# Mechanisms of cycloheximide-induced apoptosis in liver cells

Alice V. Alessenko*, Peter Ya. Boikov, Galina N. Filippova, Alexey V. Khrenov, Anatoliy S. Loginov, Elena D. Makarieva

*Institute of Biochemical Physics RAS, 4 Kosygin str., Moscow 117977, Russia*

Received 23 July 1997; revised version received 25 August 1997

**Abstract Cycloheximide in sublethal doses caused apoptosis in liver cells in vivo, inducing c-*myc*, c-*fos*, c-*jun* and *p53* genes and accumulation of sphingosine, a toxic product of the sphingomyelin cycle. These data support the hypothesis that continuous synthesis of labile protective proteins is required to restrain apoptosis in liver; sphingosine might be important in mediating cycloheximide-induced apoptosis as an endogenous modulator of protein kinase C activity.**



*Key words:* Apoptosis; Cycloheximide; Sphingosine; c-*myc* gene expression; c-*fos* gene expression; c-*jun* gene expression; *p53* gene expression

## 1. Introduction

Apoptosis, programmed cell death, has been shown to play a key role in normal development, differentiation, glandular atrophy following hormonal withdrawal and maturation of the immune system. Its hallmark biochemical feature is endonuclease activation, giving rise to internucleosomal DNA fragmentation. There are also characteristic morphological changes, including chromatin condensation, nuclear fragmentation, shrinkage, the formation of dense chromatin masses and apoptotic bodies. Cytoplasmic structures are relatively preserved (reviewed in [1,2]).

Apoptosis is an active process which is governed by a signal transduction pathway. Up-regulation of c-*myc*, c-*fos* and *p53* genes is associated with transmission of the apoptotic signal [3,4]. Utilization of the sphingomyelin pathway for induction of the apoptotic response has already been demonstrated in a large variety of mammalian cells [5–8]. Interference with apoptosis appears to be one of the mechanisms leading to unrestrained growth and development of cancer. Conversely, several chemotherapeutic agents as well as ionizing radiation impact their antitumor activities by initiating apoptosis [2,9]. While the nature of drug interactions with the cellular target has been extensively studied, the mechanism by which chemotherapeutic agents induce the apoptotic pathway remains unclear.

Cycloheximide (CHI) is a well-known inhibitor of protein synthesis and synergistically increases tumor necrosis factor-α (TNF-α) cytotoxicity [10]. In mouse and rat thymocytes and in many other cell types, apoptosis requires protein and RNA synthesis, suggesting the existence of 'death proteins' [11]. In contrast, the HL-60 human leukemia cell line and certain TNF-susceptible lines have been shown to undergo increased apoptosis when macromolecular synthesis is inhibited, suggesting that in these cases labile 'protective proteins' may exist [12]. Probably, regulation of apoptosis differs with cell type and stage of differentiation and does not always require protein synthesis.

Here we demonstrate that cycloheximide in sublethal doses causes apoptosis in liver cells in vivo, inducing c-*myc*, c-*fos*, c-*jun* and *p53* gene expression and accumulation of sphingosine, a toxic product of the sphingomyelin cycle, in liver cell nuclei.

## 2. Materials and methods

Wistar rats weighing 120–150 g were treated with CHI (Serva) at single doses of 0.01, 0.05, 0.1 and 0.3 mg per 100 g of body weight; these doses are known to inhibit [$^3$H]leucine incorporation into proteins in a dose-dependent manner.

The livers were removed at various time intervals after administration of CHI.

Nuclear fraction from liver cells was prepared as described [13]. The purity of nuclear preparations were assessed by electron microscopy and marker enzyme assays.

RNA was isolated from rat livers with the guanidine isothiocyanate method [14]. Total RNA (25 μg) was fractionated in 1% agarose gel in denaturing conditions, transferred to nitrocellulose filters, and hybridized with plasmids containing inserts of the c-*myc*, c-*fos*, c-*jun* or *p53* genes and labelled by nick-translation.

Lipids from liver and nuclei were isolated as described [15].

Sphingosine was analyzed by reverse-phase HPLC as its fluorescent derivatives (2H-isoindoles) as described [16].

Analysis of DNA fragmentation was performed by agarose gel electrophoresis.

## 3. Results

### 3.1. *Inhibition of protein synthesis by various doses of cycloheximide*

CHI at a sublethal dose (0.3 mg/100 g) inhibits protein synthesis to 95% of the control level. Lower doses (0.1 and 0.05 mg/100 g) decreased the inhibiting effect of CHI on protein synthesis to 60%. 0.01 mg/100 g of CHI had no effect on protein synthesis (Fig. 1).

