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Augmenter of liver regeneration: An important intracellular survival factor for hepatocytes☆

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Abstract

Background/Aims—Augmenter of liver regeneration (ALR), a protein synthesized and stored in hepatocytes, is associated with mitochondria, and possesses sulfhydryl oxidase and cytochrome *c* reductase activities. We sought to determine the effects of ALR depletion in hepatocytes by antisense oligonucleotide transfection.

Methods—Rat hepatocytes in primary culture were transfected with antisense oligonucleotide for ALR mRNA (ALR-AS) or scrambled oligonucleotide. Various analyses were performed at times up to 24 h after transfection.

Results—Treatment with ALR-AS caused a decrease in ALR mRNA, cellular depletion of ALR protein primarily from mitochondria, and decreased viability. Flow cytometric analysis of ALR-AS-transfected hepatocytes stained with annexin-V_{cy3} and 7-aminoactinomycin D revealed apoptosis as the predominant cause of death up to 6 h; incubation beyond this time resulted in necrosis in addition to apoptosis. ALR-AS-transfection caused release of mitochondrial cytochrome *c*, activation of caspase-3, profound reduction in the ATP content, and cellular release of LDH. Inhibition of caspase-3 inhibited the early phase of ALR-AS-induced death but not the late phase that included ALR and LDH release.

Conclusions—These results suggest that ALR is critically important for the survival of hepatocytes by its association with mitochondria and regulation of ATP synthesis.

Keywords

Augmenter of liver regeneration; Antisense; Hepatocytes; Apoptosis; Necrosis

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1. Introduction

Augmenter of liver regeneration (ALR), originally identified in the soluble fractions of hypertrophic rat livers [1–3], was purified from weanling rat liver [4,5], and its gene cloned in rat, mouse and human [6,7]. The mitogenic and antiatrophic activities of native and cloned ALR were demonstrated in partially hepatectomized rats [1] and in dogs with portacaval shunt [2–4,6,7]. ALR also inhibits the lytic activity of hepatic natural killer cells [8,9], which is critical for unabated liver regeneration [10]. Presence of hepatotrophic activity in weanling and regenerating [1–3,11] but not in the unmodified adult animal livers [1,3] suggested that ALR is not synthesized by the latter. However, we found that ALR mRNA and protein are expressed similarly in weanling and unmodified adult rat livers in hepatocytes [12]. Together, these observations suggest that ALR may have multiple intracellular and extracellular functions. Indeed, results of experiments with short form (15 kDa) [13–15] differ substantially from that with long form (22 kDa) ALR [12].

The C-terminus of mammalian ALR is 40% identical with ERV1P (essential for respiration and vegetative growth 1 protein) [16], product of the *Saccharomyces cerevisiae* gene *ERV1* [17,18]. ERV1p and ALR are flavin-containing sulfhydryl oxidases localized in the mitochondrial intermembrane space [19–21]. ERV1p is necessary for the growth and survival of the yeast as indicated by complete loss of mitochondrial genome and death upon disruption of the *ERV1* gene [17,22]. We hypothesized that in hepatocytes, ALR might play role functionally equivalent to ERVlp. Transfection of primary hepatocytes with antisense oligonucleotide for ALR mRNA (ALR-AS) led to mitochondrial and cellular depletion of ALR, profound loss of ATP, mitochondrial release of cytochrome *c*, cellular release of LDH and apoptotic/necrotic death of hepatocytes.

2. Materials and methods

2.1. Preparation of hepatocytes

Hepatocytes were prepared as described previously [23], and suspended (0.25×10^6 cells/ml) in William's medium E supplemented with 2 mM L-glutamine, penicillin/streptomycin, 10% FBS, and 10^{-6} M insulin. Cells were plated at a density of $0.063 \times 10^6/cm^2$, the medium renewed 3 h later, and the cells used following overnight incubation.

2.2. Transfection with ALR-AS

The sequence of phosphorothioated ALR-AS-oligonucleotide complementary to nucleotide sequence 24–43 downstream from the start codon of ALR mRNA was 5′-GACTGCCGCGAGGGAAACCT-3′; the sequence of scrambled oligonucleotide was 5′-ACTGACAGATCGGGCAAGCC-3′. To increase stability [24], five bases of the oligonucleotides at both 5′ and 3′ ends were replaced with 2′-*O*-meth RNA, internal dCs were replaced with 5-Me-dC, FAM-labeling was added at the 5′ end and a hairpin loop was introduced at the 3′ end. Lipofectamine-2000 (8 μl/ml; Invitrogen) or the oligonucleotides (final concentration 1.2 μM) in lipofectamine-2000 were incubated separately in 200 μl of Opti-MEM medium (GibcoBRL, Grand Island, NY) for 30 min. Eight hundred microliters of Opti-MEM was added and the combined solution was overlaid onto the cells. After 5 h, 1 ml of William's medium E containing 20% FBS was added to each well. The efficiency of transfection was determined by fluorescence microscopy.

2.3. Live cell imaging

Collagen-coated (10%) 40-mm coverslips with cultured hepatocytes were inserted in a FCS2 closed heated (37 °C) live cell chamber (Bioptics, Bulter, PA), which was placed on a Nikon TE300 inverted microscope (Melville, NY). Media were perfused through the chamber at a

rate of 0.5 ml/h. Using a 40× dry objective, five stage positions were imaged with transmitted light every 3 min. The acquired images were processed with Metamorph software (Universal Imaging Corporation, Downingtown, PA).

2.4. Flow cytometric analysis

The medium containing detached cells was aspirated, and the attached cells were harvested using 0.025% trypsin. The two cell fractions were pooled, washed twice with PBS by centrifugation at 450*g* (10 min, 4 °C), fixed in ice-cold 70% ethanol for 3 h, and washed with Ca^{2+}-/Mg^{2+}-free HBSS containing 1% BSA. The cells were suspended in 0.5 ml of propidium iodide solution (50 μg/ml propidium iodide, 1 mg/ml sodium citrate, 100 μg/ml RNase I and 0.1% Triton X-100). After 30 min at 37 °C, the cells were analyzed by flow cytometry in a fluorescence-activated cell sorter (Epics XL.MCL, Beckman–Coulter) using EXPO32 software.

To distinguish apoptosis and necrosis, the cells were harvested (see above), washed with PBS, and suspended in buffer A (10 mM Hepes, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4) at 1×10^6 cells/ml. Annexin-V_{cy3} (4 μg/ml) and 7-aminoactinomycin D (7-AAD) (5 μg/ml) were added to 100 μl of the cell suspension. After gentle mixing, the suspension was incubated at room temperature for 15 min in dark, followed by the addition of 400 μl of buffer A. Flow cytometry was performed within 1 h.

2.5. Determination of viability, ATP and cell death markers

The viability was determined by the MTT assay [25]. The cells were harvested as described above for ATP determination using Cell Viability Assay Kit-ATP (Sigma Chemical Co., St. Louis, MO). Cytosolic cytochrome *c* was measured using the Quantikine murine immunoassay kit (R&D Systems, Minneapolis, MN). Caspase-3 activity was determined using caspase fluorescent assay kit (BD Biosciences-Clontech, San Jose, CA). LDH was measured using spectrophotometric assay kit (Stanbeo Laboratory, Boerne, TX).

2.6. Determination of ALR mRNA and protein

After treatments, the culture medium was aspirated and centrifuged to separate detached cells. ALR in the medium was measured by ELISA [12]. The attached cells were harvested by trypsin treatment; the two cell fractions were combined and centrifuged (l000*g*/5 min). Total RNA was extracted from the cells and analyzed for ALR mRNA by semiquantitative RT-PCR as described previously [12].

For mitochondrial preparation, hepatocytes (pooled attached and detached fractions) were homogenized in 2 mM Hepes, pH 7.4, containing 0.22 M mannitol, 70 mM sucrose, 0.1 mM EDTA, and 1% fatty acid-free BSA. The homogenate was centrifuged at 800*g* for 10 min, followed by centrifugation of the supernatant at 11,000*g* for 15 min. The pellet was washed and suspended in RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA) containing 25 μl/ml protease inhibitor cocktail (Sigma) and 0.5 mM phenylmethylsulfonylfluoride. After 15 min on ice, the lysate was centrifuged (10,000*g*/10 min); ALR in the supernatant was determined by ELISA and Western blot analysis [12].

For cytosolic preparation, hepatocytes were homogenized in 10 mM Tris–HCl, pH 8.0, containing 60 mM KCl, 1 mM EDTA, 100 μM PMSF and 25 μl/ml protease inhibitor cocktail. After 10 min on ice, NP40 (25 μl/ml) was added; the homogenate was vortexed vigorously for 10 s, centrifuged (2500 rpm/4 min) followed by centrifugation of the supernatant at 14,000 rpm for 5 min. ALR in the supernatant was quantified by ELISA [12].

2.7. Statistical analysis

Statistical significance was determined by ANOVA-Duncan analysis using SPSS program. A *p* value of 0.05 was considered statistically significant.

3. Results

3.1. Effect of recombinant rat ALR (rrALR) on hepatocytes

Fig. 1A shows purity of the rrALR by Coomassie blue staining and Western blot analysis [12]. The rrALR did not affect the DNA synthesis in hepatocytes at either 24 h or 48 h (Fig. 1B); in contrast, TGF-α caused a robust increase in the DNA synthesis at both points. The lack of response of rat hepatocytes to 22 kDa-rrALR is consistent with absence of ALR-specific receptors in them [12].

