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Biochimica et Biophysica Acta 1553 (2002) 232–237

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Effects of cold exposure in vivo and uncouplers and recouplers in vitro on potato tuber mitochondria

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Received 11 June 2001; received in revised form 25 October 2001; accepted 21 November 2001

Abstract

Effects of cold exposure in vivo and treatment with laurate, carboxyatractylate, atractylate, nucleotides, and BSA in vitro on potato tuber mitochondria have been studied. Cold exposure of tubers for 48–96 h resulted in some uncoupling that could be reversed completely by BSA and partially by ADP, ATP, UDP, carboxyatractylate, and atractylate. UDP was less effective than ADP and ATP, and atractylate was less effective than carboxyatractylate. The recoupling effects of nucleotides were absent when the nucleotides were added after carboxyatractylate. GDP, UDP, and CDP did not recouple mitochondria from either the control or the cold-exposed tubers. This indicates that the cold-induced fatty acid-mediated uncoupling in potato tuber mitochondria is partially due to the operation of the ATP/ADP antiporter. As to the plant uncoupling protein, its contribution to the uncoupling in tuber is negligible or, under the conditions used, somehow desensitized to nucleotides. © 2001 Published by Elsevier Science B.V.

Keywords: Uncoupling protein; ADP/ATP antiporter; Adenosine 5′-diphosphate; Uridine 5′-diphosphate; Guanosine 5′-diphosphate; Fatty acid; Carboxyatractylate; Atractylate; Plant mitochondria; Cold adaptation

1. Introduction

The uncoupling effect of long-chain non-esterified fatty acids has been studied in mitochondria for almost half a century (for reviews, see [1,2]). This process plays an important role in urgent heat production and some other physiological and pathological processes. It is now well established that some mitochondrial anion carriers are involved in fatty acid-induced uncoupling: thermogenin (UCP1) in brown adipose tissue mitochondria, the ADP/ATP antiporter, the aspartate/glutamate antiporter, and the dicarboxylate carrier in mitochondria of muscle and liver. These carriers increase the proton conductivity of the inner mitochondrial membrane in the presence of rather low concentrations of fatty acids [1–3]. It has been suggested that anion carriers facilitate the translocation of fatty acid anions through the hydrophobic barrier of the inner mitochondrial membrane [1,4]. During recent years, several uncoupling proteins (UCPs) other than UCP1 have been discovered in many tissues (for review, see [3]).

Abbreviations: $\Delta\Psi$, transmembrane electric potential difference; BSA, bovine serum albumin; CAtr, carboxyatractylate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HEPES, *N*-2-(hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); EGTA, ethylene glycol-bis(2aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; UCP, uncoupling protein; PUMP, plant uncoupling mitochondrial protein

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0005-2728/01/$ – see front matter © 2001 Published by Elsevier Science B.V.
PII: S0005-2728(01)00244-4

Macri, Vianello and coauthors [5,6] found that in plant mitochondria (from pea stem and sunflower hypocotyl) the ADP/ATP antiporter participates in fatty acid-induced uncoupling. They reported that inhibitors of the ADP/ATP antiporter – carboxyatractylate (CAtr), atractylate, and ADP – suppress palmitate-induced uncoupling [5,6]. The participation of the ADP/ATP antiporter in fatty acid-induced uncoupling has also been shown for potato tuber mitochondria [7].

On the other hand, Vercesi and coauthors found a UCP-like plant uncoupling mitochondrial protein (PUMP) in potato [8–11] (see also [12]). UCPs have also been identified in plant mitochondria from other species [12,14,15]. In some of these experiments, atractylate was added to avoid ADP/ATP antiporter-mediated uncoupling [9,11]. However, in experiments on liver and heart mitochondria it was found that atractylate suppresses the fatty acid-induced uncoupling only partially, while CAtr produces a much stronger recoupling effect [16,17]. It should be stressed that low fatty acid concentrations were used in these studies. At higher concentrations, even the CAtr recoupling effect was low.