### 3.2. *Relationship between the levels of c-myc, c-fos, c-jun and p53 gene expression in rat liver and the extent of protein synthesis inhibition by cycloheximide*

The 95% inhibition of protein synthesis by a sublethal dose of CHI induced short-term superexpression of c-*myc*, c-*fos* and c-*jun* genes (Fig. 2). Transcripts of these genes were found during a period of 2–4 h after CHI injection. At the stage of restoration of protein synthesis the mRNA content produced by these genes was sharply reduced. The *p53* tumor suppressor gene, which is directly involved in the process of programmed cell death [4], is also activated by a sublethal dose of CHI (Fig. 2). In contrast to c-*fos* and c-*myc*, the expression of the *p53* gene is weaker and lasts for a longer time during the periods of inhibition, restoration, and activation of pro-

*Corresponding author: Fax: (7) (95) 137 41 01.
E-mail: aless@center.chph.ras.ru


*PII* S0014-5793(97)01161-7

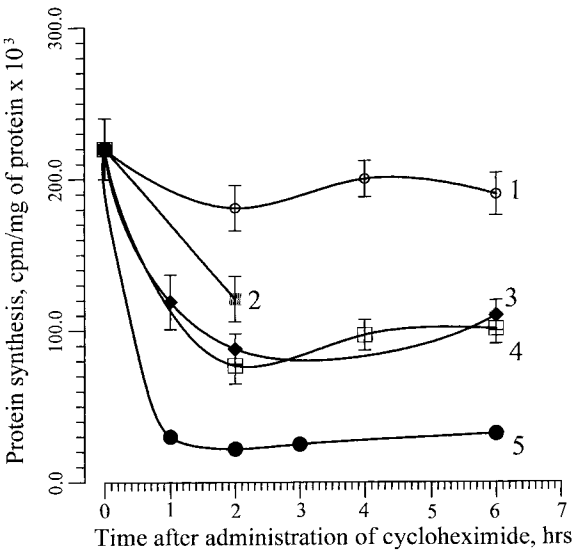


Fig. 1. Time course of changes in protein synthesis in rat liver cells after administration of CHI: 1, 0.01; 2, 0.025; 3, 0.05; 4, 0.1; 5, 0.3 (mg/100 g).

tein synthesis (Fig. 2). The decrease of protein inhibition by CHI at doses of 0.1 and 0.05 mg/100 g was accompanied by a decrease of c-*myc* and c-*fos* expression. At a dose of 0.01 mg/100 g CHI failed to inhibit protein synthesis and stimulate c-*myc*, c-*fos* and *p53* gene expression [17,18].

### 3.3. *Protein synthesis inhibition induces apoptosis in liver cells in vivo*

Unlike thymocytes and T-cell lines in which protein synthesis inhibition has been shown to prevent induced apoptosis [11,19], we found that CHI at a concentration of 0.3 mg/100 g (sublethal dose) caused an increase in DNA fragmentation at all time points. Gel electrophoresis showed clear DNA ladders with no evidence of random-sized DNA fragments (Fig. 3), indicating the occurrence of apoptosis but not necrosis.

### 3.4. *Morphology of apoptotic liver cells*

By electron microscopy apoptotic liver cells isolated 6 h after the administration of CHI showed the condensation of chromatin into a more uniform electrodense mass (Fig. 4A), the formation of apoptotic bodies (Fig. 4B), condensation of cytoplasm and zeiosis (blebbing attributed to untethering of the plasma membrane from the cytoskeleton). Disruption of the plasma membrane was not observed.

### 3.5. *Changes in the content of sphingosine in rat liver cells and nuclei after treatment with CHI*

It has been found that exposure to sphingosine induced DNA fragmentation and morphological changes characteristic of apoptosis in various cell lines [20–22]. We examined changes in free sphingosine level in the whole hepatocytes and their nuclei during treatment of rats with various doses of CHI (0.01, 0.05 and 0.3 mg/100 g) for indicated time intervals. The sphingosine level in whole hepatocytes increased concomitantly with an increase of c-*myc* and c-*fos* gene expression (Fig. 5).