3.2. Effect of ALR-AS on cellular ALR and viability

ALR-AS-treatment caused time-dependent loss of ALR from cells with concomitant increase in the extra-cellular medium (Fig. 2A and B). This effect was associated with progressive loss of viability (Fig. 2C). The relatively high basal level of ALR release without apparent loss from cultured hepatocytes is consistent with our previous report [12]. Phase contrast microscopy showed rounding and detachment of hepatocytes by ALR-AS but not scrambled-ODN treatment (Fig. 3A). Flow cytometric cell cycle analysis (Fig. 3B) showed that 35% and 33% of ALR-AS-transfected hepatocytes were in G0/G1 and G2/M phases, respectively, as compared with 20% and 70% control cells, indicating strong growth arrest and increased apoptosis [26]. Scrambled ODN-treatment induced only marginal changes. Continuous acquisition of the live cell images of ALR-AS-transfected hepatocytes showed beginning of their shrinking and detachment within 2–3 h. In the image captured at 5.5 h (Fig. 3C), several hepatocytes exhibited apoptotic characteristics (rounding, loose contact with neighboring cells and surface, and cytoplasmic blebs). Only few scrambled-ODN-treated hepatocytes showed evidence of morphological changes indicative of apoptosis at 12 h. Together these results suggest that ALR is essential for physiological function and survival of hepatocytes.

We determined ALR mRNA and protein levels at 12 h time point considering near-maximal loss of ALR and viability (Fig. 2). ALR-AS-treatment caused nearly complete loss of ALR mRNA, (Fig. 4A). There was substantial loss of ALR from ALR-AS-treated cells when the whole cell homogenate was analyzed (Fig. 4B); tracking the subcellular distribution showed that cellular ALR loss was predominantly from the mitochondrial compartment. The decrease in the cytosolic ALR was modest, but the loss of mitochondrial ALR was very strong both by ELISA (Fig. 4B) and Western blot analysis (Fig. 4C).

Whether ALR-AS causes necrosis secondary to apoptosis was determined in hepatocytes labeled with annexin-V_{cy3} [marker of phosphatidylserine externalization during apoptosis [27,28], and 7-AAD [permeant marker of presumptively necrotic cells [29]. At 24 h of ALR-AS treatment, apoptotic cells (Fig. 5A, lower right quadrant) increased threefold (Fig. 5B), while the number of cells in late apoptosis/necrosis phase (upper right quadrant) (Fig. 5A) increased fourfold (Fig. 5B). Relatively small changes were seen in scrambled ODN-transfected cells (Fig. 5A and B).

3.3. Apoptotic signaling, ATP loss and LDH release

To evaluate more precisely the role of apoptosis in ALR-AS-treated hepatocytes, we quantified cytosolic cytochrome *c* and caspase-3-like activity. Both cytosolic cytochrome *c* and caspase-3-like activity increased robustly between 6 and 12 h in ALR-AS-treated cells, with peak values at 12 h (Fig. 6). Scrambled ODN-treated hepatocytes showed a smaller progressive increase in cytosolic cytochrome *c* between 6 and 24 h, and relatively small increase in the

caspase-3-1ike activity. The ATP content of ALR-AS-treated cells decreased progressively between 3 h and 24 h, with the sharpest decrease between 3 and 6 h (Fig. 7A). Scrambled ODN-treated cells showed a small progressive decrease, while in control cells ATP content was unchanged. LDH was released at 12 h and later from ALR-AS-transfected cells (Fig. 7B), with no change in scrambled ODN-treated cells. Together these results confirm that both apoptosis and necrosis contribute to the loss of ALR-AS-treated hepatocytes.

Next we determined the time-course of the loss of ALR and ATP from mitochondria (Fig. 8). Within 1 h after ALR-AS treatment, there was substantial loss of mitochondrial ALR (maximal at 6 h), followed by ATP loss with significant decrease at 3 h and maximal between 12 and 24 h.

Finally, we determined if caspase-3 inhibition blocks ALR-induced apoptosis/necrosis of ALR-treated hepatocytes. Pretreatment and presence of DEVD-fmk during ALR-AS-treatment inhibited depletion of ALR, and LDH release till 6 h (Fig. 9A–C) and inhibited substantially the loss of viability till 12 h (Fig. 9D). But after this, the loss of ALR, LDH and viability was nearly similar in incubations without and with DEVD-fmk (Fig. 9). These results suggest that the initial phase of ALR-AS-induced cells death (apoptotic) is inhibited by caspase-3 inhibition, but not the continued ALR loss leading to necrosis.

4. Discussion

In this report we present evidence supporting ALR's role as an intracellular survival factor for hepatocytes. The 22 kDa rrALR did not stimulate DNA synthesis in rat hepatocytes unlike in human hepatocytes with recombinant human ALR dimer (rhALR) [13,15]. On a molar basis hepatocyte DNA synthesis stimulated by rhALR was much stronger than that by the powerful hepatocyte mitogens EGF and TGF-α [13]. The effects of rhALR and the most potent hepatocyte mitogen HGF on the DNA synthesis in human hepatocytes were also comparable [15]. The discrepancy may be because we used the entire rrALR molecule (198 amino acids; starting sequence MAAPS–) and not the short ALR molecule (125 amino acids) with starting sequence of MRTQ– ("M" representing amino acid "74" in native ALR) [7]. In previous studies [13,15], dimer of the human ALR C-terminus sequence starting with MRTQ– ("M" representing amino acid "71" in native ALR [7]) expressed in the bacterial system was used. The monomer (short ALR) has mw of about 15 kDa while the cDNA for native ALR encodes a protein with mw of about 22 kDa [6,7]. The crystal structure [30] and the FAD-linked sulfhydryl oxidase and cytochrome *c* reductase activities of ALR [31] were deduced based on the assumption that it is present as a dimer of the short ALR. Unlike the rat and mouse ALR monomers, each 15 kDa subunit of human ALR contains two additional nonconserved free cysteine residues (C74 and C85) [31]. Again whether the long human ALR performs the same function is unclear [31]. However, we note that both the full-length 22 kDa and 15 kDa carboxy terminal portion of the ERV1 protein, which contains the conserved CXXC motif, were found to bind FAD and perform the sulfhydryl oxidase function [19]. Interestingly, the full-length ERV1 protein (22 kDa/189 amino acid residues), but not its carboxy terminal 15 kDa domain that forms dimer [19], contains leader sequence for import into mitochondria, and thus the N-terminal domain is clearly required for mitochondrial translocation of ERV1p [32]. Putative targeting sequences for mitochondria have also been found in the homologous mammalian proteins [32]. ALR is colocalized with GFAP in neurons in all brain regions, specifically in the nucleus and the external envelope of mitochondria; Western blot analysis demonstrated at least 2 dimers, which under reducing condition migrated as two bands with MW of 21 and 23 kDa [33]. The rat ALR was found to exist as high molecular weight species (about 38–42 kDa) and can very well be the dimers of the full-length ALR [12]. A 15 kDa ALR isoform was reported to be present in the human cancer cell line HepG2 [14], but whether it dimerises before eliciting biological actions is not known.

Transfection of rat hepatocytes with ALR-AS decreased their viability profoundly due to apoptosis and necrosis. Significant release of cytochrome *c*, but not of LDH, at 6 h indicates that apoptosis is a primary mechanism of cell death at this time point. Thereafter, the release of LDH increased suggesting that necrosis was a major cause of cell death after 6 h. In the late stages of apoptosis also cell membrane may become permeable thus allowing the release of LDH [34–36]. The involvement of both cell death processes was confirmed by the presence of annexin-5cy3-positive/7-AAD-negative and annexin-5cy3-positive/7-AAD-positive cells after ALR-AS treatment. While ALR depletion caused apoptosis and necrosis of hepatocytes, interestingly, only the apoptotic phase of cell death was strongly but not completely inhibited by caspase-3 inhibition. DEVD-fmk inhibited but did not prevent ALR loss as well as LDH release from ALR-AS-treated cells. Thus the necrotic phase of ALR depletion-induced cell death seems to be independent of prior apoptosis. Also caspase-independent apoptosis at early times (compare Figs. 6B and 9D) [37] may not be ruled out. A small effect of the scrambled-ODN observed at late time points might be nonspecific as these effects are not accompanied by any cellular or mitochondrial ALR loss compared to the control.