In most experiments, PUMP-mediated uncoupling was evaluated by the suppression of fatty acid-induced uncoupling by GTP [8], GDP [13], and ATP [8–10]. However, these nucleotide effects are hardly conclusive. It is known that in some cases ATP significantly increases succinate dehydrogenase activity of plant mitochondria [18], which can result in an increase in the transmembrane electric potential difference ($\Delta\Psi$) when succinate is used as the respiratory substrate. Moreover, ATP possesses some CAtr-sensitive recoupling activity in liver mitochondria, where no UCP is found [16].

This study was devoted to the fatty acid-induced uncoupling and nucleotide-, CAtr- and atractylate-induced recoupling in potato tuber mitochondria where PUMP mRNA expression is known to be low [12].

2. Materials and methods

2.1. Isolation of mitochondria

Intact potato tubers (*Solanum tuberosum* L.) were stored for 3–4 months at 8°C and then 1–2 months at 22°C in the dark. For cold treatment, they were kept at 4°C for 48–96 h.

Mitochondria were isolated using a Percoll self-generated gradient method as modified by Wagner [19,20]. Tuber tissue (400 g) was homogenized in a squeezer with 400 ml homogenization medium and filtered through steel wire gauze with 0.5 mm holes. The homogenization medium contained 350 mM mannitol, 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM *N*-2-(hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES; pH 7.4), 2 mM cysteine, 0.5% polyvinylpyrrolidone and bovine serum albumin (BSA; 3 mg/ml). Unbroken tissue, cell debris, nuclei, and etioplasts were separated by centrifugation at $4000\times g$ for 5 min. The mitochondrial pellet was obtained from the supernatant after a second centrifugation ($12\,000\times g$, 10 min). It was purified on a 21% Percoll self-generating gradient, containing 350 mM mannitol, 1 mM EDTA, 20 mM HEPES (pH 7.2) and BSA (3 mg/ml). Mitochondrial suspension (2 ml) in 350 mM mannitol and 20 mM HEPES (pH 7.2) were placed on the top of the 40 ml gradient and centrifuged for 40 min at $20\,000\times g$. The mitochondrial fraction located close to the gradient bottom was collected, diluted with the same buffer, and precipitated with centrifugation at $12\,000\times g$, 10 min. The pellet was washed with medium containing 350 mM mannitol and 20 mM HEPES (pH 7.2) and resuspended in 100 μl of the same medium.

Mitochondrial protein was measured by the biuret method.

2.2. Recording of oxygen consumption and membrane potential

$\Delta\Psi$ was estimated using the safranin O method [21]. The difference in optical densities between 555 nm and 523 nm (ΔA) was recorded on an Aminco DW-2000 spectrophotometer. Mitochondria were added to 2 ml incubation medium which contained 250 mM sucrose, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 0.5 mM ethylene glycol-bis(2aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 3 mM KH_2PO_4 (pH 7.4), and 7 μM safranin O. Mitochondrial protein concentration was 0.4 mg/1 ml. A mixture of 100 μM TMPD and 5 mM

ascorbate, or 5 mM succinate, was used as the oxidation substrate.

Oxygen consumption was recorded by a Clark-type oxygen electrode and LP-9 polarograph; the same incubation medium but without safranin was used. The incubation temperature was 28°C.

Under the conditions used the isolated mitochondria showed a respiratory control ratio of about 3 with succinate as the substrate.

2.3. Chemicals

D-Mannitol, MOPS, lauric acid, oligomycin, succinate, glutamate, CAtr, atractylate, EDTA, EGTA, ADP, ATP, GDP, UDP, CDP, and delipidized BSA (cat. No. A-6003) were from Sigma, rotenone from Serva, 2,4-*p*-dinitrophenol from Fluka, and IDP from Reanal. Sucrose was twice precipitated from a concentrated solution in bidistilled water with distilled ethanol. A stock solution of 20 mM lauric acid in ethanol was used.

