CHX after 4 hr of incubation.

1 and 2 hr after addition of CHX. After 3 hr, the DEVD-like activity was approx. 5-fold increased compared to control cells (Figs. 3 and 4). Caspase-1-like (YVAD-like) activity was also measured, but during the incubation period CHX exposure did not result in any increase in YVAD-like activity (not shown), suggesting that this

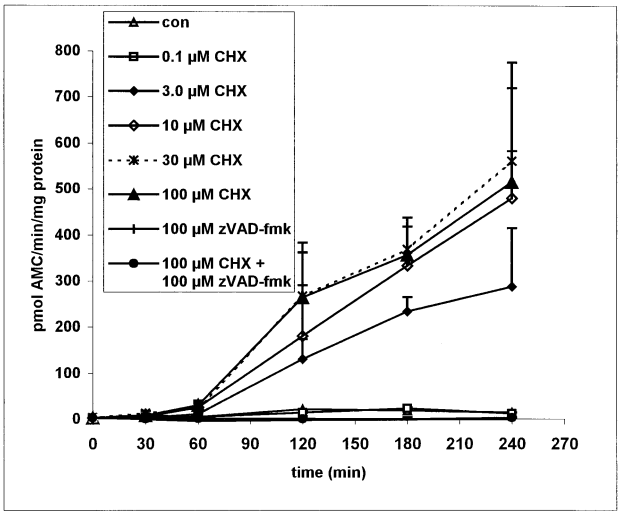

FIG. 3. Dose-dependent induction of DEVD-AMC cleavage in CHX-treated hepatocytes. Freshly isolated rat hepatocytes were preincubated for 45 min at 37° before CHX (0.1–100 μM) was added. Caspase inhibitor zVAD-*fmk* was added during the last 30 min of the preincubation period. At the indicated time points, aliquots were removed from the cell incubations for determination of the DEVD-AMC caspase activity. The enzyme activity is expressed as pmol AMC per mg protein per minute. Data shown are means ± SD and the results of three independent experiments.

caspase subfamily was not involved in the CHX-mediated apoptosis in rat hepatocytes.

The caspase inhibitor zVAD-*fmk* completely prevented the increase of DEVD-like activity (Figs. 3 and 4). Inhibition of caspases by preincubation of the hepatocytes with zVAD-*fmk* or DEVD-*cho* completely prevented the CHX-

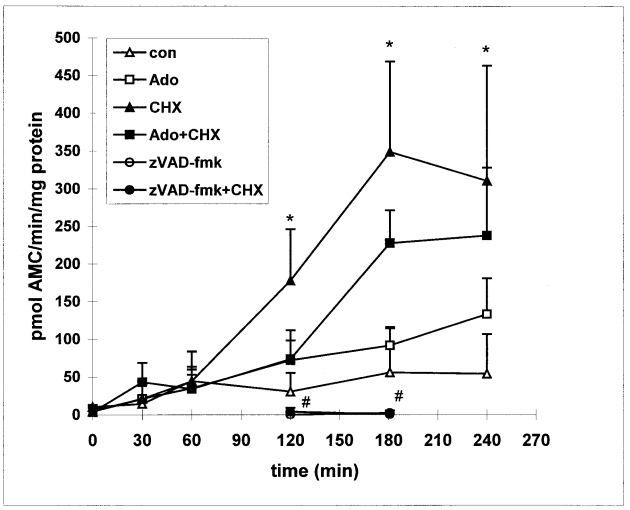

FIG. 4. Effect of adenosine on DEVD-AMC cleavage in CHX-treated hepatocytes. Freshly isolated rat hepatocytes were preincubated in the absence or presence of Ado (100 μM) for 30 min. CHX (100 μM) was added to the cells. At the indicated time points, aliquots were removed from the cell incubations for determination of the DEVD-AMC caspase activity. The enzyme activity is expressed as pmol AMC per mg protein per minute. Data shown are means ± SD and the results of three independent experiments.