Depletion of ATP in ALR-AS-transfected hepatocytes suggests possible association of ALR with mitochondrial function. Mitochondria playa critical role in apoptosis, which cannot occur in the setting of cellular ATP depletion [38–43]. Both an apoptosis-dependent increase in ATP consumption and a separate decrease in ATP synthesis may contribute to the lethal ATP depletion, global loss of cellular integrity, and necrosis [44] of ALR-AS-treated hepatocytes. In support of this supposition, ERV1P is shown to be essential for the biogenesis of mitochondria [17,22,32], normal mitochondrial morphology and stable maintenance of mitochondria in *S. cerevisiae* [16,45]. Disruption of the *ERV-1* gene results in complete loss of mitochondrial genome and death of the yeast following a few replications [22]. Thus the significant association of ALR with mitochondria and its progressive loss that preceded mitochondrial ALR depletion and cytochrome *c* release during treatment with ALR-AS are consistent with the notion that ALR, like ERV-1P, might regulate oxidative phosphorylation, which is critical for the function and survival of the cells. In intact rats, ALR modulates hepatocyte mitochondrial function by increasing both nuclear-encoded mitochondrial transcription factor A and the oxidative phosphorylation capacity of the liver [46]. It is well known that a number of hepatically active mediators have no distinct effect in hepatocytes, but elicit the release of secondary mediators from nonparenchymal cells. Indeed, our preliminary data (not shown) indicate that rat Kupffer cells specifically bind radioiodinated 22 kDa-rrALR. These data provide both a convincing positive control for the undetectable binding seen in hepatocytes, and a mechanism for ALR-elicited hepatocyte-targeted mediator release from this nonparenchymal population.

Although ALR and ERV-1 are essential for the survival of hepatocytes and *S. cerevisiae*, respectively, a major difference is that ALR is abundantly expressed in hepatocytes [12], but the expression of ERV-1 is very low in the yeast [45]. Despite the low level of ERV-1 expression, null mutation in the gene in the haploid yeast strain stopped their replication after 3–4 days [17]; it was suggested that this effect was due to high *in vivo* stability of the ERV-1 mRNA and protein. Lisowsky [22], using expression of the ERV1 gene under the *GAL1-10* promoter, found complete loss of mitochondrial genome after the promoter was shut off. The cells grew for a limited time under these conditions, but showed arrest in cell-division cycle as the terminal phenotype suggesting the essential requirement but stability of the ERV1 mRNA and protein. The ALR mRNA levels were almost completely reduced and cellular and the mitochondrial release of ALR protein was significantly increased in ALR-AS-treated cells suggesting that the depletion of ALR from mitochondria is associated with hepatocyte death.

In summary, our results indicate a fundamental role of ALR in the survival of primary hepatocytes. Whether the death caused by the inhibition of its synthesis and specific release

from the mitochondria has clinical relevance needs further investigation. Based on the results presented herein, we propose that there is a dynamic exchange between the mitochondrial and newly synthesized and post-translationally modified cytosolic ALR (Fig. 10). Inhibition of ALR synthesis leads to decreased uptake but does not stop its mitochondrial release. This results in reduced ATP synthesis, mitochondrial injury, release of cytochrome *c* and apoptosis. Progressive depletion of ALR and further decrease in ATP causes necrosis, which is independent of caspase-mediated apoptotic phase.

Abbreviations

7-AAD	7-aminoactinomycin D
ALR	augmenter of liver regeneration
ERV	essential for respiration and vegetative growth
AS	antisense
rrALR	recombinant rat ALR
rhALR	recombinant human ALR.

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References

1. LaBrecque DR, Pesch LA. Preparation and partial characterization of hepatic regenerative stimulator substance (HSS) from rat liver. J Physiol 1975;248:273–284. [PubMed: 1151784]
2. Starzl TE, Jones AF, Terblanche J, Usui S, Porter KA, Mazzoni G. Growth-stimulating factor in regenerating canine liver. Lancet 1979;1:127–130. [PubMed: 84151]
3. Francavilla A, Hagiya M, Porter KA, Polimeno L, Ihara I, Starzl TE. Augmenter of liver regeneration: its place in the universe of hepatic growth factors. Hepatology 1994;20:747–757. [PubMed: 8076931]
4. Francavilla A, Barone M, Van Thiel DH, Mazzaferro V, Prelich J, Starzl TE. Further steps of HSS (hepatic stimulatory substance) purification. Dig Dis Sci 1991;36:674–680. [PubMed: 2022170]
5. Francavilla A, Ove P, Polimeno L, Coetzee M, Makowka L, Rose J, et al. Extraction and partial purification of a hepatic stimulatory substance in rats, mice, and dogs. Cancer Res 1987;47:5600–5605. [PubMed: 3664466]
6. Hagiya M, Francavilla A, Polimeno L, Ihara I, Sakai H, Seki T, et al. Cloning and sequence analysis of the rat augmenter of liver regeneration (ALR) gene: expression of biologically active recombinant ALR and demonstration of tissue distribution. Proc Natl Acad Sci USA 1994;91:8142–8146. (Proc Natl Acad Sci USA 1995;92:3076 (Correction)). [PubMed: 8058770]
7. Giorda R, Hagiya M, Seki T, Shimonishi M, Sakai H, Michaelson J, et al. Analysis of the structure and expression of the augmenter of liver regeneration (ALR) gene. Mol Med 1996;2:97–108. [PubMed: 8900538]
8. Francavilla A, Vujanovic NL, Polimeno L, Azzarone A, Iacobellis A, Deleo A, et al. The in vivo effect of hepatotrophic factors augmenter of liver regeneration, hepatocyte growth factor, and insulin-like growth factor-II on liver natural killer cell functions. Hepatology 1997;25:411–415. [PubMed: 9021955]
9. Tanigawa K, Sakaida I, Masuhara M, Hagiya M, Okita K. Augmenter of liver regeneration (ALR) may promote liver regeneration by reducing natural killer (NK) cell activity in human liver diseases. J Gastroenterol 2000;35:112–119. [PubMed: 10680666]
10. Vujanovic NL, Polimeno L, Azzarone A, Francavilla A, Chambers WH, Starzl TE, et al. Changes of liver-resident NK cells during liver regeneration in rats. J Immunol 1995;154:6324–6338. [PubMed: 7759871]

11. Francavilla A, Starzl TE, Porter K, Scotti-Foglieni C, Michalopoulos GK, Carrieri G, et al. Screening for candidate hepatic growth factors by selective portal infusion after Eck's fistula. Hepatology 1991;14:665–670. [PubMed: 1916668]
12. Gandhi CR, Kuddus RH, Subbotin VM, Prelich J, Rao AS, DeLeo A, et al. A fresh look at augmenter of liver regeneration (ALR) in rats. Hepatology 1999;29:1435–1445. [PubMed: 10216127]
13. Wang G, Yang X, Zhang Y, Wang Q, Chen H, Wei H, et al. Identification and characterization of receptor for mammalian hepatopoietin that is homologous to yeast ERV1. J Biol Chem 1999;274:11469–11472. [PubMed: 10206950]
14. Wang Y, Lu C, Wei H, Wang N, Chen X, Zhang L, et al. Hepatopoietin interacts directly with COP9 signalosome and regulates AP-1 activity. FEBS Lett 2004;572:85–91. [PubMed: 15304329]
15. Dayoub R, Thasler WE, Bosserhoff AK, Singer T, Jauch KW, Schlitt HJ, et al. Regulation of polyamine synthesis in human hepatocytes by hepatotrophic factor augmenter of liver regeneration. Biochem Biophys Res Commun 2006;345:181–187. [PubMed: 16677602]
16. Hofhaus G, Stein G, Polimeno L, Francavilla A, Lisowsky T. Highly divergent amino termini of the homologous human ALR and yeast scERV1 gene products define species specific differences in cellular localization. Eur J Cell Biol 1999;78:349–356. [PubMed: 10384986]
17. Lisowsky T. Dual function of a new nuclear gene for oxidative phosphorylation and vegetative growth in yeast. Mol Gen Genet 1992;232:58–64. [PubMed: 1552903]
18. Lisowsky T, Weinstat-Saslow DL, Barton N, Reeders ST, Schneider M. A new human gene located in the PKD1 region of chromosome 16 is a functional homologue to ERV-1 of yeast. Genomics 1995;29:690–697. [PubMed: 8575761]
19. Lee J, Hofhaus G, Lisowsky T. Ervlp from *Saccharomyces cerevisiae* is a FAD-linked sulfhydryl oxidase. FEBS Lett 2000;477:62–66. [PubMed: 10899311]
20. Lisowsky T, Lee JE, Polimeno L, Francavilla A, Hofhaus G. Mammalian augmenter of liver regeneration protein is a sulfhydryl oxidase. Dig Liver Dis 2001;33:173–180. [PubMed: 11346147]
21. Lange H, Lisowsky T, Gerber J, Muhlenhoff U, Kispal G, Lill R. An essential function of the mitochondrial sulfbydryl oxidase Erv1p/ALR in the maturation of cytosolic Fe/S proteins. EMBO Rep 2001;2:715–720. [PubMed: 11493598]
22. Lisowsky T. ERV1 is involved in the cell-division cycle and the maintenance of mitochondrial genomes in *Saccharomyces cerevisiae*. Curr Genet 1994;26:15–20. [PubMed: 7954891]
23. Kuddus RH, Nalesnik MA, Subbotin VM, Rao AS, Gandhi CR. Enhanced synthesis and reduced metabolism of endothelin-1 (ET-1) by hepatocytes: a mechanism of increased ET-l levels in cirrhosis. J Hepatology 2000;33:725–732.
24. Neckers LM. Oligodeoxynucleotide inhibitors of function: mRNA and protein interactions. Cancer J Sci Am 1998;4:S35–S42. [PubMed: 9619269]
25. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. J Immunol Methods 1983;65:55–63. [PubMed: 6606682]
26. Diez-Fernandez C, Sanz N, Alvarez AM, Wolf A, Cascales M. The effect of non-genotoxic carcinogens, Phenobarbital and clofibrate, on the relationship between reactive oxygen species, antioxidant enzyme expression and apoptosis. Carcinogenesis 1998;19:1715–1722. [PubMed: 9806150]
27. Vermes I, Haanen C, Rutelingsperger CPM. A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescence labeled annexin V. J Immunol Methods 1995;180:39–52. [PubMed: 7622868]
28. Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Ab1. J Exp Med 1995;182:1545–1556. [PubMed: 7595224]
29. Philpott NJ, Turner AJ, Scopes J, Westby M, Marsh JC, Gordon-Smith EC, et al. The use of 7-amino actinomycin D in identifying apoptosis: simplicity of use and broad spectrum of application compared with other techniques. Blood 1996;87:2244–2251. [PubMed: 8630384]
30. Wu CK, Dailey TA, Dailey HA, Wang BC, Rose JP. The crystal structure of augmenter of liver regeneration: a mammalian FAD-dependent sulfhydryl oxidase. Protein Sci 2003;12:1109–1118. [PubMed: 12717032]