3. Results and discussion

Cold treatment of potato tubers resulted in a reduction of the mitochondrial $\Delta\Psi$. This was seen from the fact that the initial safranin response level (before the first addition of a recoupler) was lower in the mitochondria from the cold-exposed tubers than that from control tubers (cf. A and B in Figs. 1–3). Addition of BSA to isolated mitochondria increased $\Delta\Psi$, and this increase was much larger in the case of the cold-treated samples. These data are consistent with the assumption that the cold increases the level of mitochondrial free fatty acids as mediators of thermoregulatory uncoupling [8,22]. The sensitivity of $\Delta\Psi$ to added laurate was found to be greater in mitochondria isolated from cold-treated potato tubers than in control mitochondria (Figs. 1–3). Addition of 60 μM laurate to mitochondria isolated from cold-treated potato tubers resulted in a 1.5-fold higher effect on the safranin response (ΔA) compared with mitochondria from control tubers. In the control and cold-exposed tubers, the values were 0.028 ± 0.004 ($n = 5$) and 0.043 ± 0.005 ($n = 7$), respectively.

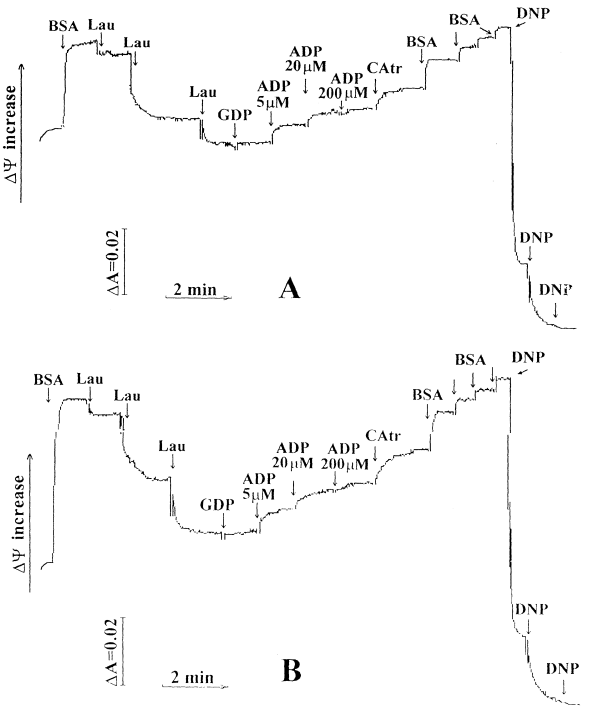

Fig. 1. Effects of cold exposure and some in vitro uncouplers and recouplers on potato tuber mitochondrial $\Delta\Psi$. (A) Control tubers; (B) cold-treated tubers. Incubation medium contained 250 mM sucrose, 10 mM MOPS, 0.5 mM EGTA, 3 mM KH_2PO_4 (pH 7.4), 100 μM TMPD, 5 mM ascorbate, oligomycin (2 μg/mg protein), 2 μM rotenone, 7 μM safranin O, mitochondria (0.4 mg/ml). Additions: 20 μM laurate (Lau), 200 μM GDP, 2 μM CAtr, 100 μM 2,4-*p*-dinitrophenol (DNP) and BSA, 0.5 mg/ml.

The uncoupling effect of laurate was insensitive to 200 μM GDP (Fig. 1). This nucleotide was still completely ineffective when its concentration was increased to 750 μM (not shown in the figure). On the other hand, ADP caused some recoupling. A measurable effect was seen when 5 μM ADP was added. CAtr further increased $\Delta\Psi$ when added after ADP addition. Glutamate (4 mM) and 2 mM malonate did not increase $\Delta\Psi$ (not shown). We showed earlier that in pea stem mitochondria under the same experimental conditions glutamate did not increase $\Delta\Psi$ [23].

Addition of CAtr before lauric acid caused a recoupling which was more pronounced in mitochondria from the cold-treated potato. Nucleotides had no effect when added after CAtr (Fig. 2). In other assays, it was found that 1 mM GDP, ATP and UDP, when added after CAtr, did not increase $\Delta\Psi$

