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ORIGINAL ARTICLE

The Level of ALR is Regulated by the Quantity of Mitochondrial DNA

Tibor Balogh[1] · **Tamás Lőrincz**[1] · **Ibolya Stiller**[2] · **József Mandl**[3] · **Gábor Bánhegyi**[3] · **András Szarka**[1,2]

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Abstract Augmenter of liver regeneration (ALR) contributes to mitochondrial biogenesis, maintenance and to the physiological operation of mitochondria. The depletion of ALR has been widely studied and had serious consequences on the mitochondrial functions. However the inverse direction, the effect of the depletion of mitochondrial electron transfer chain and mtDNA on ALR expression has not been investigated yet. Thus mtDNA depleted, ρ^0 cell line was prepared to investigate the role of mitochondrial electron transfer chain and mtDNA on ALR expression. The depletion of mtDNA has not caused any difference at mRNA level, but at protein level the expression of ALR has been markedly increased. The regulatory role of ATP and ROS levels could be ruled out because the treatment of the parental cell line with different respiratory inhibitors and uncoupling agent could not provoke any changes in the protein level of ALR. The effect of mtDNA depletion on the protein level of ALR has been proved not to be liver specific, since the phenomenon could be observed in the case of two other, non-hepatic cell lines. It seems the level of mtDNA and/or its products may have regulatory role on the protein level of ALR. The up-regulation of ALR can be a part of the adaptive response in ρ^0 cells that preserves the structural integrity and the transmembrane potential despite the absence of protein components encoded by the mtDNA.

Keywords Augmenter of liver regeneration · Mitochondrial DNA · ρ^0 cells · Regulation

✉ András Szarka
szarka@mail.bme.hu

[1] Department of Applied Biotechnology and Food Science, Laboratory of Biochemistry and Molecular Biology, Budapest University of Technology and Economics, Szent Gellért tér 4, Budapest 1111, Hungary

[2] Pathobiochemistry Research Group of Hungarian Academy of Sciences, Semmelweis University, P.O. Box 260, Budapest 1444, Hungary

[3] Department of Medical Chemistry, Molecular Biology and Pathobiochemistry Pathobiochemistry, Semmelweis University, POB 260, Budapest 1444, Hungary

Introduction

Augmenter of liver regeneration (ALR) has been first described as a hepatotrophic factor as it has promoted liver regeneration following partial hepatectomy [1]. ALR protein has been sequenced and cloned in rat [2], mouse and human [3]. ALR is expressed in almost all rat and mouse tissues in a relatively low abundance, but in high abundance in the liver and in the testis [2, 3]. The human ALR and the ERV1 (essential for respiration and vegetative growth) from *Saccharomyces cerevisiae* have been shown high homology with 42 % identical amino acid residues [4]. ERV1 gene is essential for cell viability and for the biogenesis of functional mitochondria. Immediate and complete loss of mitochondrial genome and a specific arrest in the cell-division cycle have been observed after the gene has been switched off [5, 6]. The chimeric construct of ERV1 and the human ALR gene could complement the temperature sensitive ERV1 mutant and restored near normal viability suggesting that the human gene is not just a structural but also a functional homologue of the yeast ERV1 [4]. Later it has been revealed that both proteins possess an additional amino-terminal sequence with mitochondrial import sequence and have a molecular size of approximately 22 kDa [3, 7]. Both ERV1 and ALR are localized predominantly in the intermembrane space (IMS) of mitochondria and act as a sulfhydryl oxidase [8, 9]. The ERV1/ALR proteins are important elements of the

mitochondrial disulphide relay system localized in the IMS. The depletion of ERV1 has prevented the mitochondrial protein import [10]. ERV1 is oxidized by cytochrome c which in turn passes its electrons via cytochrome c oxidase to molecular oxygen [11, 12]. Substrates of the mitochondrial disulphide relay system include many proteins relevant to cytochrome c oxidase biogenesis [13, 14], as well as many Tim chaperones [15]. Sequencing of the ALR gene revealed a c.581 G/A homozygous mutation in an inbred Moroccan family with three siblings affected by congenital cataract, progressive muscular hypotonia, sensorineural hearing loss, and developmental delay [16].