31. Farrell SR, Thorpe C. Augmenter of liver regeneration: a flavin-dependent sulfhydryl oxidase with cytochrome *c* reductase activity. Biochemistry 2005;44:1532–1541. [PubMed: 15683237]
32. Lisowsky T. Removal of an intron with unique 3′ branch site creates an amino-terminal protein sequence directing the scERV1 gene product to mitochondria. Yeast 1996;12:1501–1510. [PubMed: 8972573]
33. Tury A, Mairet-Coello G, Lisowsky T, Griffond B, Fellmann D. Expression of the sulfhydryl oxidase ALR (augmenter of liver regeneration) in adult rat brain. Brain Res 2005;1048:87–97. [PubMed: 15916753]
34. Morita M, Watanabe Y, Akaike T. Protective effect of hepatocyte growth factor on interferon-gamma-induced cytotoxicity in mouse hepatocytes. Hepatology 1995;21:1585–1593. [PubMed: 7768503]
35. Crenesse D, Laurens M, Gugenheim J, Heurteaux C, Cursio R, Rossi B, et al. Intermittent ischemia reduces warm hypoxia-reoxygenation-induced JNK(1)/SAPK(1) activation and apoptosis in rat hepatocytes. Hepatology 2001;34:972–978. [PubMed: 11679968]
36. Ahlemeyer B, Klumpp S, Krieglstein J. Release of cytochrome *c* into the extracellular space contributes to neuronal apoptosis induced by staurosporine. Brain Res 2002;934:107–116. [PubMed: 11955473]
37. Chipuk JE, Green DR. Do inducers of apoptosis trigger caspase-independent cell death? Nat Rev Mol Cell Biol 2005;6:268–275. [PubMed: 15714200]
38. Strasser A, O'Connor L, Dixit VM. Apoptosis signaling. Annu Rev Biochem 2000;69:217–245. [PubMed: 10966458]
39. Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998;281:1312–1316. [PubMed: 9721091]
40. Kim JS, He L, Qian T, Lemasters JJ. Role of the mitochondrial permeability transition in apoptotic and necrotic death after ischemia/reperfusion injury to hepatocytes. Curr Mol Med 2003;3:527–535. [PubMed: 14527084]
41. Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell 1996;86:147–157. [PubMed: 8689682]
42. Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME. Apoptosis signaling by death receptors. Eur J Biochem 1998;254:439–459. [PubMed: 9688254]
43. Lemasters JJ. The mitochondrial permeability transition: from biochemical curiosity to pathophysiological mechanism. Gastroenterology 1998;115:783–786. [PubMed: 9742002]
44. Leist M, Single M, Castoldi AF, Kuhnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med 1997;85:1481–1486. [PubMed: 9126928]
45. Becher D, Kricke J, Stein G, Lisowsky T. A mutant for the yeast scERVI gene displays a new defect in mitochondrial morphology and distribution. Yeast 1999;15:1171–1181. [PubMed: 10487920]
46. Polimeno L, Capuano F, Marangi LC, Margiotta M, Lisowsky T, Ierardi E, et al. The augmenter of liver regeneration induces mitochondrial gene expression in rat liver and enhances oxidative phosphorylation capacity of liver mitochondria. Dig Liver Dis 2000;32:510–517. [PubMed: 11057927]

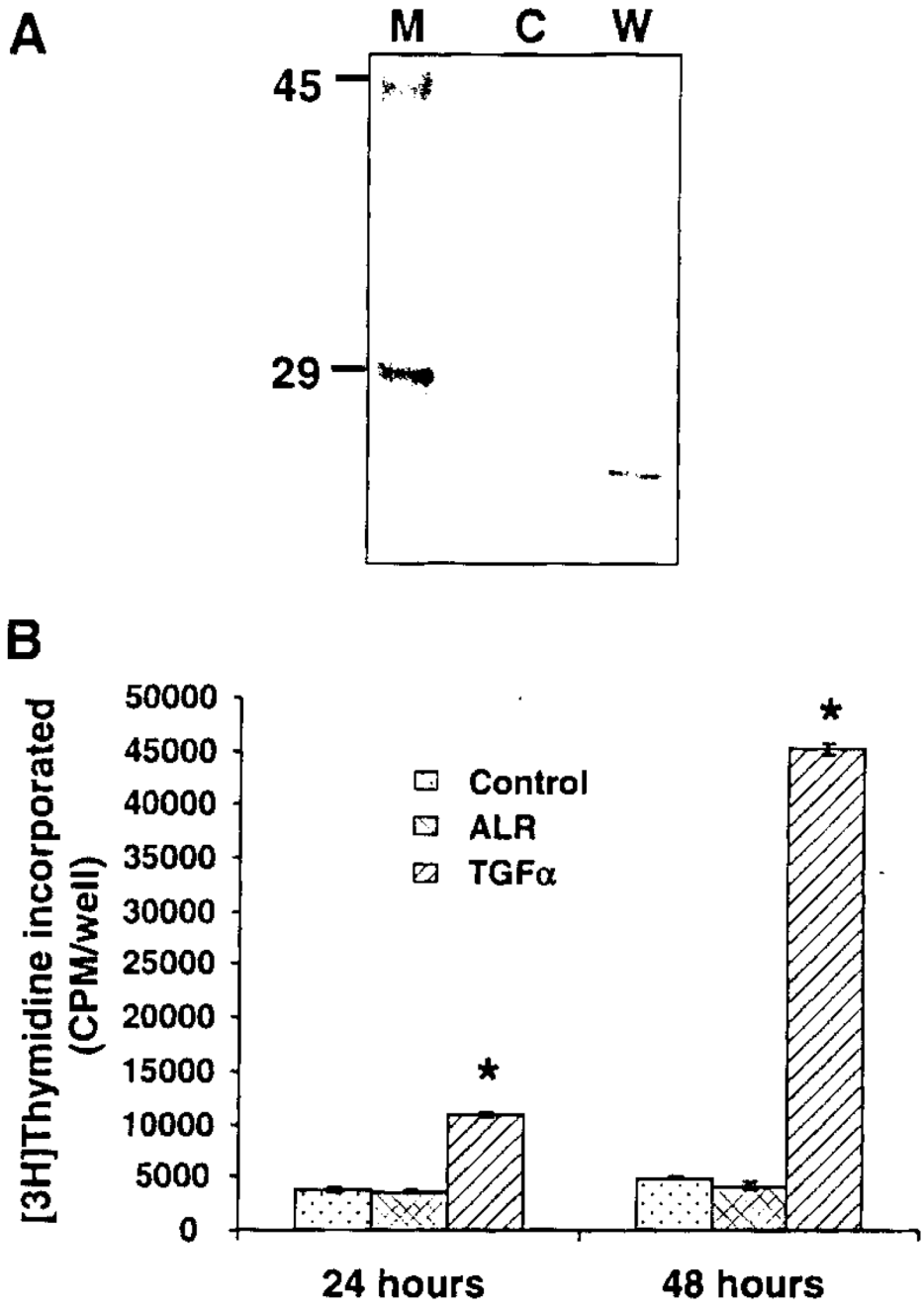

Fig. 1.

Effect of rrALR on DNA synthesis in hepatocytes. (A) Coomassie blue staining and Western blot analysis of recombinant rat ALR (rrALR) using anti-rrALR antibody show a single band with molecular weight of about 22 kDa. (B) Hepatocytes were stimulated under serum-free condition with 1 μg/ml ALR or 10 ng/ml TGF-α for 24 and 48 h. DNA synthesis was then measured by [^{3}H]thymidine incorporation assay. $^*p < 0.001$ vs control.