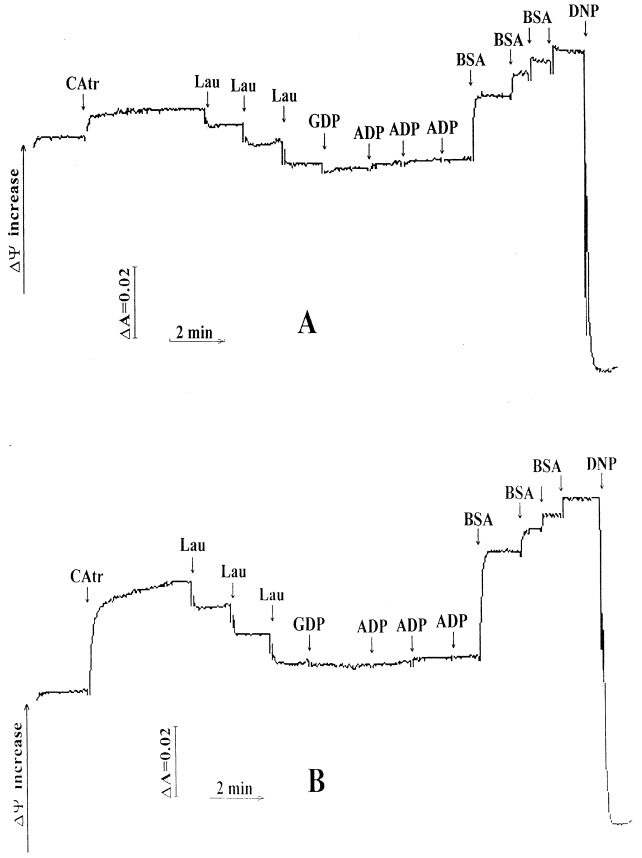

Fig. 2. Recoupling effect of CAtr on mitochondria from control (A) and cold-treated (B) potato tubers. Additions: 200 μM dinitrophenol (DNP). Other conditions and additions as in Fig. 1.

either. GDP added before laurate was also ineffective (not shown).

Atractylate, another inhibitor of the ATP/ADP antiporter, at 10 μM concentration had a recoupling effect which was, however, smaller than that of CAtr. Treatment with CAtr after atractylate produced an additional $\Delta\Psi$ increase (Fig. 3A,B). ADP still increased $\Delta\Psi$ when added after 10 μM atractylate and laurate (Fig. 3C).

The recoupling effect of some nucleotides other than ADP and GDP was also studied. ATP (200 μM) could effectively substitute for ADP (not shown). On the other hand, 250 μM CDP and IDP (not shown) as well as 5 μM UDP (Fig. 4) did not increase the $\Delta\Psi$ in the presence of laurate. However, 250 μM UDP produced a recoupling effect that was of the same magnitude as that of 20 μM ADP (Fig. 4). Under the same in vitro conditions, UDP proved to be ineffective as a recoupler in rat liver mitochondria (not shown). UDP at a concentration of 250 μM did not increase $\Delta\Psi$ of the potato mitochondria in the presence of CAtr.

In some experiments, we studied the effect of atractylate on nucleotide transport by the ADP/