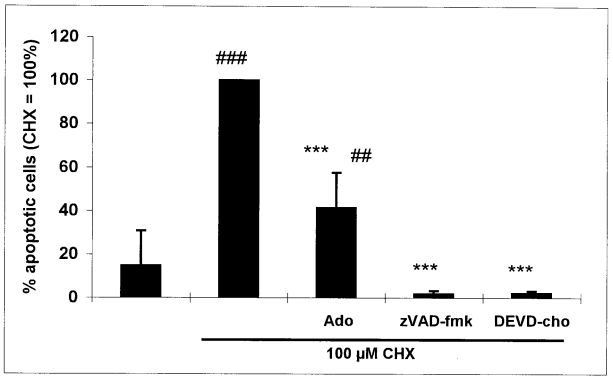

FIG. 5. Effect of adenosine and caspase inhibitors zVAD-*fmk* and DEVD-*cho* against CHX-induced apoptosis. Freshly isolated rat hepatocytes were preincubated for 45 min at 37°, and zVAD-*fmk*, DEVD-*cho*, or Ado (100 μM) was added during the last 30 min of incubation. The incubation period of 4 hr started with the addition of 100 μM CHX. The caspase inhibitors alone did not induce any effects in the hepatocytes. The CHX-induced apoptosis was set each time at 100% to compare the percentage reduction by zVAD-*fmk*, DEVD-*cho*, and Ado. CHX induced apoptosis in 16.6% ± 5.7% of the cells. Data are means ± SD of 6 separate experiments. $^{###}P < 0.001$ and $^{##}P < 0.005$ for the induction of apoptosis by CHX compared to control hepatocytes. $^{***}P \leq 0.0001$, a significant reduction in apoptosis compared to CHX-induced apoptosis.

induced apoptosis, confirming the involvement of caspases (Fig. 5). On the contrary, Ado (100 μM) reduced the CHX-induced DEVD-like activity only slightly (Fig. 4).

Protection by Adenosine against CHX-Induced Apoptosis

Addition of Ado (30–300 μM) to hepatocytes did not cause apoptosis in freshly isolated rat hepatocytes (Table 1). Instead, it was capable of protecting the hepatocytes against CHX-induced apoptosis. At a concentration of 30 μM, the protection by Ado was not statistically significant, but at 100 and 300 μM, a 52% and 62% decrease, respectively was observed, resulting in an increase in the percentage of viable cells (Table 1).

Mechanism of Adenosine-Mediated Protection against CHX-Induced Apoptosis

The adenosine kinase inhibitor AMDA (20 μM) [24] was not able to prevent or influence the Ado-mediated protection against CHX-induced apoptosis (Fig. 6). However, XAC, a non-selective antagonist of adenosine receptors, (5 μM) [25], nearly completely abolished the Ado-mediated protection against CHX-induced apoptosis (Fig. 7) (to a level of 90% of the CHX-induced apoptosis), indicating that one of the adenosine P1 receptors is involved in protection against CHX-apoptosis.

Mitochondrial Membrane Potential during CHX-Mediated Apoptosis

Flow cytometric analysis was used to measure the $\Delta\Psi$ of the viable and apoptotic cells after the various treatments.

TABLE 1. Adenosine protection against CHX-induced apoptosis in hepatocytes

Control	Viable cells (%)	Apoptotic cells (%)	Dead cells (%)
Adenosine			
0	75 (6)	3 (3)	20 (3)
30 μM	78 (4)	4 (3)	18 (4)
100 μM	79 (4)	5 (4)	16 (3)
300 μM	75 (7)	5 (4)	22 (8)
100 μM CHX			
Adenosine			
0	65 (9)*	18 (7)*	17 (4)
30 μM	72 (7)†	13 (6)*	15 (2)*
100 μM	75 (5)‡	10 (6)‡	16 (1)
300 μM	70 (9)	7 (3)†	23 (6)

Hepatocytes were incubated in the absence (upper table) or presence (lower table) of 100 μM CHX. Cells were preincubated with 30, 100, and 300 μM Ado for 30 min before CHX was added. After 4 hr, samples were taken and the percentages of viable, apoptotic, and dead cells were determined by flow cytometric analysis of the cells labelled with ANV and PI.

*$P < 0.02$ for a statistically significant effect compared to non-exposed, control hepatocytes.