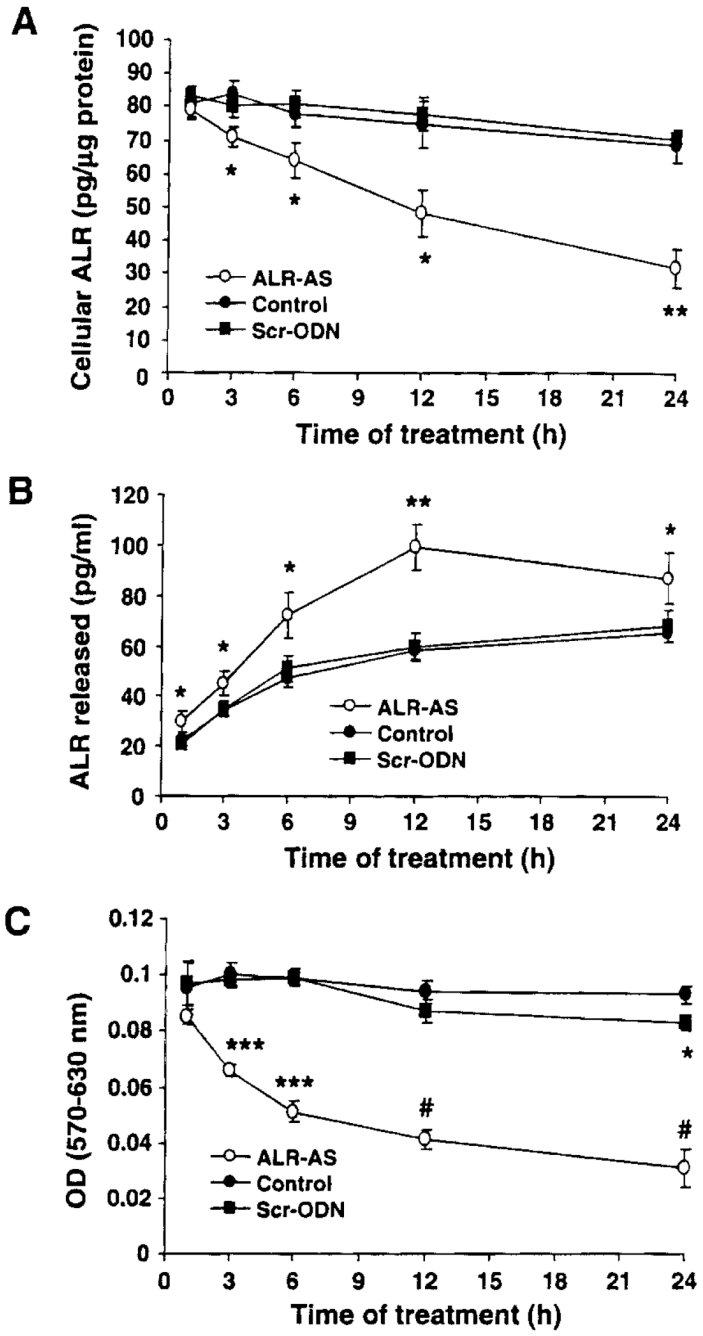

Fig. 2.

Time-course of ALR-AS-induced loss of cellular ALR and viability. Hepatocytes were transfected with ALR-AS or scrambled oligonucleotides as described in Section 2. At indicated time points, the release of ALR into extracellular medium (A), cell-associated ALR (B), and cell viability (C) was measured. Results are means ± SD of triplicate determinations from a representative experiment. $^{*}p < 0.05$ vs control; $^{**}p < 0.01$ vs control; $^{***}p < 0.005$ vs control; $^{\#}p < 0.001$ vs control.

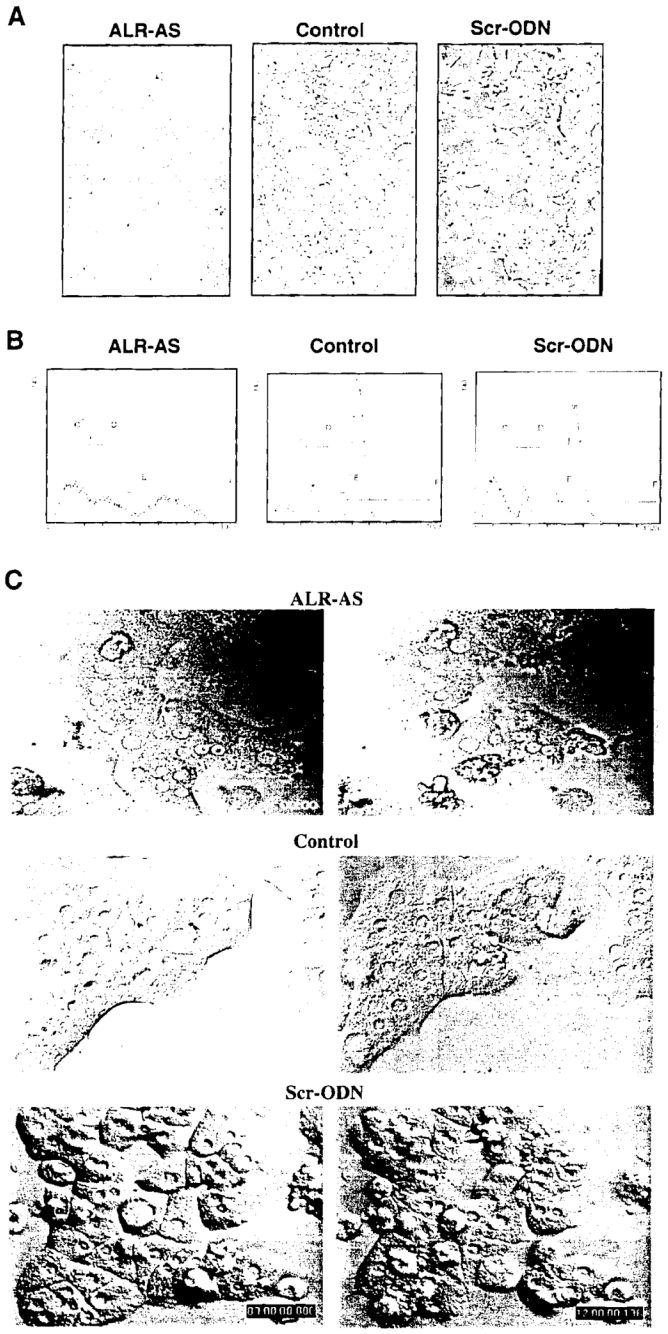

Fig. 3.

Morphology and live cell images of hepatocytes after transfection with ALR-AS. Hepatocytes were transfected with ALR-AS or scrambled oligonucleotides as described in Section 2. (A) Phase contrast images of ALR-AS-treated, control and scrambled ODN-treated cells at 24 h are shown. (B) Cells were harvested at 24 h after transfection with ALR-AS or scrambled ODN and subjected to flow cytometry after labeling with propidium iodide. The subG0 phase, G0/G1, S, and G2/M phases of the cell cycle were analyzed by diploid staining profiles and ModFit software program. Forward and side light scatter characteristics were used to exclude cell debris (size and granularity) from the analysis. The percentage of cell aggregates (doublets) and other background were also eliminated using peak vs integral gating of cells. C, G0 subphase; D,

G0/G1 phase; E, S phase; and F, G2/M phase. Values for the percentage of cells in various phases with standard deviations from three-independent experiments are presented in the text. (C) Images of ALR-AS-treated, control, and scrambled ODN-treated hepatocytes were acquired every 3 min as described in Section 2. Representative images of hepatocytes acquired at the start of the experiment and at about 5.5 h (ALR-AS-treated), and 12 h (control or scrambled ODN-treated) are shown.