The transfection of primary hepatocytes with antisense oligonucleotide for ALR mRNA 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 [17]. Similar effects could be observed in the recently generated liver-specific conditional ALR knockout (ALR-L-KO) mice. Markedly lower number of mitochondria per hepatocyte could be detected in ALR-L-KO mice at 2 weeks [18]. Majority of them showed swollen, abnormal shape with increased spacing or loss of cristae. The extent of these morphological changes decreased by the elevation of ALR at later times. Similarly the ATP level was extremely low in ALR-L-KO livers at 2 weeks then it increased by 4 weeks and remained constant (at a significantly lower level than in wild type mice) at 8 weeks [18]. The expression of the ATP synthase subunit Atp5g1 and the mitochondrial transcription factor A (TFAM) were also low at 2 weeks then increased at 4 and 8 weeks, but remained lower than in the wild type animals.

Although the majority of mitochondrial proteins are encoded in nuclear DNA (nDNA), mitochondria have their own circular DNA (mtDNA). mtDNA encodes 13 subunits for the electron transfer chain complexes I, III, IV and V, furthermore the essential elements of the mitochondrial translation apparatus (2 rRNAs, and 22 tRNAs) [19].

The close relationship of ALR and mitochondria, mitochondrial biogenesis seems evident. As it was pointed out ALR contributes to mitochondrial biogenesis, maintenance and to the physiological operation of mitochondria [5, 6, 16–18]. The electrons from the mitochondrial relay system (composed of MIA40 and ALR) are channelled to the mitochondrial electron transfer chain [11, 12]. The consequence of the depletion of mitochondrial electron transfer chain (mETC) and mtDNA on ALR has been investigated in hepatic and non-hepatic ρ^0 (mtDNA depleted) cell lines. This way we could gain information on the effect of both ATP and mtDNA depletion on the regulation of ALR expression.

Materials and Methods

Reagents and Antibodies

Growth media, fetal bovine serum, non-essential amino acids, ethidium bromide, sodium pyruvate, uridine, rotenone, antimicyn A, sodium azide and 2,4-dinitrophenol were purchased from Sigma (St. Louis, MO, USA). 100× antibiotic/antimycotic solution, glutamine, Trypsin-EDTA were obtained from GIBCO, Life Technologies. Anti-GFER (ALR) and MIA40 rabbit polyclonal and peroxidase-conjugated goat anti-rabbit polyclonal antibodies were all purchased from Proteintech Group (Chicago, IL, USA). PCR primers for COX II, β-globin, ALR and 18S rRNA were purchased from Sigma (St. Louis, MO, USA). All the other chemicals used were of analytical grade.

Cell Culture

HepG2 human hepatocyte carcinoma, MCF7 human breast adenocarcinoma, SH-SY5Y human neuroblastoma cell lines were obtained from Sigma (St. Louis, MO, USA), and grown at 37 °C, under 5 % CO_2 atmosphere. The HepG2 cell line was cultured in EMEM supplemented with 10 % fetal bovine serum, 2 mM glutamine, 1 % non-essential amino acids and antibiotic/antimycotic solution. The MCF7 cell line was cultured in DMEM supplemented with 10 % fetal bovine serum, 2 mM glutamine and antibiotic/antimycotic solution. Finally the SH-SY5Y cell line was cultured in Ham's F12:EMEM (1:1) supplemented with 15 % fetal bovine serum, 2 mM glutamine, 1 % non-essential amino acids and antibiotic/antimycotic solution. Hereafter, these media will be referred to as 'complete media'.