The sphingosine level in liver cells 2 h after injection of a sublethal dose of CHI was roughly 2-fold greater than that in

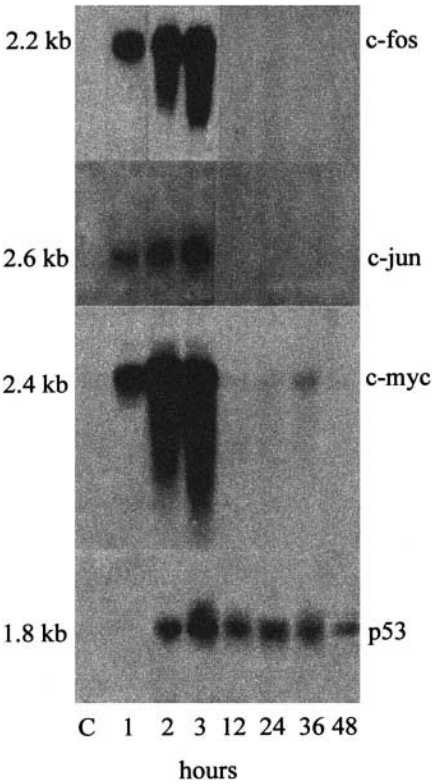


Fig. 2. Time course of c-*fos*, c-*jun*, c-*myc* and *p53* gene expression after injection of CHI in a sublethal dose.

untreated cells. The sphingosine content in control liver cells was 55 ± 15 ng/mg of protein. On the other hand, the level of free sphingosine in livers isolated from rats injected with CHI at doses which did not sharply increase gene expression did not show any elevation. Sphingosine accumulated considerably in liver cell nuclei 2 h after CHI injection at a sublethal dose, exceeding the control values almost 3-fold (Fig. 5). The


Fig. 3. Time course of apoptotic DNA degradation in liver cells after injection of CHI in a sublethal dose. Lane 1, control; lanes 2–7, 1, 2, 3, 4, 6 and 10 h after injection respectively.

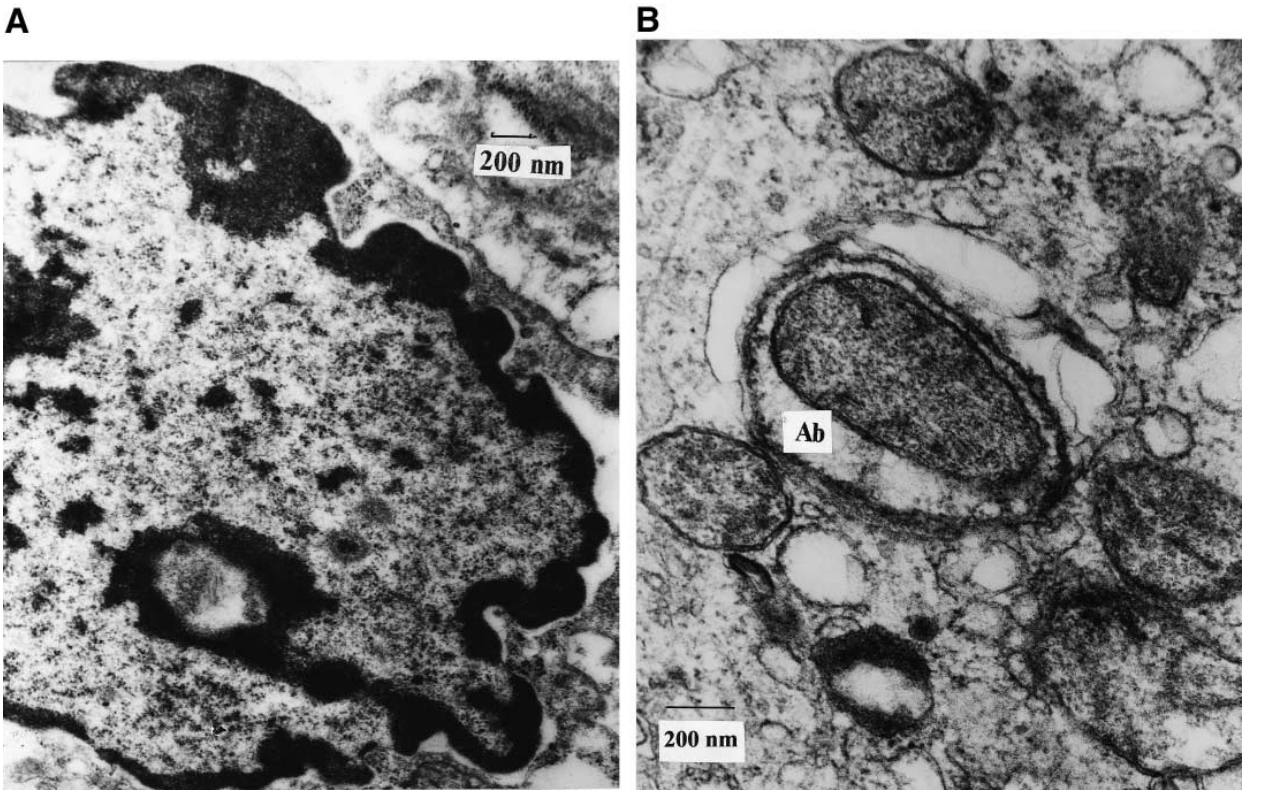


Fig. 4. Electron micrographs of apoptotic changes observed in mouse liver cells 6 h after injection of a sublethal dose of CHI. A: Condensation of chromatin in the nucleus. B: Formation of apoptotic bodies, which consist of intact mitochondrion and a fragment of cytoplasm surrounded by the unit membrane.

content of free sphingosine in control nuclei was 49 ± 9 ng/mg of protein.

