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NADH-generating substrates reduce peroxyl radical toxicity in RL-34 cells

J. Antosiewicz[1], J.H. Spodnik[2], M. Teranishi[5], A. Herman-Antosiewicz[4], Ch. Kurono[5], T. Soji[5], M. Woźniak[3], A. Borkowska[1], T. Wakabayashi[3]

[1]*Department of Bioenergetics and Physiology of Exercise, Medical University of Gdańsk, Poland*
[2]*Department of Anatomy and Neurobiology, Medical University of Gdańsk, Poland*
[3]*Medical Chemistry, Medical University of Gdańsk, Poland*
[4]*Department of Molecular Biology, University of Gdańsk, Poland*
[5]*Department of Anatomy, Nagoya City University Medical School, Japan*

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There is general agreement that oxidative stress may induce apoptotic and necrotic cell death. Recently it has been shown that NADH can be considered an important antioxidant as it reacts with peroxyl and alkoxyl radicals under in vitro conditions. Therefore, in the present study we hypothesized that an increase in intracellular NADH using specific substrates will protect RL-34 cells against cytotoxicity of 2'-azobis (2-amidinopropane) dihydrochloride (AAPH), which is a peroxyl radical generating compound. Cells treated for 24 hours with 6.0 mM AAPH were severely damaged: mitochondria were vacuolated, and the level of free radicals significantly increased. Both apoptotic and necrotic cells were detected (11.1% and 11.4%, respectively) even after 5 hours of treatment. Pretreatment of the cells with substrates which increase the intracellular level of NADH, such as lactate, beta-hydroxybutyrate, and ethanol, distinctly inhibited AAPH-induced reactive oxygen species (ROS) formation and cell death. On the other hand, acetoacetate (AcA), which decrease the intracellular level of NADH, had opposite effects. Interestingly, NADH-generating substrates augment, while AcA reduced superoxide radical formation induced by AAPH. These results may suggest that although NADH generating substrates may exert some deleterious effects within a cell by inducing reductive stress, they diminish alkoxyl or peroxyl radical cytotoxicity. The protection is associated with a decrease in ROS formation measured by dichlorofluorescein, but with an increase in superoxide radical formation. (Folia Morphol 2009; 68, 4: 247–255)

Key words: AAPH, ethanol, apoptosis

INTRODUCTION

The role of reactive oxygen species (ROS) in apoptotic and necrotic cell death is well established. There is an increasing amount of evidence that ROS not only damage cell structure, which leads to cell death, but can also activate specific signalling pathways which can determine the way in which a cell will die. Activation of some signalling pathways is usually associated with oxidation of specific proteins, which eventually induce specific signalling pathways. For example, during oxidative stress, tioredoxin has been shown to dissociate from apoptosis signal-regulating kinase 1 (ASK1), which causes activation of ASK1 and c-jun terminal kinase (JNK) [15, 16].

Address for correspondence: J. Antosiewicz, PhD, Department of Bioenergetics and Physiology of Exercise, Medical University of Gdańsk, Dębinki 1, 80–210 Gdańsk, Poland, tel: +48 58 349 14 50, e-mail: jant@amg.gda.pl

On the other hand, ROS inactivates phosphatease, which is responsible for switching off JNK [9]. Therefore, the overall result of oxidative stress is prolonged JNK activation, which can lead to apoptosis or necrosis. Cysteine is a protein amino acid, the most vulnerable to oxidation; however, it has been shown that oxidation of a specific cysteine in a protein by ROS, like hydrogen peroxide, is a fast reaction when cysteine is dissociated with a cysteine thiolate anion (Cys-S-). Although, in the majority of the proteins cysteine does not dissociate in physiological conditions due to high pKa of sulphydryl groups, which is around 8.5. In such conditions, cysteine can be oxidized by stronger oxidants like hydroxyl or peroxyl radicals. Therefore, signalling initiated by ROS will be dependent on the kind of oxidant formed. GTP binding protein has been shown to be activated by oxidation in the presence of hydrogen peroxide and iron but not by H_2O_2 alone [19]. It has been reported that NADH can react non-enzymatically with singlet oxygen [3], thiyl radicals of cysteine and glutathione [7], peroxyl, and alkoxyl radical [20]. Considering the fact that NADH reacts with several free radical species very efficiently, and that the reaction can be enhanced by binding the coenzyme to some enzymes e.g. lactate dehydrogenase or glyceraldehyde-3-phosphate dehydrogenase [5, 6], we have assumed that such chemical reactions may also take place in the living cell. Free radical mediated oxidation of NADH is a one-electron process which leads to the formation of NAD radical. Under aerobic conditions, the NAD radical reacts at a diffusion-controlled rate with oxygen, resulting in the formation of superoxide. Thus, the protective effect of NADH against free radicals may be expected when it reacts with stronger radicals, e.g., alkoxyl and peroxyl radicals, converting them into weaker radicals, e.g., superoxide (Fig. 1). The reaction rate constant of superoxide, an anion radical, with protein amino acids and other cell compounds is much lower when compared to radicals like peroxyl and alkoxyl. Therefore, we hypothesized that increased intracellular NADH concentration will protect RL-34 cells against peroxyl radical cytotoxicity.