Preparation and Maintenance of ρ^0 Cell Lines

The generation of ρ^0 cells has been achieved by the protocol of [20] King and Attardi. To deplete the mitochondrial DNA (prepare ρ^0 cell lines) the selected cell lines (HepG2, MCF7, SH-SY5Y) were chronically exposed to ethidium bromide in complete medium supplemented with pyruvate and uridine. HepG2 and MCF7 cells were incubated in the complete medium supplemented with 50 ng/ml ethidium bromide, 100 μg/ml sodium pyruvate and 50 μg/ml uridine for at least 2 weeks. As it was earlier described by Trimmer et al. [21] the SH-SY5Y cells were treated with 5 μg/ml ethidium bromide for approximately 16 weeks, and the medium was supplemented with 100 μg/ml sodium pyruvate and 50 μg/ml uridine as well. The elimination of mtDNA was verified by real time PCR analysis as described below.

The ratio of mitochondrial and nuclear DNA during the ethidium-bromide treatment has been monitored by real-time PCR (PikoReal, Thermo Scientific). The COX II gene has

been chosen as DNA sequence encoded by the mtDNA. The following primer pair has been applied for the amplification of COX II gene: FW 5′-CATCCTAGTCCTCATCGCCCTCC-3′ and REV 5′-GGGCATGAAACTGTGGTTTGCTCC-3′. The β-globin gene has served as reference DNA sequence encoded by the nuclear DNA. The following primer pair has been applied for the amplification of β-globin gene FW 5′-TTTCCCACCCTTAGGCTGCTG-3′ and REV 5′-GGGAAAGAAAACATCAAGCGTCCCA-3′.

Total DNA for the subsequent PCR amplification has been isolated from both wild type and ρ^0 cells by using Wizard SV Genomic DNA Purification System (Promega, Madison, WI, USA). Each sample has been analysed in replicates of three. The amplification protocol for both genes were as follows: initial denaturation at 95 °C for 3 min, then denaturation step at 95 °C for 10 s, combined annealing and extension step at 60 °C for 30 s (40 PCR cycle).

Inhibition of Mitochondrial Respiratory Chain Complexes

In order to block the different mitochondrial respiratory complexes, HepG2 cells were incubated for 24 h in complete media, supplemented with 100 μg/ml sodium pyruvate, 50 μg/ml uridine and with the inhibitors of different respiratory complexes. The following inhibitors have been applied: rotenone for Complex I, at final concentration of 0.025 μM, thenoyltrifluoroacetone (TTFA) for Complex II, at final concentration of 200 μM, antimycin A for Complex III, at final concentration of 0.0025 μM, sodium-azide for Complex IV at final concentration of 250 μM. Furthermore the well-known uncoupling agent, 2,4-dinitrophenol (DNP) was used at a final concentration of 100 μM to inhibit the ATP production of the mitochondrial ATP-synthase (Complex V). The viability of the treated cells has been tested with trypan blue staining. The rate of dead cells remained below 5 % in all cases. O_2-consumption of the cells were measured with a Clarck type oxygen electrode (Hansatech Instruments) to verify the effectiveness of inhibitor-treatments.

Western blotting

Cells have been harvested, and lysed in lysis buffer (150 mM NaCl; 1 % NP40; 0.1 % SDS; 50 mM Tris-HCl, pH 8). The homogenized samples were incubated at 4 °C for 30 min and centrifuged at 14,000 g for 15 min at 4 °C to separate the protein fraction (supernatant) from the cellular debris (pellet). Protein determination was carried out using the Pierce BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's protocol. 50 μg amounts of total protein samples were loaded onto 15 % polyacrylamide gel under reducing conditions and transferred to a 0.45 μm nitrocellulose membrane using a Cleaver electrophoresis and Western-blot apparatus (Cleaver Scientific, Warwickshire, UK). After Ponceau staining the membranes were blocked with Saturating-solution (5 % non-fat dry milk in TBS-Tween (0.05 M Tris; 0.15 M NaCl; 0.05 % Tween-20)) by swinging it continuously for 2 h. Then the membranes were incubated overnight at 4 °C with primary antibodies diluted in Saturating-solution. Subsequently the membranes were incubated with HRP-conjugated secondary antibodies (1 h, at room temperature). Finally ECL reagent was used to detect the signals on the light-sensitive film (AGFA Healthcare, Mortsel, Belgium).