†$P < 0.02$ and ‡$P < 0.005$ for the statistically significant effect of ADO compared to hepatocytes exposed to CHX alone. Data are means ± SD (in parentheses) of five independent experiments.

Dead cells did not have a $\Delta\Psi$ and were excluded (as described previously in [22]). The fluorescent compound TMRM, a cationic fluorophore that accumulates into mitochondria, was used to determine $\Delta\Psi$ during CHX-mediated apoptosis. To discriminate between viable and

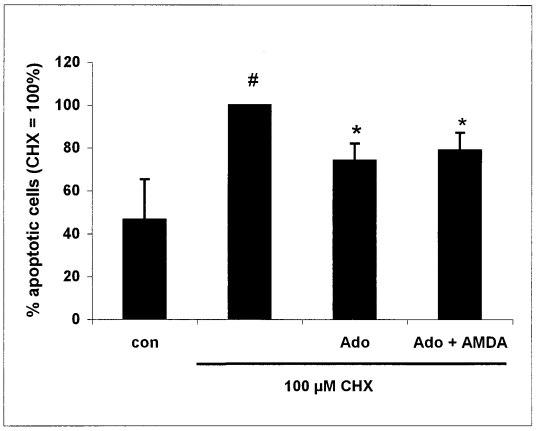

FIG. 6. Effect of adenosine kinase inhibitor AMDA on Ado-mediated protection. Freshly isolated rat hepatocytes were preincubated for 45 min at 37°, and Ado (100 μM) and AMDA (20 μM) were added during the last 30 min of the preincubation. The incubation period of 4 hr started with the addition of 100 μM CHX. The inhibitor alone did not induce any effects in the hepatocytes. The CHX-induced apoptosis was set each time at 100% to compare the effect of the different treatments. The CHX induced apoptosis in 19.6.6% ± 10% of the cells. Data are means ± SD in triplicate out of 2 separate experiments. $^{#}P < 0.05$ for the induction of apoptosis by CHX compared to control hepatocytes. $^{*}P \leq 0.05$, a significant reduction in apoptosis compared to CHX-induced apoptosis.

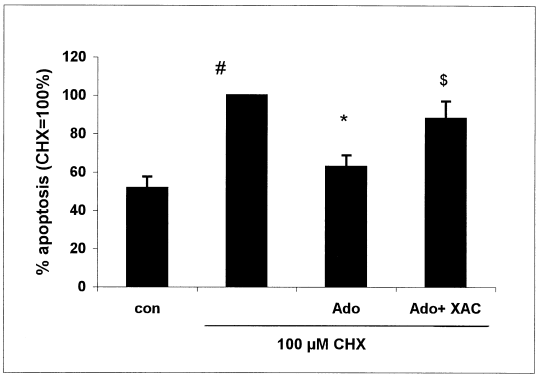

FIG. 7. Effect of adenosine receptor antagonist XAC on Ado-mediated protection. Freshly isolated rat hepatocytes were pre-incubated for 45 min at 37°, and Ado (100 µM) and XAC (5 µM) were added during the last 30 min of the preincubation. The incubation period of 4 hr started with the addition of 100 µM CHX. The antagonist alone did not induce any effects in the hepatocytes. The CHX induced apoptosis was set each time at 100% to compare the effect of the different treatments. The CHX induced apoptosis in 16.7% ± 7% of the cells. Data are means ± SD in triplicate out of 2 separate experiments. $^{\#}P < 0.05$ for the induction of apoptosis by CHX compared to control hepatocytes. $^{*}P \leq 0.05$, a significant reduction in apoptosis compared to CHX induced apoptosis. $^{\$}P \leq 0.05$, a significant reduction in Ado-mediated protection. The ado + XAC group was not statistically different from the CHX alone group.