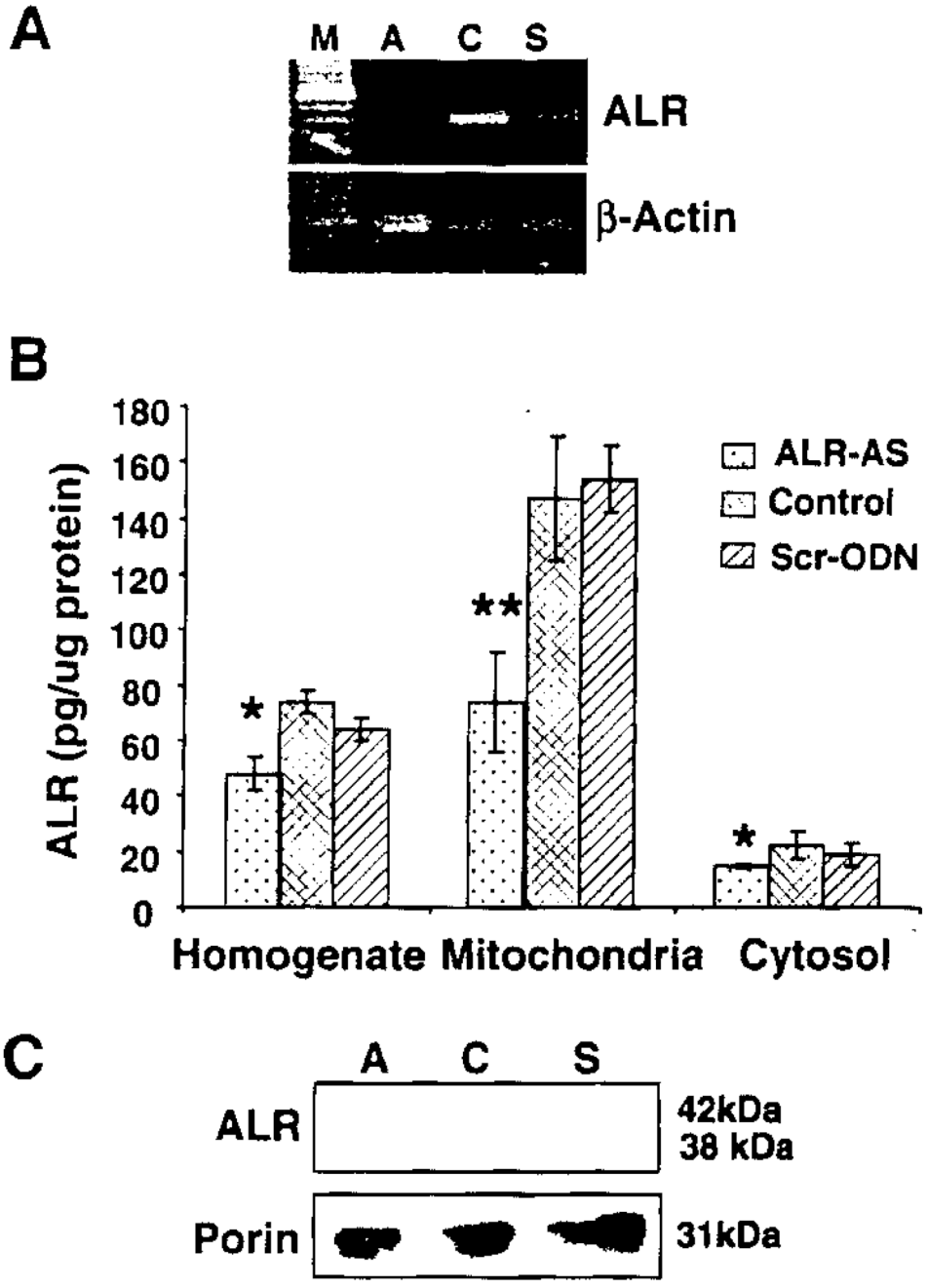

Fig. 4.

Effect of ALR-AS on ALR mRNA and protein. (A) mRNA from the whole cells of ALR-AS-treated (A), control (C) and scrambled ODN-treated (S) hepatocytes were extracted after 12 h incubation period. RT-PCR was performed as described in Section 2. The expression of β-actin (mRNA control) is shown to indicate equal loading. (B) The whole homogenate, mitochondria and cytosol were analyzed for the ALR levels by ELISA after various treatments for 12 h. Results shown are means ± SD of triplicate determinations from a representative experiment. $^{*}p < 0.05$ vs control; $^{**}p < 0.025$ vs control. (C) Protein from the mitochondria of ALR-AS-treated (A), control (C) and scrambled ODN-treated (S) hepatocytes was extracted

after 12 h incubation and Western blot analysis were performed as described in Section 2. Porin expression is shown to indicate similar loading of mitochondrial protein.

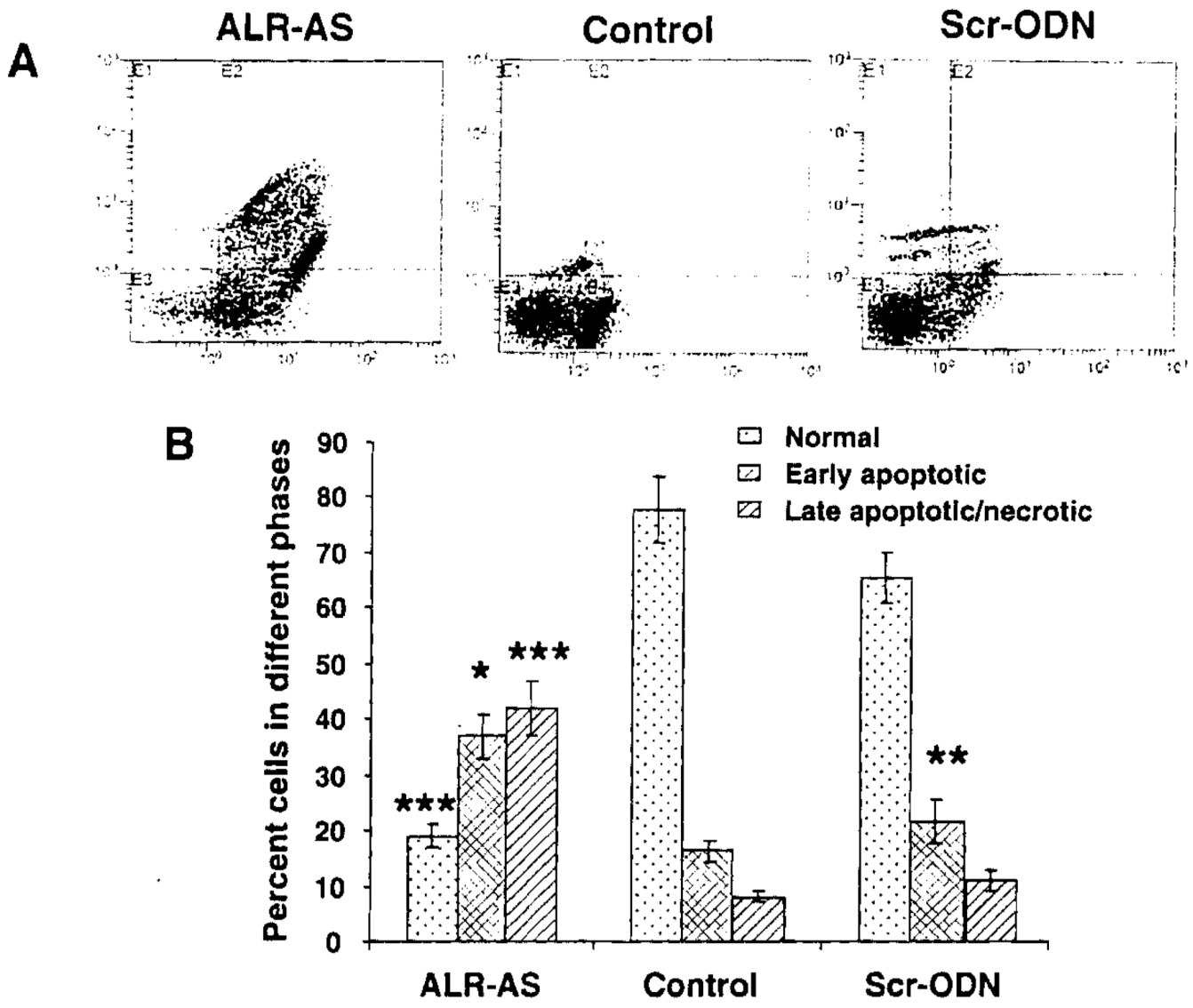

Fig. 5.

Flow cytometry of annexin-V_{cy3}- and 7-AAD-labeled hepatocytes. Control cells and cells treated with scrambled ODN or ALR-AS were harvested at 24 h and labeled with annexin-V_{cy3} and 7-AAD. (A) Flow cytometry was then performed as described in Section 2; a representative profile is shown. E1, E2, E3 and E4 represent cell debris, necrotic cells (annexin-V_{cy3}-positive/7-AAD-positive), normal cells (annexin-V_{cy3}-negative/7-AAD-negative) and apoptotic cells (annexin-V_{cy3}-positive/7-AAD-negative), respectively. (B) Histogram showing percentage of normal cells, and cells in early apoptotic and late apoptotic/necrotic phases. For each measurement, 10,000 events were counted. Each value is average of duplicate determinations from two separate experiments ± SD. $^{*}p < 0.01$ vs control; $^{**}p < 0.05$ vs control; $^{***}p < 0.005$ vs control.