RT-PCR Analysis

Total RNA was purified using innuPREP RNA Mini Kit (Analytikjena AG, Thuringia, Germany). 2 μg of total RNA was reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's protocol. Transcript levels of ALR and 18S rRNA as internal control were quantified by PikoReal real-time PCR (Thermo Scientific) and Sensifast™ SYBR No-ROX Kit (Bioline London, UK). The ALR cDNA transcript was amplified with the forward primer 5′-CAC AAT GAA GTG AAC CGC AAG-3′ and reverse primer 5′-CAC CCA ACT GAG ACA CAA CAG-3′. The amplification of 18S rRNA cDNA was carried out by using the forward primer 5′-GTA ACC CGT TGA ACC CCA TT-3′ and reverse primer 5′-CCA TCC AAT CGG TAG TAG CG-3′ [22].

Results

Development of ρ^0 Cell Lines

Long-term treatment of HepG2 human hepatocyte carcinoma, MCF7 human breast adenocarcinoma and SH-SY5Y human neuroblastoma cell lines with ethidium bromide resulted in generation of cells devoid of mtDNA and lacking a functional respiratory chain. The ratio of mitochondrial and nuclear DNA (the number of mtDNA/cell) during the ethidium-bromide treatment has been followed up by real-time PCR. This ratio has been fallen steeply in all cell lines (Fig. 1). Virtually at the end of sampling no mtDNA could be detected (Fig. 1).

The success of the preparation of ρ^0 cell lines has been reinforced by checking the respiratory activity. As it was expected the oxygen consumption has significantly fallen by the decrease of mtDNA/cell ratio (data not shown). This way the elimination of functional respiratory chain has been verified.

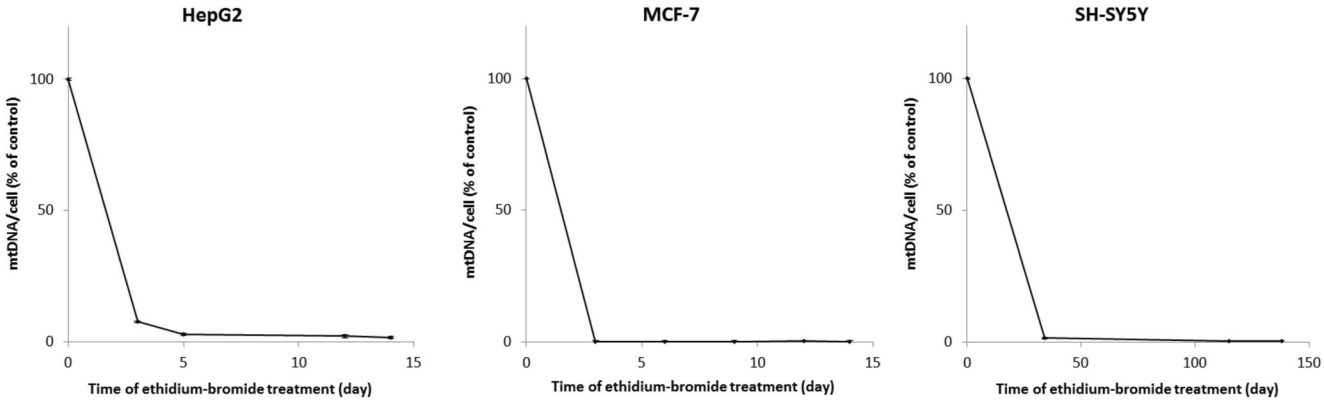

Fig. 1 The changes of mtDNA content of HepG2, MCF-7 and SH-SY5Y cell lines on long time exposure to ethidium bromide. The ethidium bromide treatment has started on parallel cell cultures at the same time. Cells of parallel tissue culture plates have been harvested at different stages of the treatment and total cellular DNA has been isolated. mtDNA content has been determined by real-time PCR analysis

The Effect of mtDNA Depletion on the Expression of ALR

ALR plays an important role in the biogenesis of functional mitochondria and in the maintenance of mitochondrial genome [5, 6, 16–18]. However there is no information about the possible regulatory role of mtDNA and mETC on the expression of ALR. Thus the consequence of the depletion of mtDNA and mETC on the expression of ALR has been investigated in hepatic and non-hepatic mtDNA depleted cell lines. After the successful generation of ρ^0 cell lines from the three different parental cell lines (HepG2, MCF7, SH-SY5Y) the expression of ALR (at the mRNA level) has been determined by real-time PCR. The depletion of mtDNA caused no significant difference in the mRNA level of all investigated cell lines (Table 1).