apoptotic cells, the externalisation of PS by apoptotic cells was used. In non-exposed cells, two populations of cells with a $\Delta\Psi$ were found: cells with a high $\Delta\Psi$ (100%) that were ANV-negative (80%, the viable cells) and a population of ANV-positive cells (4%, apoptotic cells) with a low $\Delta\Psi$, their $\Delta\Psi$ being reduced by 50% compared to the ANV-negative cells. Exposure to CHX (100 µM) for 4 hr increased the population of cells with a low $\Delta\Psi$ (24%). The treatment with CHX had no effect on the $\Delta\Psi$ of the population of viable cells. Treatment of the hepatocytes with zVAD-*fmk* (100 µM) prevented PS exposition and the decrease in $\Delta\Psi$. Ado (100 µM) reduced the percentage of apoptotic cells with a low $\Delta\Psi$ (as in Fig. 5). Similarly, viable cells showed no reduction in $\Delta\Psi$, indicating that Ado prevented the CHX-induced exposition of PS and the decrease in the $\Delta\Psi$ simultaneously.

DISCUSSION

CHX has been reported to induce DNA fragmentation, characteristic of apoptosis in liver after *in vivo* administration to rats [3], suggesting that it is a candidate for induction of apoptosis *in vitro*. It is for this reason that we first characterised the effects of CHX in freshly isolated rat hepatocytes. The results show that CHX rapidly induces apoptosis in hepatocytes. CHX caused chromatin condensation, bleb formation, DEVD-like caspase activation, exposition of PS, and a decrease in $\Delta\Psi$. Two other protein synthesis inhibitors, puromycin and emetine, similarly induce chromatin condensation in rat hepatocytes [26], suggesting that the inhibition of protein synthesis probably stimulates the apoptotic processes. The apoptotic effect of protein synthesis inhibition was also found in other cell types, such as the HL-60 cell line [1] or a lymphoid CEM cell line [2]. However, CHX does not always have a pro-apoptotic effect in hepatocytes: it inhibited apoptotic processes in rat hepatocytes exposed to transforming growth factor β (TGF-β) [27].

CHX activated caspase-3-like proteases (CPP32/DEVD-like), but not caspase-1-like proteases (ICE/YVAD-like). The group of caspase-3-like proteases consists of at least five caspases that are all involved in apoptosis: caspase-3 (CPP32/YAMA), −6 (Mch2), −7 (Mch3/ICE-LAP3/CMH-1), −8 (Mch5/FLICE-1/MACH-1), and −10 (Mch4/FLICE -) [28]. During apoptosis caspases are activated; *in vitro* it has been shown that caspase-10 can act autocatalytically and can also cleave caspases-3 and-7 [29] and that caspase-3 can cleave caspase-7 [30] and caspase-6 [31]. It is possible that a similar cascade is activated during CHX-mediated processes. The detection of caspase-3-like protease activity indicates that other caspases could be activated as well, but the method used did not allow us to distinguish between the various forms of caspase-3-like proteases.

Because no effect of CHX on caspase-1-like proteases was found, it seems that these are non-essential for apoptosis. This has also been observed in apoptotic immune cells; moreover, caspase-1-deficient mice develop normally without apparent defects in the apoptotic process [32]. In contrast, caspase-3 (CPP32)-deficient mice died at 1–3 weeks of age and showed a decrease in apoptosis of brain cells, indicating a critical role for caspase-3 in apoptosis. However, thymocytes isolated from these mice underwent apoptosis normally, indicating that in these cells other caspases, such as caspase-6 or-7, were mediating the effects [33].

Recent studies have shown that mitochondria are important for the apoptotic process. A decrease in mitochondrial membrane potential was triggered by several apoptosis-inducing agents in a variety of cell lines, with this decrease preceding nuclear apoptosis [8, 34–36]. We also observed that the $\Delta\Psi$ in apoptotic (ANV-positive) hepatocytes was decreased during CHX-induced apoptosis. Previously, a similar result was observed when apoptosis was induced in rat hepatocytes by exposure to activated natural killer cells [22].

Pretreatment of the hepatocytes with caspase inhibitors zVAD-*fmk* or DEVD-*cho* abolished apoptosis completely. The increase in caspase-3-like protease activity by CHX was also completely prevented, as were the other apoptotic events, i.e. PS externalisation, decrease in $\Delta\Psi$, formation of blebs, and nuclear apoptosis (chromatin condensation and nuclear fragmentation). This indicates that these are located downstream of caspase activation, the latter being a terminal event in apoptosis.