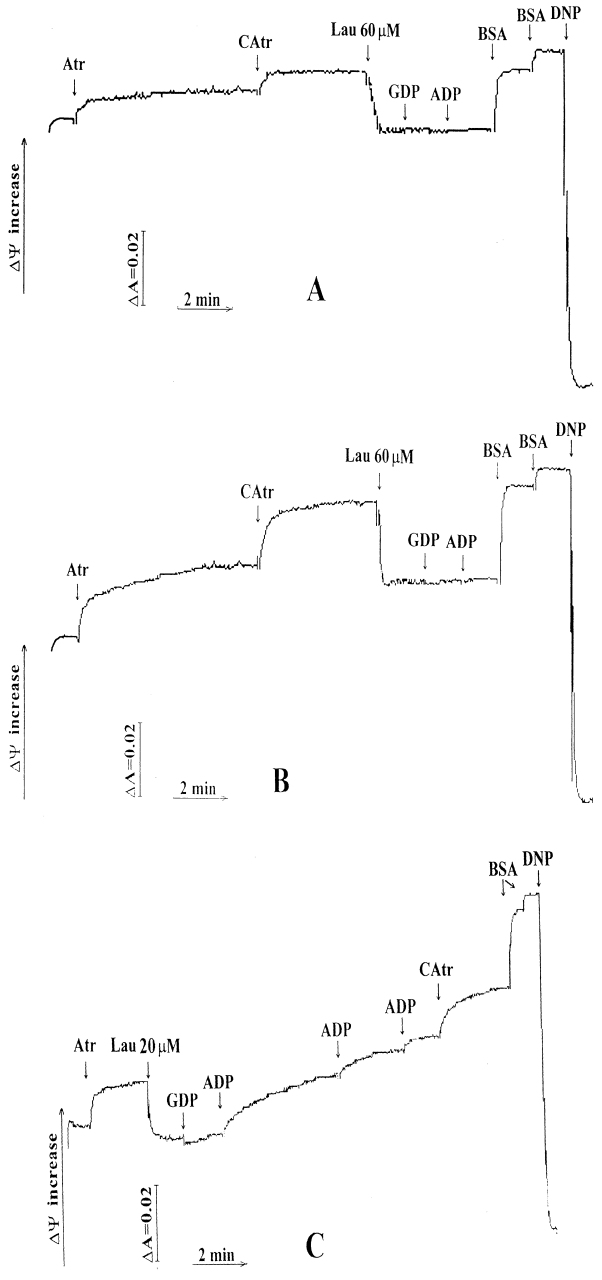

Fig. 3. Recoupling effect of atractylate on mitochondria from control (A) and cold-treated (B,C) potato tubers. For conditions and additions, see Fig. 2. Atr, 10 μM atractylate; however, in C BSA was added after CAtr only.

ATP antiporter. We examined a $\Delta\Psi$ decrease caused by ADP addition to phosphate-, magnesium-, TMPD- and ascorbate-containing medium without oligomycin. It was shown that ADP addition caused cyclic $\Delta\Psi$ decreases whereas UDP does not induce such an effect (Fig. 5A). Atractylate (10 μM) failed to completely arrest the ADP-dependent $\Delta\Psi$ decrease, whereas this could be done by the subsequent addition of oligomycin (Fig. 5B).

Lauric acid did not decrease and CAtr, ADP, ATP, and UDP did not increase the respiration rate when 5 mM ascorbate in the presence of 100 μM TMPD and oligomycin (2 μg/mg protein) was used as the oxidation substrate. Essentially similar data were obtained when succinate was used as a substrate (not shown). Thus, the $\Delta\Psi$ lowering by laurate as well as its elevation by CAtr and nucleotides were not due to inhibition or stimulation of respiration, respectively.

In a number of papers, PUMP activity was estimated by GDP-, GTP- or ATP-induced $\Delta\Psi$ increase [8–10,13]. However, this method seems to be valid only if these nucleotides were added in the presence

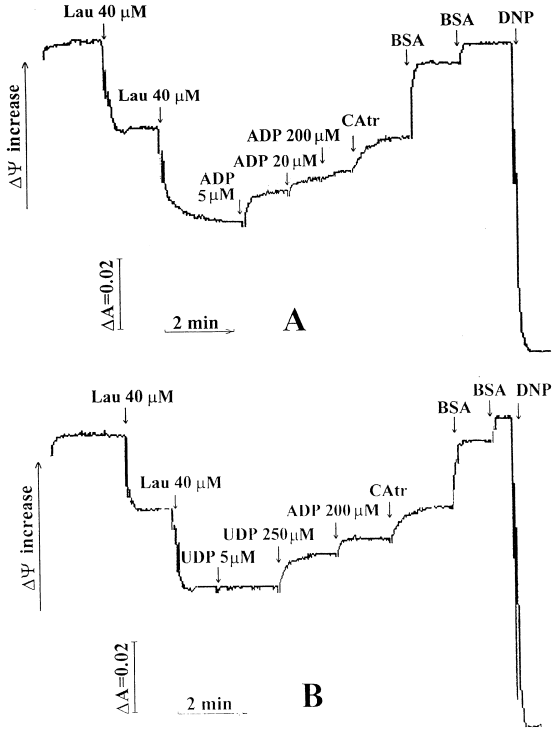

Fig. 4. Effect of nucleotides on laurate-induced uncoupling in potato tuber mitochondria from cold-treated plants. Oligomycin is present. For conditions and additions, see Figs. 2 and 5.