After the determination of the effect of mtDNA depletion on mRNA level of ALR, the protein level of ALR has also been investigated. The expression of ALR at the protein level has significantly elevated due to the depletion of mtDNA. Since ALR had been described first time as a hepatotrophic factor [1] it has great importance that the protein level of ALR has also increased in the non-hepatic (MCF7 human breast adenocarcinoma and SH-SY5Y human neuroblastoma) mtDNA depleted cell lines (Fig. 2). The level of MIA40, the other member of the mitochondrial disulphide relay system has also been investigated. However the protein level of this protein has not been changed due to the depletion of mtDNA (Fig. 2).

Table 1 The effect of mtDNA depletion on the expression (mRNA level) of ALR

Cell line	Relative ALR expression
HepG2 parental	1.00 ± 1,11
HepG2 ρ0	0.48 ± 1,28
SH-SY5Y parental	1.00 ± 1,42
SH-SY5Y ρ0	0.94 ± 1,25
MCF7 parental	1.00 ± 1,44
MCF7 ρ0	1.86 ± 1,82

The Effect of Different Respiratory Chain Inhibitors on the Expression of ALR

To gain further information on the elevated expression of ALR in the ρ^0 cells and on the regulatory role of mtDNA in the expression of ALR, parental HepG2 cells were treated with the inhibitors of different mitochondrial electron transfer chain complexes. This way similar to the ρ^0 cells ATP production could be reduced. The inhibition of complex I with rotenone blocks the electron supply to ubiquinol that beyond the inhibition of ATP production attenuates the ROS generation, whereas antimicyn A, an inhibitor of complex III, accelerates oxidant production. Hence both the possible regulatory role of ATP and reactive oxygen species (ROS) could be investigated. To make the picture complete the complex II inhibitor TTFA, the complex IV inhibitor Na-azide and the uncoupling agent 2,4-dinitrophenol were also applied. The latter compound beyond the depletion of ATP also reduce the generation of ROS. The effect of all the inhibitors, uncoupling agent on the respiration of the treated cells has been investigated by the measurement of the oxygen consumption of the cells. The effect of all the applied compounds corresponded to the expectations (data not shown).

Interestingly neither of the compounds could cause the elevation of ALR expression contrariwise it was observed in the case of ρ^0 cells (Fig. 3 vs Fig. 2).

Discussion

ALR is linked to the mitochondrion by several ties. As it was pointed out ALR contributes to mitochondrial biogenesis, maintenance and to the physiological operation of

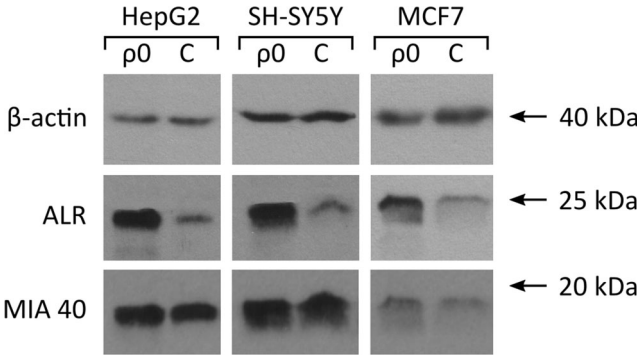

Fig 2 The effect of mtDNA depletion on the expression of ALR and MIA 40. The protein level of ALR and MIA40 in mtDNA depleted (ρ0) cells has been compared with the corresponding protein level of parental control cells (C). The mtDNA content of hepatic (HepG2) and non-hepatic (SH-SY5Y and MCF7) cells has been depleted by long term ethidium bromide treatment. Then the cells were harvested and total protein content was isolated. 50 μg of protein extracts were analysed by SDS-PAGE under reducing conditions. After blotting, membranes were immunostained with anti-ALR or anti-MIA40 polyclonal antibody. β-actin was used as an internal loading control. The figure illustrates a representative result of five independent experiments