## 4. Discussion

Internucleosomal cleavage of DNA is a result of an apoptotic-specific signal transduction which involves lipid second messengers, protein kinases, protein phosphatases, proteases and oncoproteins. We have shown that regulation of apoptosis in liver cells is different from that in thymocytes, because the inhibitor of protein synthesis CHI is able to induce apoptotic events in liver while apoptosis in thymocytes was inhibited by CHI. CHI-induced apoptosis in liver was detected by gel electrophoresis of internucleosomal DNA fragments and morphological changes in nuclei and chromatin structure by electron microscopy. CHI-mediated apoptotic changes in hepatocytes were accompanied by the overexpression of c-*myc*, c-*fos* and c-*jun* genes and long-term expression of *p53* gene. The protein products of these genes contribute significantly in transducing the apoptotic signal. Concomitantly with this elevation of gene expression the sphingosine level also increased in the whole hepatocytes and their nuclei. Sphingosine, a metabolite of sphingomyelin turnover, can elicit a variety of cellular responses such as inhibition of growth factor action, modulation of receptor function, inhibition of calmodulin-dependent enzymes and promotion of antitumor activity [22]. It has been found that sphingosine functions as an inhibitor of protein kinase C (PKC) and plays an important role in TNF-induced apoptosis [6,7,20,21]. PKC inhibitors strongly induce apoptosis in certain cell types [23]. Sphingosine regulates among other biological processes growth suppression which is PKC-independent [22]. For example, sphingosine induces dephosphorylation of the Rb protein independent of PKC inhibition [24]. The potent and specific activation of Rb by

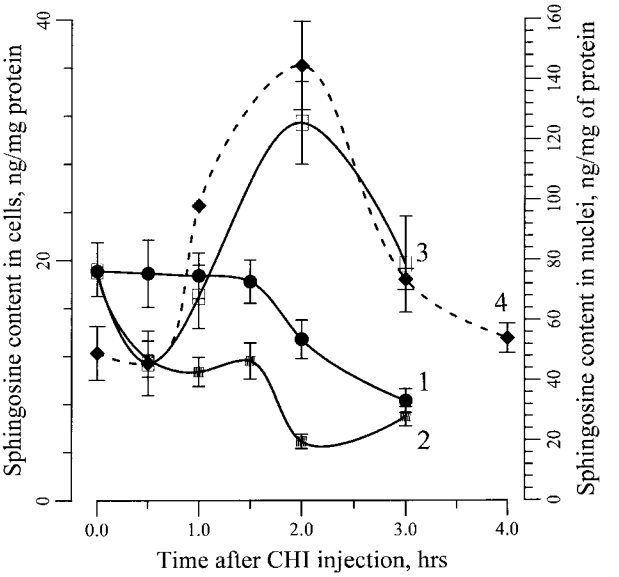


Fig. 5. Time course of changes in free sphingosine level in hepatocytes during treatment of rats with different doses of CHI (1, 0.01; 2, 0.05; 3, 0.3 mg/100 g) and time course of changes in free sphingosine level in liver cell nuclei after injection of a sublethal dose of CHI (4).

sphingosine correlates with inhibition of cell growth and with arrest at $G_0/G_1$ of the cell cycle. *Bcl*-2 is known to inhibit apoptosis in response to a wide spectrum of agents, like chemotherapeutic agents, TNF-α, ionizing radiation and activation of the FAS receptor [25]. Recently, it was shown that apoptosis induced by sphingosine and *N*,*N*-dimethylsphingosine was accompanied by a concomitant decrease of *bcl*-2 expression at both RNA and protein levels in HL-60 cells, while expression of *bcl*-XL and *bax* mRNA was not affected [26]. These results suggest that sphingosine may function as an endogenous mediator of apoptotic signaling.

The results of our experiments suggest that continuous synthesis of labile 'protective' proteins is necessary to restrain apoptosis in liver. Sphingosine might be important in mediating CHI-induced apoptosis as an endogenous modulator of PKC and inhibitor of *bcl*-2 expression.

*Acknowledgements:* The authors would like to thank Katya Gupalo for her editorial assistance. This study was supported by the Russian Foundation for Basic Research (Grant 95-04 12209a).

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