Treatment of the hepatocytes with adenosine reduced the percentage of apoptotic cells after treatment with CHX by approx. 50%; i.e. PS exposition and decreases in $\Delta\Psi$ and

nuclear apoptosis were abolished in half of the hepatocytes that otherwise probably would have become apoptotic. In addition, the caspase 3-like activity was only slightly reduced by adenosine, indicating that adenosine functions between the activation of caspase(s) and the apoptotic processes such as translocation of PS and the decrease in $\Delta\Psi$. The question as to whether adenosine might inhibit the cleavage of some substrates or influence apoptosis by another process remains to be solved. It is not clear yet whey adenosine does not prevent apoptosis in all hepatocytes. A possible explanation could be that the liver consists of different hepatocyte populations that might react differently to adenosine.

Most effects of adenosine are receptor-mediated. Recently, adenosine was reported to delay the spontaneous apoptotic process induced in human neutrophils by activation of the adenosine A_{2a} receptor [37]. HL-60 leukaemia cells and U-937 lymphoma cells were protected against adenosine A_3 receptor antagonist-induced apoptosis by agonists of the adenosine A_3 receptor [38]. Here, it is shown that in hepatocytes as well adenosine receptors are involved in mediating protection against apoptosis. However, the intracellular effects influencing specific apoptotic events following receptor stimulation by adenosine have never been investigated. Therefore, a mechanism of the adenosine-mediated delay in apoptosis remains to be elucidated, although it may be linked to intracellular ATP concentrations. Lund *et al.* [39] showed that adenosine increases intracellular ATP levels in hepatocytes. Apoptosis is an active process that needs energy for apoptotic processes such as chromatin condensation, PS translocation, and the activation of caspase 3 by cytochrome *c* [40–42]. On the other hand, decreasing ATP levels were a signal for the Bo cell line to undergo apoptosis [43], while human T cells stimulated with a specific apoptotic signal, such as Fas or staurosporine, underwent necrosis when ATP was depleted [44], indicating that ATP levels do play a critical role in pathways of cell death.