mitochondria [5, 6, 16–18]. The electrons from the mitochondrial disulphide relay system (composed of MIA40 and ALR) are channelled to the mETC [11, 12].The consequences of the depletion of ALR on the mitochondrial functions have been studied widely. However the inverse direction, the effect of the depletion of mtDNA and mETC on ALR expression has not been investigated yet. Thus we aimed at the elucidation of the role of mETC and mtDNA on ALR expression.

The depletion of mitochondrial electron transfer chain could be achieved by the selective depletion of mtDNA. Long-term treatment of laboratory cell lines with ethidium bromide resulted in the generation of cells devoid of mtDNA and lacking a functional respiratory chain [20]. In the first set of experiments mtDNA depleted, ρ^0 cell line has been prepared from HepG2 human hepatocyte carcinoma parental cell line. The selective loss of mtDNA and the lack of functional respiratory chain has been followed by real-time PCR (Fig. 1) and by the measurement of cellular oxygen consumption.

Since ALR is also involved in liver regeneration [1, 23] two other, non-hepatic (MCF7 human breast adenocarcinoma and SH-SY5Y human neuroblastoma) cell lines were chosen to prepare ρ^0 cell lines. The loss of mtDNA could be verified by real-time PCR in the case of these latter cell lines too (Fig. 1).

In the second turn of the experiments, the possible change in the mRNA level of ALR due to the loss of mtDNA and the lack of functional respiratory chain has been determined by real-time PCR. Interestingly the depletion of mtDNA did not cause any difference in the mRNA level of the ALR in the investigated cell lines (Table 1).

However the depletion of mtDNA caused a markedly difference in the expression of ALR at the protein level (Fig. 2). The effect of mtDNA depletion on the protein level of ALR has been proved to be not liver specific, since the phenomenon could also be observed in the case of the other two, non-hepatic (MCF7 and SH-SY5Y) cell lines. The elevation of the protein level of ALR did not accompanied by the elevation