We observed that AAPH-induced cell apoptosis and necrosis were significantly inhibited by treatment with NADH-generating substrates like lactate, beta-hydroksy-butyrate, and ethanol. These effects of the substrates were associated with decreased ROS formation, measured by dichlorofluorescein (DCF) oxidation, and increased superoxide radical formation.

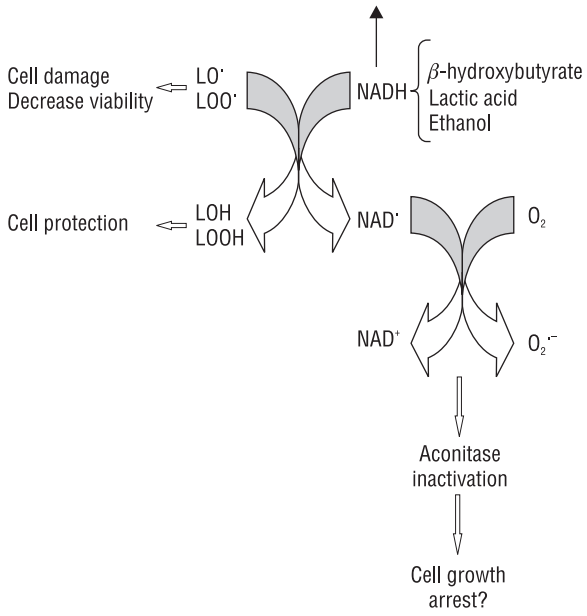

Figure 1. Postulated improving effects of ethanol on alkoxyl and peroxyl radical-induced cell injury via elevation of intracellular level of NADH; LO — alkoxyl radical; LOO — peroxyl radical; NAD· — NAD radical.

MATERIAL AND METHODS

Cell culture

Rat liver cell line RL-34 cells (JCRB 0247) were obtained from the Health Science Research Resources Bank (HSRRB) (Osaka, Japan). Cells were maintained in DMEM supplemented with 10% heat-inactivated calf serum, 4 mM L-glutamate, 0.45% glucose, and kanamycin (60 mg/L) as described previously [10].

Treatment of cells with various chemicals

2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was used as the peroxyl radical generator [18]. The chemicals expected to increase or decrease the intracellular level of NADH, employed in the present study, were: ethanol (20 mM), lactate (20 mM), and β-hydroxybutyrate (20 mM) for the former, and acetoacetate (10 mM) for the latter.

Flow cytometric analysis of intracellular levels of free radicals

Cells grown on 10-cm culture dishes were stained with 5 μM carboxy-H_2-DCFDA and were kept for 60 minutes in the culture chamber as described previously [10]. They were then harvested by trypsinization. The cells were then washed and resuspended in PBS and immediately submitted for analysis using a Coulter Elite FACSCAN (Coulter Corporation)

with excitation and emission setting of 495 and 525 nm, respectively. Gating was performed to remove cellular debris before the data were collected. Furthermore, changes in the intracellular level of superoxide were also detected using dihydroethidium (DHE) (Molecular Probes). DHE has been shown specifically to react with superoxide, yielding fluorescent ethidium. For this purpose, the cells grown on culture dishes were incubated with 5 μM DHE for 45 min at 37°C as described previously [25], then washed and resuspended in PBS and submitted for flow cytometric analysis.

Aconitase and NAD(P)H assay

Aconitase activity was determined in fresh cell lysates prepared by resuspending the cells in 50 mM Tris-HCl and 0.5% Triton X-100 solution. The assay was performed in a 0.12 mL reaction mixture containing 50 mM Tris, 5 mM sodium citrate, 0.6 mM MnCl2, 0.2 mM NADP pH 7.4, and 0.5 units of porcine heart isocitrate dehydrogenase. The reaction was started by addition of the cell extracts. The mixture was incubated for 7 minutes at 25°C, after which the linear absorbency change at 340 nm at 25°C was measured for 10 minutes.

NAD(P)H determination in the alkaline cell extract was performed according to the method described previously [26].

Determination of lipid peroxidation

Oxidative stress induced by AAPH was also monitored by measuring the amount of thiobarbituric acid reactive substances (TBARS), as described peviously [4]. RL-34 cells were treated for 24 hours with various chemicals, harvested by trypsinization, and used for the measurement of TBARS.