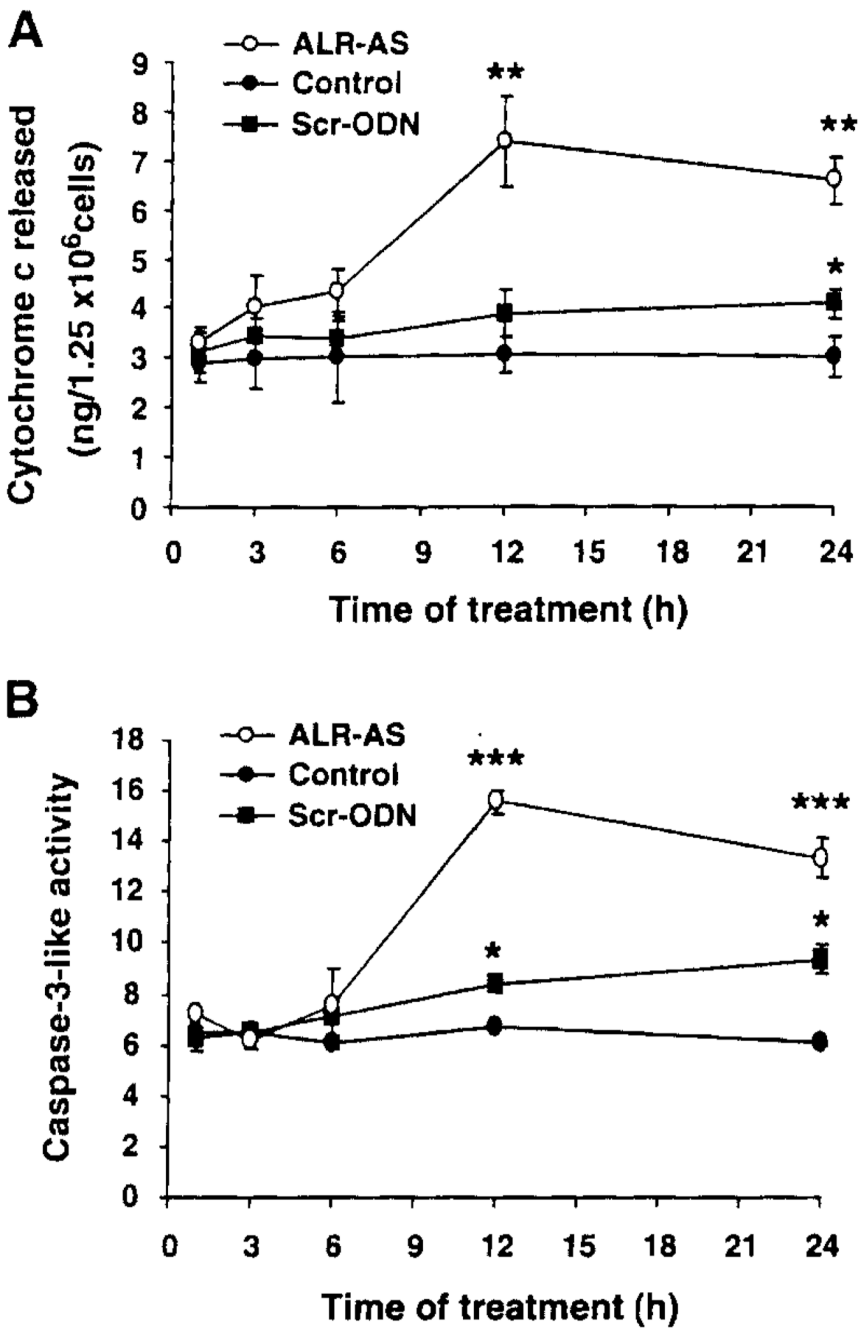

Fig. 6.

Effect of ALR-AS on cytochrome *c* release and caspase-3-like activity. Cells were treated with ALR-AS, lipofectamine carrier (control) or scrambled ODN for indicated time periods. Mitochondrial release of cytochrome *c* (A) and caspase-3-like activity (B) were determined as described in Section 2. Results are means ± SD of triplicate determinations from a representative experiment. $^{*}p < 0.05$ vs control; $^{**}p < 0.01$ vs control; $^{***}p < 0.001$ vs control.

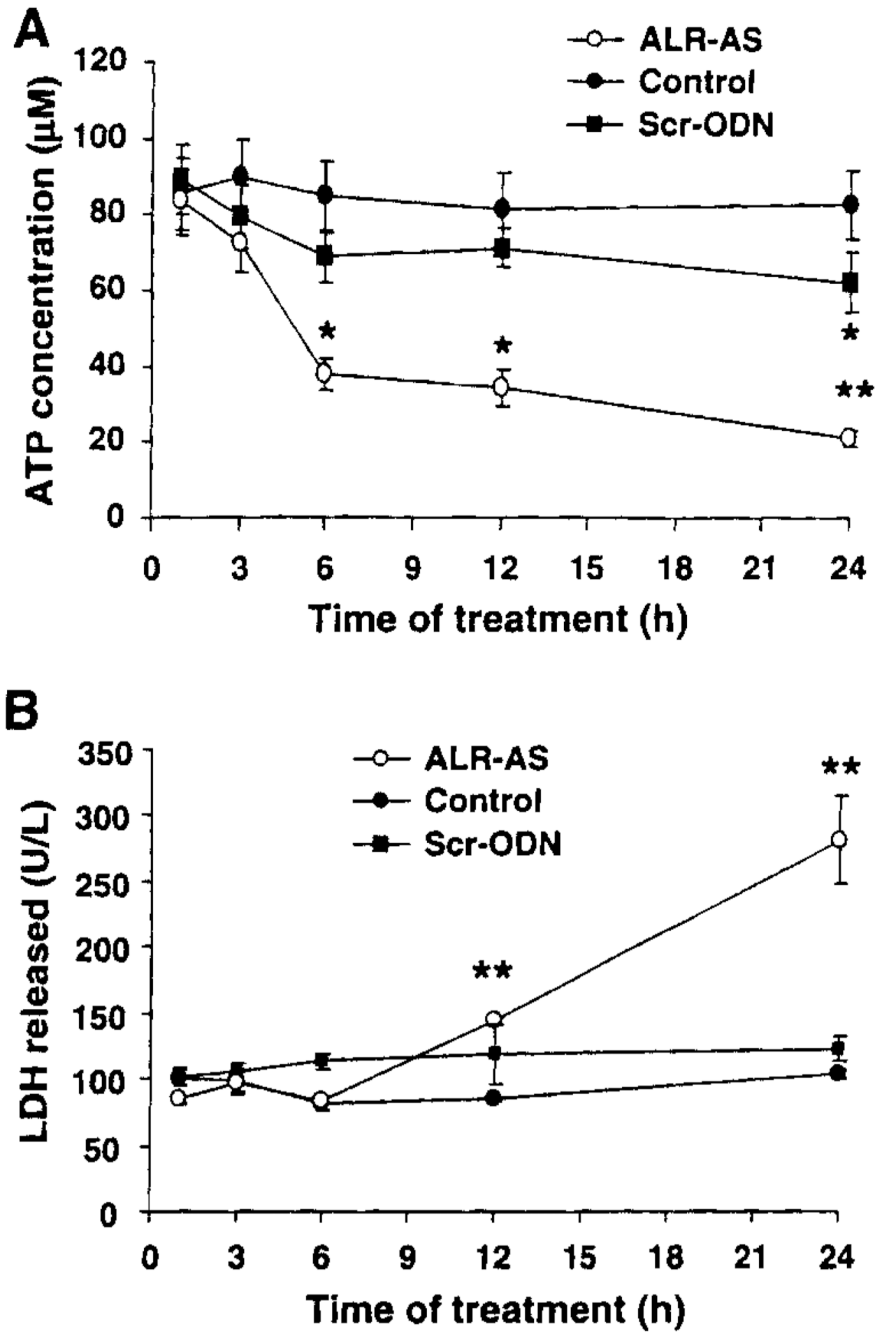

Fig. 7.

Effect of ALR-AS on the ATP content and LDH release in hepatocytes. Cells were treated with ALR-AS, PBS or scrambled ODN for indicated time periods, and cellular ATP content (A) and release of LDH (B) were measured as described in Section 2. Results are means ± SD of triplicate determinations from a representative experiment. $^{*}p < 0.05$ vs control; $^{**}p < 0.001$ vs control.

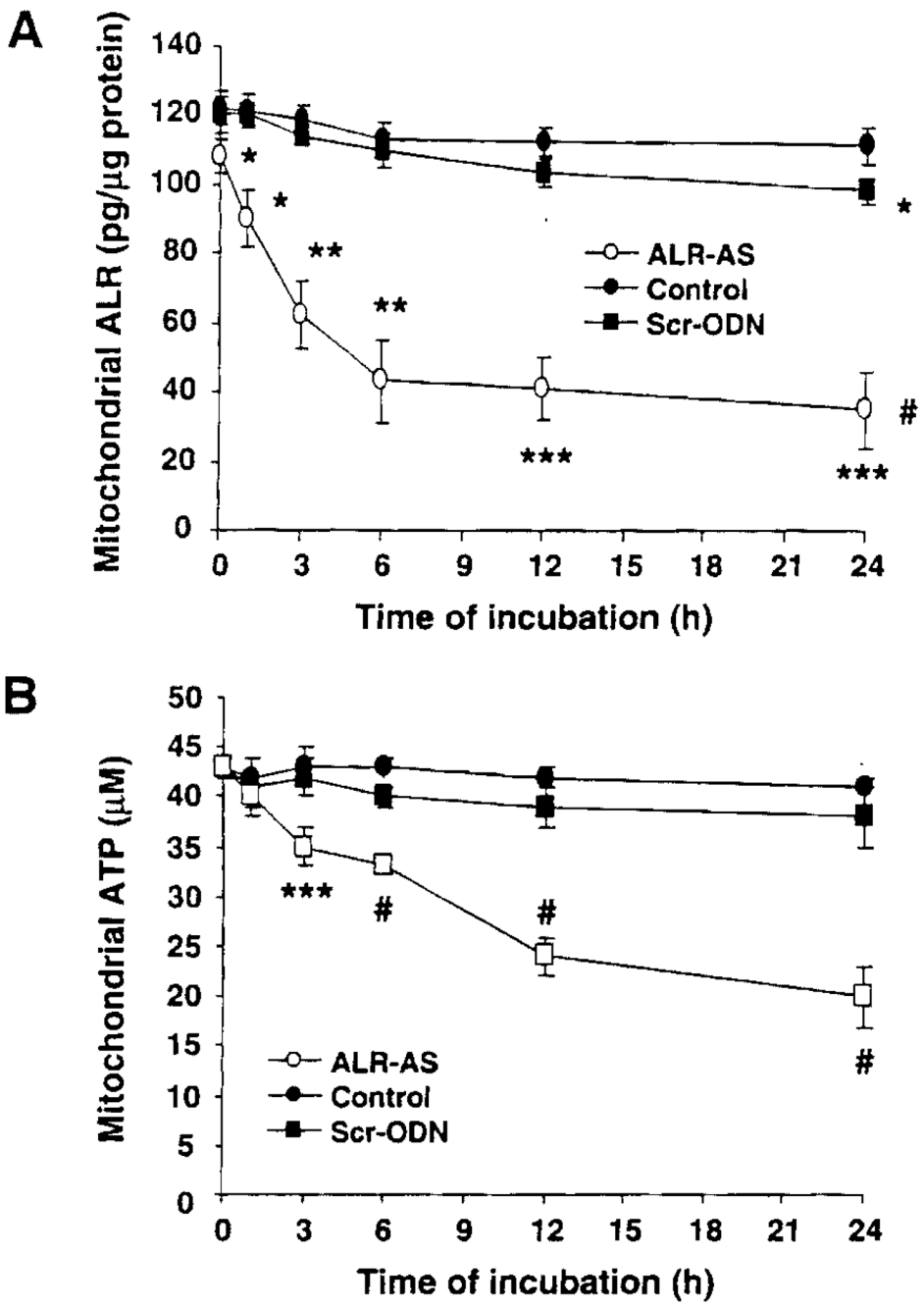

Fig. 8.