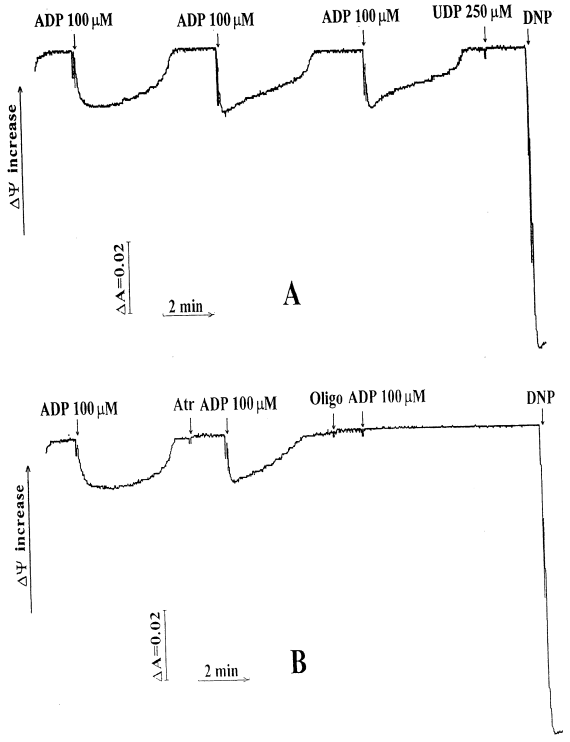

Fig. 5. Effects of ADP and UDP on $\Delta\Psi$ in of mitochondria from cold-treated potato, added to the incubation medium containing no oligomycin. The incubation medium contained 250 mM sucrose, 10 mM MOPS, 0.5 mM EGTA, 3 mM KH_2PO_4, 3 mM $MgCl_2$ (pH 7.4), 100 μM TMPD, 5 mM ascorbate, 2 μM rotenone, 7 μM safranin O and mitochondria (0.4 mg/ml). 100 μM ADP, 250 μM UDP, 10 μM atractylate (Atr) and oligomycin (2 μg/mg protein) were added where indicated.

of CAtr, arresting the ADP/ATP antiporter-mediated uncoupling. In our case, 250 μM ADP did not induce an increase in $\Delta\Psi$ when added after CAtr (Figs. 2 and 3). GDP was ineffective even without CAtr. Cold exposure, which is known to increase the expression of PUMP [11,24], failed to induce any GDP recoupling in spite of the fact that some BSA-sensitive uncoupling and sensitization of mitochondrial $\Delta\Psi$ to laurate could be seen. Such relationships might be explained if in the potato tuber mitochondria the initial UCP level is very low [12] and the cold exposure fails to increase it to a measurable value. Another possibility is that the cold exposure not only increases the UCP level but also desensitizes UCP to nucleotide recoupling. This effect was suggested to be responsible for a paradoxical observation made recently in our group when cold-induced uncoupling was studied in rat skeletal muscle mito-

chondria. It was found that 24 h cold exposure of rat results in (i) a 3-fold increase in the amount of the UCP3 protein, (ii) an increase in the BSA-induced uncoupling, (iii) an increase in the uncoupling efficiency of added laurate and (iv) a decrease in GDP recoupling [25].

It is noteworthy that glutamate and malonate did not possess any recoupling activity in the potato tuber mitochondria. This means that the contribution of the aspartate/glutamate antiporter [26] and the dicarboxylate carrier [27] to the fatty acid uncoupling in these mitochondria is negligible.

In conclusion, the data presented indicate that (i) cold exposure of potato tubers results in a fatty acid-mediated uncoupling which is partially reversed by CAtr, the ATP/ADP antiporter inhibitor, and (ii) purine nucleotides added after CAtr do not induce further recoupling. The latter observation suggests that nucleotide-sensitive, UCP-mediated H^+ conductance does not contribute to thermoregulatory uncoupling in the potato tuber mitochondria.

Acknowledgements

We are grateful to Dr. R. Lozier for editing the style of the manuscript. This study was supported by grants 01-04-48648, 01-04-48431, and 00-15-97799 from the Russian Foundation for Basic Research, YSF 01/1-0030 from INTAS, RBO-863 from the Ludwig Institute for Cancer Research, and KO 597 from the Federal Program 'Integration'.

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