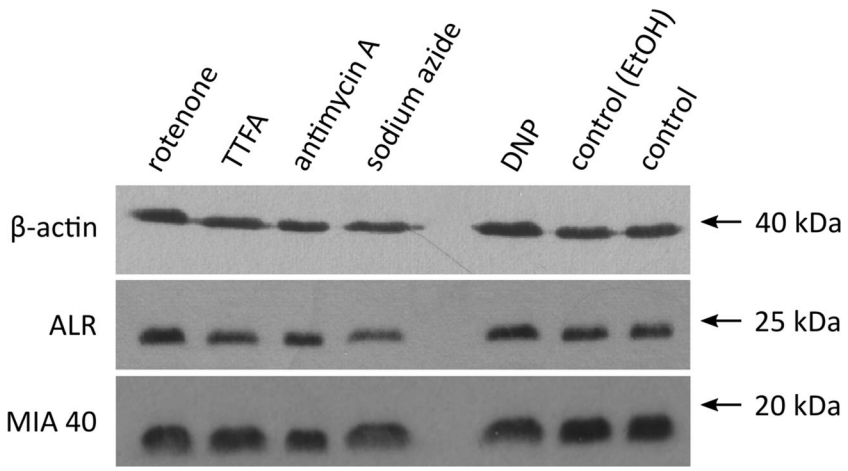

Fig 3 The effect of different respiratory chain inhibitors on the expression of ALR and MIA40. HepG2 cells were treated with the complex I inhibitor rotenone (0.025 μM), the complex II inhibitor TTFA (200 μM), the complex III inhibitor antimycin A (0.0025 μM) and the complex IV inhibitor sodium azide (250 μM). 2,4-dinitrophenol (DNP, 100 μM) was applied as an uncoupling agent. The water-insoluble reagents were dissolved in ethanol thus an additional EtOH control was also applied that contains 0.45 *v/v*% EtOH (the highest concentration of EtOH that occurred in the medium of inhibitor treated cells). After 24 h of treatment total protein was isolated from the cells. 50 μg amounts of protein extracts were analysed by SDS-PAGE under reducing conditions. After blotting, membranes were immunostained with anti-ALR or MIA40 polyclonal antibody. β-actin was used as an internal loading control. The figure illustrates a representative result of three independent experiments

of MIA40, the other member of the mitochondrial disulphide relay system (Fig. 2).

What can be the regulatory factor behind this significant change in the expression of ALR?

The first possible choice can be the level of ATP. It is well known that the depletion of ALR leads to the reduction of ATP level [17, 18]. Thus the reduced ATP level due to the lack of functional mitochondrial electron transfer chain in ρ^0 cell lines may provoke the elevation of the protein level of ALR. The possibility of ATP as a regulatory factor for ALR expression should have ruled out because the reduced ATP level due to the treatment of the parental cell line with different respiratory inhibitors and uncoupling agent could not provoke any elevation in the protein level of ALR (Fig. 2).

The second candidate for the regulation of ALR might have been the elevated level of reactive oxygen species since the dysfunction of the electron transport chain in ρ^0 cells was associated with an increase in ROS generation [24]. It is a well-known way to increase the intramitochondrial/intracellular ROS generation by the blockage of the electron transport chain (complex III) by antimicyn A [25]. However the elevation of ROS by the blockage of complex III has not accompanied by the elevation of ALR (Fig. 3). As the reduction of ROS level due to the treatment of the parental cells by the uncoupling agent 2,4-dinitrophenol has not caused any alteration in the level of ALR (Fig. 3).

The observed caspase-3 activation due to the silence or knock out of ALR raise the third possibility that ALR has an anti-apoptotic role in the case of ρ^0 cells. This possibility cannot be ruled out since ρ^0 cells showed resistance against apoptosis [26].

The fact that the protein level of the other member of the mitochondrial disulphide relay system, MIA40 has not changed together with the level of ALR suggest that the elevation of ALR (Fig. 2) is independent from the mitochondrial oxidative folding.

It seems that the level of mtDNA and/or its products may have a regulatory role on the protein level of ALR. Earlier, the up-regulation of two nuclear mitochondrial biogenesis genes NRF-1 and TFAM has been reported in the case of ρ^0 cells [24]. The loss of mtDNA in ρ^0 cells is associated with significant distortion of mitochondrial structure, disorganized inner and outer membranes, resulting in the appearance of 'ghost-like' mitochondria [27]. However, the mitochondrial biogenesis (the number of mitochondrial scaffolds) and membrane potential is not affected by the depletion of mtDNA [24, 27, 28]. Since it is well-known that ALR contributes to mitochondrial biogenesis and maintenance [5, 6, 16–18] the liver independent up-regulation of ALR can be a part of the adaptive response in ρ^0 cells that preserves the structural integrity of the mitochondrial inner membrane as well as the transmembrane electrochemical gradient despite the absence of protein components encoded by the mtDNA. Hence ALR may contribute to the maintenance of mitochondrial functions in the case of mtDNA loss or mutations in different mitochondrial diseases and aging. The machinery of mitochondrial biogenesis and maintenance is only partially characterized yet. Here we present data that ALR can be an important element of this machinery and the mtDNA can be an important regulatory factor for ALR expression at the protein level.

The present observations might also have important implications in human pathology. ALR proved to be hepatoprotective in human hepatocytes treated with different proapoptotic agents, including ethanol [29]. Liver-specific deletion of ALR resulted in the development of steatohepatosis and hepatocellular carcinoma in mice [18]. These and other findings suggest that ALR is a possible hepatoprotective factor, which needs further confirmation in clinical studies.

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