Effect of ALR-AS on mitochondrial ALR and ATP. Cells were treated with ALR-AS, PBS or scrambled ODN for indicated time periods. Mitochondria were then prepared, and their ALR (A) and ATP (B) contents were measured. Results are means ± SD of triplicate determinations from a representative experiment. $^{*}p < 0.05$ vs control; $^{**}p < 0.01$ vs control; $^{***}p < 0.005$ vs control; $^{\#}p < 0.001$ vs control.

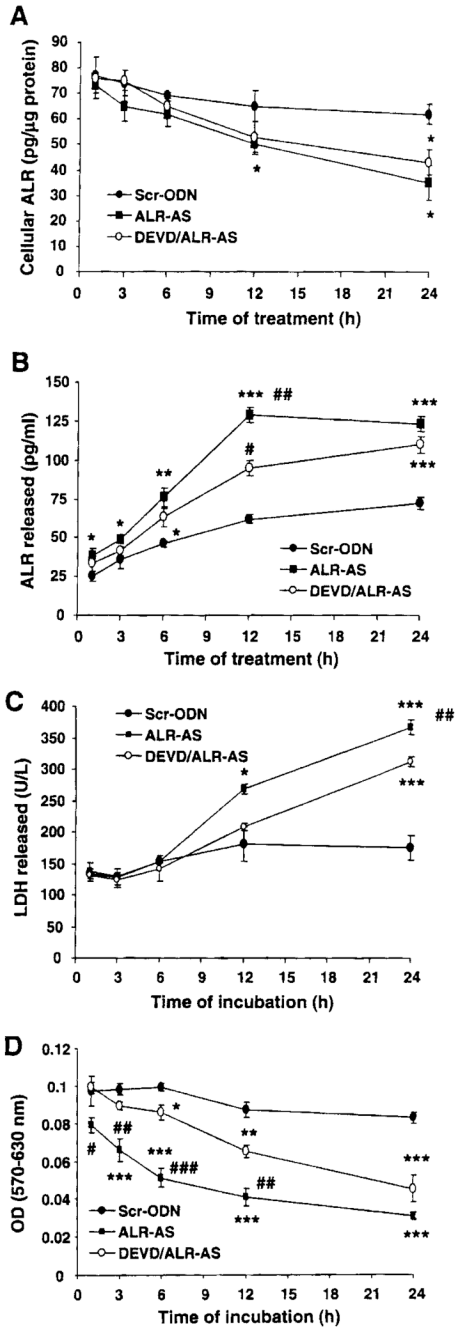

Fig. 9.

Effect of caspase-3 inhibitor DEVD-fmk on ALR release and cellular ALR, LDH release and viability of hepatocytes during ALR-AS treatment. Cells preincubated with 5 μM DEVD-fmk or carrier for 30 min. The medium was aspirated and the cells were then treated with scrambled ODN or ALR-AS (without and with DEVD-fmk) as described in Section 2. At indicated time points, ALR release in the medium (A), cellular ALR (B), extracellular release of LDH (C) and cell viability (D) were determined. Results are means ± SD of triplicate determinations from a representative experiment. $^{*}p < 0.05$ vs control; $^{**}p < 0.01$ vs control; $^{***}p < 0.001$ vs control; $^{\#}p < 0.005$ vs control; $^{\#\#}p$, 0.05 vs DEVD-fmk/ALR-AS; $^{\#\#\#}p < 0.005$ vs DEVD/ALR-AS.

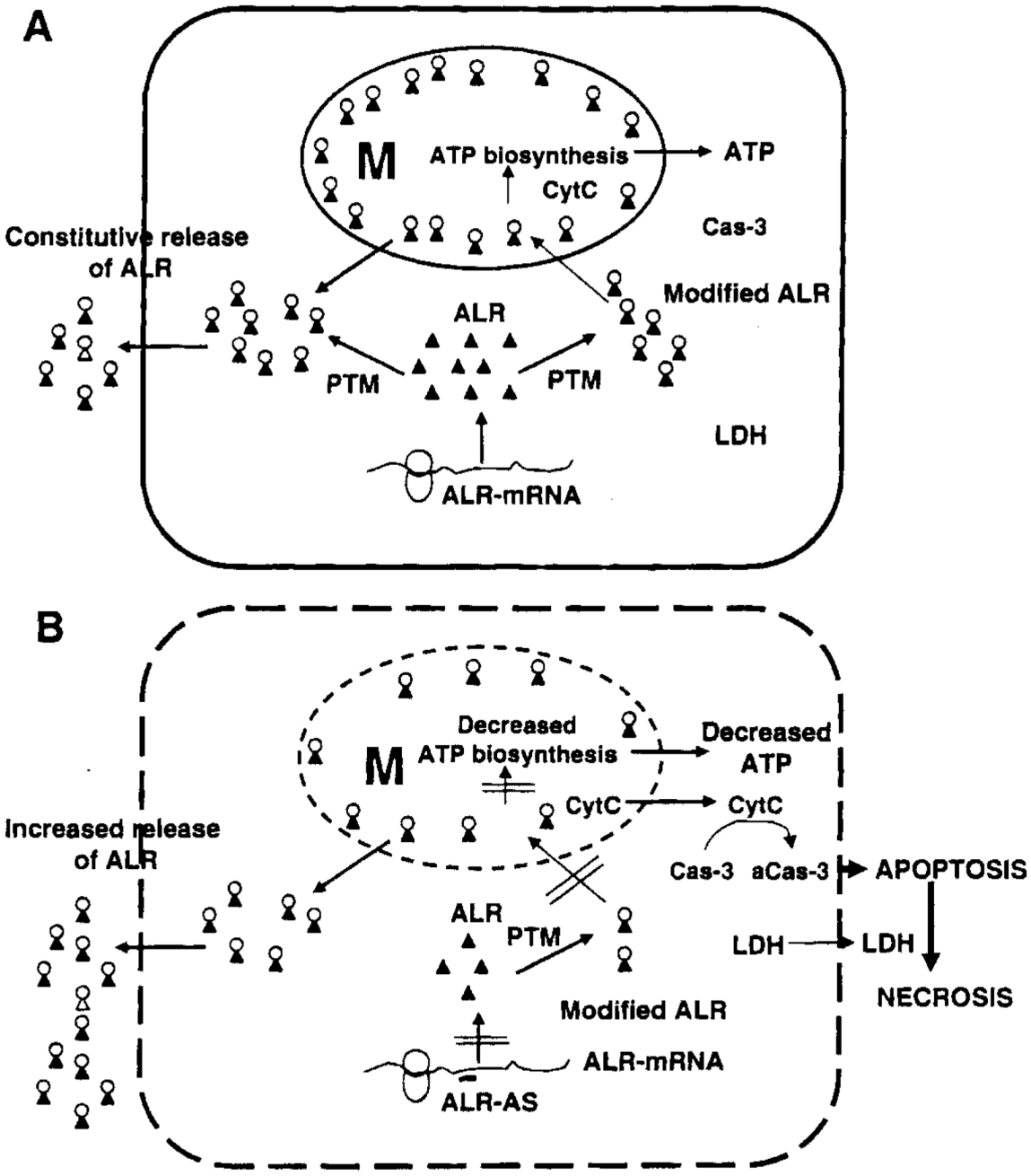

Fig. 10.

Schematic representation of ALR-AS-induced death of hepatocytes. (A) In physiology, ALR is constitutively synthesized and post-translationally modified. The modified ALR is transported into mitochondria and is involved in ATP synthesis (oxidative phosphorylation). ALR uptake and release by mitochondria is a dynamic process. The modified (and unmodified) ALR is constitutively released by the cells for actions at distant sites. (B) Upon inhibition of ALR synthesis by ALR-AS transfection, uptake but not the release of ALR by mitochondria (and the cells) is prevented. This leads to inhibition of ATP synthesis, release of cytochrome *c*, activation of caspase-3 and apoptosis. Further reduction in ALR levels causes profound loss of ATP, loss of cell membrane integrity (LDH release) and necrosis. M, mitochondria; PTM,

post-translational modification; CytC, cytochrome *c*; cas-3, caspase-3; aCas-3, activated caspase-3. Other organelles such as nucleus, endoplasmic reticulum, etc. are not shown for clarity.