References

1. Martin SJ, Lennon SV, Bonham AM and Cotter TG, Induction of apoptosis (programmed cell death) in human leukemic HL 60 cells by inhibition of RNA or protein synthesis. *J Immunol* **145:** 1859–1867, 1990.
2. Meisenholder GW, Martin SJ, Green DR, Nordberg J, Babior BM and Gottlieb RA, Events in apoptosis. Acidification is downstream of protease activation and BCL-2 protection. *J Biol Chem* **271:** 16260–16262, 1996.
3. Faa G, Ledda Columbano GM, Ambu R, Congiu T, Coni P, Riva A and Columbano A, An electron microscopic study of apoptosis induced by cycloheximide in rat liver. *Liver* **14:** 270–278, 1994.
4. Alessenko AV, Boikov PY, Filippova GN, Khrenov AV, Loginov AS and Makarieva ED, Mechanisms of cycloheximide-induced apoptosis in liver cells. *FEBS Lett* **416:** 113–116, 1997.
5. Lacronique V, Mignon A, Fabre M, Viollet B, Rouquet N, Molina T, Porteu A, Henrion A, Bouscary D, Varlet P, Joulin V and Kahn A, Bcl-2 protects from lethal hepatic apoptosis induced by an anti-Fas antibody in mice. *Nat Med* **2:** 80–86, 1996.
6. Alessenko AV, Korobko VG, Khrenov AV, Rozhnova UA, Soloviev AS and Shingarova LN, Relation of biological activity of mutant forms of recombinant human tumor necrosis factor alpha and accumulation of sphingosine in murine liver. *Biochem Mol Biol Int* **42:** 143–154, 1997.
7. Jones RA, Johnson VL, Buck NR, Dobrota M, Hinton RH, Chow SC and Kass GEN, Fas-mediated apoptosis in mouse hepatocytes involves the processing and activation of caspases. *Hepatology* **27:** 1632–1643, 1998.
8. Kluck RM, Bossy-Wetzel E, Green DR and Newmeyer DD, The release of cytochrome *c* from mitochondria: A primary site for Bcl-2 regulation of apoptosis. *Science* **275:** 1132–1136, 1997.
9. Susin SA, Zamzami N, Castedo M, Hirsch T, Marchetti P, Macho A, Daugas E, Geuskens M and Kroemer G, Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J Exp Med* **184:** 1331–1341, 1996.
10. Cronstein B, Adenosine, an endogenous anti-inflammatory agent. *J Appl Physiol* **76:** 5–13, 1994.
11. Ely SW and Berne RM, Protective effects of adenosine in myocardial ischemia. *Circulation* **85:** 893–904, 1992.
12. Mullane K and Bullough D, Harnessing an endogenous cardioprotective mechanism: Cellular sources and sites of action of adenosine. *J Mol Cell Cardiol* **27:** 1041–1054, 1995.
13. Wakade TD, Palmer KC, McCauley R, Przywara DA and Wakade AR, Adenosine-induced apoptosis in chick embryonic sympathetic neurons: A new physiological role for adenosine. *J Physiol (Lond)* **488:** 123–138, 1995.
14. Abbracchio MP, Ceruti S, Barbieri D, Franceschi C, Malorni W, Biondo L, Burnstock G and Cattabeni F, A novel action for adenosine: Apoptosis of astroglial cells in rat brain primary cultures. *Biochem Biophys Res Commun* **21:** 908–915, 1995.
15. Szondy Z, Adenosine stimulates DNA fragmentation in human thymocytes by $Ca^{(2+)}$-mediated mechanisms. *Biochem J* **304:** 877–885, 1994.
16. Kizaki H, Suzuki K, Tadakuma T and Ishimura Y, Adenosine receptor-mediated accumulation of cyclic AMP-induced T-lymphocyte death through internucleosomal DNA cleavage. *J Biol Chem* **265:** 5280–5284, 1990.
17. Tanaka Y, Yoshihara K, Tsuyuki M and Kamiya T, Apoptosis induced by adenosine in human leukemia HL 60 cells. *Exp Cell Res* **213:** 242–252, 1994.
18. Kohno Y, Sei Y, Koshiba M, Kim HO and Jacobson KA, Induction of apoptosis in HL 60 human promyelocytic leukemia cells by adenosine A(3) receptor agonists. *Biochem Biophys Res Commun* **219:** 904–910, 1996.
19. Dawicki DD, Chatterjee D, Wyche J and Rounds S, Extracellular ATP and adenosine cause apoptosis of pulmonary artery endothelial cells. *Am J Physiol* **273:** L485–494, 1997.
20. Nagelkerke JF, Barto KP and van Berkel TJ, *In vivo* and *in vitro* uptake and degradation of acetylated low density lipoprotein by rat liver endothelial, Kupffer and parenchymal cells. *J Biol Chem* **258:** 12221–12227, 1983.
21. Zoetewey JP, van de Water B, de Bont HJGM, Mulder GJ and Nagelkerke JF, Calcium-induced cytotoxicity in hepatocytes after exposure to extracellular ATP is dependent on inorganic phosphate. *J Biol Chem* **268:** 3384–3388, 1993.
22. Blom WM, de Bont HJGM, Meijerman I, Kuppen PJK, Mulder GJ and Nagelkerke JF, Interleukin-2-activated natural killer cells can induce both apoptosis and necrosis in rat hepatocytes. *Hepatology* **29:** 785–792, 1999.
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72:** 248–254, 1976.
24. Wakade AR, Kulkarni JS and Fujii JT, 2′-Deoxyadenosine selectively kills nonneuronal cells without affecting survival

and growth of chick dorsal root ganglion neurons. *Brain Res* **788:** 69–79, 1998.
25. Klotz KN, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB and Lohse MJ, Comparative pharmacology of human adenosine receptor subtypes-characterization of stably transfected receptors in CHO cells. *Naunyn Schmiedebergs Arch Pharmacol* **357:** 1–9, 1998.
26. Meijerman I, Blom WM, de Bont HGJM, Mulder GJ and Nagelkerke JF, Induction of apoptosis and changes in nuclear G-actin are mediated by different pathways: The effect of inhibitors of protein and RNA synthesis in isolated rat hepatocytes. *Toxicol Appl Pharmacol* **156:** 46–55, 1999.
27. Sanchez A, Alvarez AM, Benito M and Fabregat I, Cycloheximide prevents apoptosis, reactive oxygen species production, and glutathione depletion induced by transforming growth factor beta in fetal rat hepatocytes in primary culture. *Hepatology* **26:** 935–943, 1997.
28. Zhivotovsky B, Burgess DH, Vanags DM and Orrenius S, Involvement of cellular proteolytic machinery in apoptosis. *Biochem Biophys Res Commun* **230:** 481–488, 1997.
29. Fernandes-Alnemri T, Armstrong RC, Krebs J, Srinivasula SM, Wang L, Bullrich F, Fritz LC, Trapani JA, Tomaselli KJ, Litwack G and Alnemri ES, *In vitro* activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc Natl Acad Sci USA* **93:** 7464–7469, 1996.
30. Orth K, O'Rourke K, Salvesen GS and Dixit VM, Molecular ordering of apoptotic mammalian CED-3/ICE-like proteases. *J Biol Chem* **271:** 20977–20980, 1996.
31. Hirata H, Takahashi A, Kobayashi S, Yonehara S, Sawai H, Okazaki T, Yamamoto K and Sasada M, Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis. *J Exp Med* **187:** 587–600, 1998.
32. Kuida K, Lippke JA, Ku G, Harding MW, Livingston DJ, Su MS and Flavell RA, Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science* **267:** 2000–2003, 1995.
33. Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic P and Flavell RA, Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature* **384:** 368–372, 1996.
34. Zamzami N, Marchetti P, Castedo M, Hirsch T, Susin SA, Masse B and Kroemer G, Inhibitors of permeability transition interfere with the disruption of the mitochondrial transmembrane potential during apoptosis. *FEBS Lett* **384:** 53–57, 1996.
35. Boise LH and Thompson CB, Bcl x(L) can inhibit apoptosis in cells that have undergone Fas-induced protease activation. *Proc Natl Acad Sci USA* **94:** 3759–3764, 1997.
36. Backway KL, McCulloch EA, Chow S and Hedley DW, Relationships between the mitochondrial permeability transition and oxidative stress during ara-C toxicity. *Cancer Res* **57:** 2446–2451, 1997.
37. Walker BA, Rocchini C, Boone RH, Ip S and Jacobson MA, Adenosine A2a receptor activation delays apoptosis in human. *J Immunol* **158:** 2926–2931, 1997.
38. Yao Y, Sei Y, Abbracchio MP, Jiang JL, Kim YC and Jacobson KA, Adenosine A3 receptor agonists protect HL 60 and U 937 cells from apoptosis induced by A3 antagonists. *Biochem Biophys Res Commun* **232:** 317–322, 1997.
39. Lund P, Cornell NW and Krebs HA, Effect of adenosine on the adenine nucleotide content and metabolism of hepatocytes. *Biochem J* **152:** 593–599, 1975.
40. Kass GEN, Eriksson JE, Weis M, Orrenius S and Chow SC, Chromatin condensation during apoptosis requires ATP. *Biochem J* **318:** 749–752, 1996.
41. Kluck RM, Martin SJ, Hoffman BM, Zhou JS, Green DR and Newmeyer DD, Cytochrome *c* activation of CPP32-like proteolysis plays a critical role in a *Xenopus* cell-free apoptosis system. *EMBO J* **16:** 4639–4649, 1997.
42. Vanags DM, Pörn-Ares MI, Coppola S, Burgess DH and Orrenius S, Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. *J Biol Chem* **271:** 31075–31085, 1996.
43. Garland JM and Halestrap A, Energy metabolism during apoptosis. Bcl-2 promotes survival in hematopoietic cells induced to apoptose by growth factor withdrawal by stabilizing a form of metabolic arrest. *J Biol Chem* **272:** 4680–4688, 1997.
44. Leist M, Single B, Castoldi AF, Kuhnle S and Nicotera P, Intracellular adenosine triphosphate (ATP) concentration: A switch in the decision between apoptosis and necrosis. *J Exp Med* **185:** 1481–1486, 1997.