Flow cytometric analysis of necrosis and apoptosis

Cells cultured for 5 hours or 24 hours in the presence of various chemicals were collected from the culture dish, pelleted, and washed in PBS. To detect the necrotic changes, the cells were stained with a solution containing propidium iodide (PI) (2 μg/mL), 1% bovine serum albumin, and 0.01% sodium azide dissolved in PBS. PI can only pass through a compromised plasma membrane of necrotic cells, yielding a red fluorescence staining of nuclear DNA. To detect the apoptotic changes, the cells were stained for 30 minutes at 37°C with a solution containing PI (50 μg/mL), 0.01% Na-citrate, 0.2% Nonidet P-40, and RNAase to eliminate PI, reacted with RNA essentially according to the method described previously [24].

Protein determination

Protein was determined by the method of Lowry, using BSA as the standard [17].

Electron microscopy

A fixative containing 2% glutaraldehyde, 2% formaldehyde, and 0.1 M Na-cacodylate, pH 7.4, was used for the fixation of tissues and cells [2]. Specimens were post-fixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in Epon 812. Thin sections were cut on a Reihert ultracut N, stained with uranyl acetate and lead citrate, and examined in a Hitachi 7000 electron microscope operated at 100 kV.

RESULTS

Effects of NADH-generating substrates on AAPH-induced changes in the intracellular levels of ROS

Intracellular levels of ROS in RL-34 cells cultured for 24 hours in the presence of AAPH were detected by flow cytometry using carboxy-H_2-DCFDA. The nonfluorescent dye carboxy-H_2-DCFDA passively enters the cell and, if oxidized, forms fluorescent DCF [1]. AAPH at a concentration of 6.0 mM caused an increase in mean fluorescence intensity of DCF, indicating that the intracellular level of ROS was increased in AAPH-treated cells (Fig. 2A). On the other hand, the pretreatment of cells with ethanol (20 mM) suppressed the above-described effects of AAPH. Lactate (20 mM) and β-hydroxybutyrate (βHB) (20 mM) yielded suppressing effects similar to ethanol on AAPH-induced changes (data not shown). Pretreatment of cells with acetoacetate (AcA) (10 mM) caused a further increase in fluorescence of DCF in AAPH-treated cells (Fig. 2A). The mean fluorescence intensity of DCF in cells treated with ethanol or AcA alone was essentially the same as that of the control cells (Fig. 2A).

We also measured the intracellular level of superoxide radical. Treatment of the cells with ethanol or AcA did not change the mean ethidium fluorescence, while AAPH at a concentration of 6.0 mM caused a distinct increase in the intracellular level of superoxide radical. Pretreatment of the cells with ethanol caused a further increase in the intracellular level of superoxide in AAPH-treated cells. In addition, lactated and βHB had similar effect (result not shown). On the other hand, AcA significantly diminished AAPH-induced superoxide radical formation (Fig. 2B). An indirect method to estimate superoxide radical formation was also used, measuring

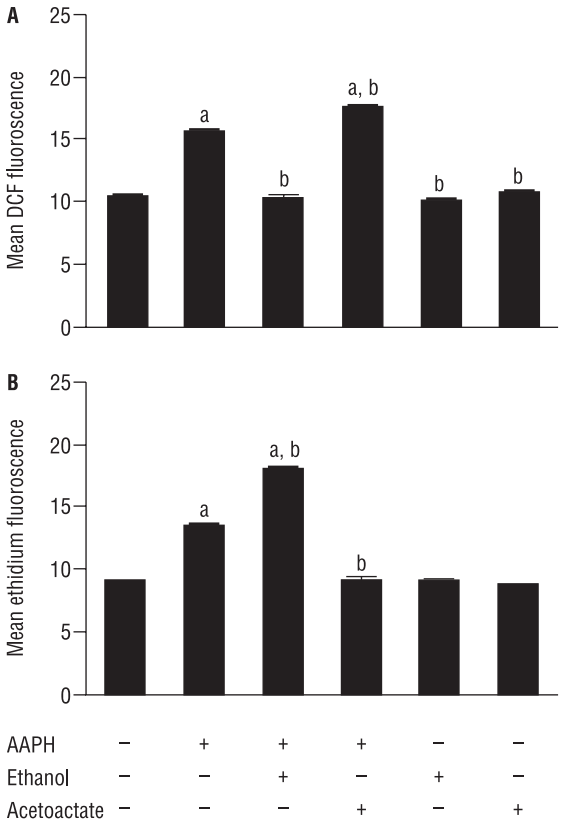

Figure 2. Ethanol as an NADH-generating substrate changes AAPH-induced reactive oxygen species generation; **A.** Mean dichlorofluorescein (DCF) fluorescence intensity; **B.** Mean ethidium fluorescence intensity in RL-34 cells treated for 24 hours with AAPH 6 mM with 20 mM ethanol and 10 mM acetoacetate, respectively. Data are mean ± standard deviation (n = 3); [a]p < 0.05, significantly different compared to PBS-treated control; [b]p < 0.05, significantly different compared to AAPH-alone treatment group by one-way ANOVA followed by Bonferroni's multiple comparison test. Similar results were observed in replicate experiments.

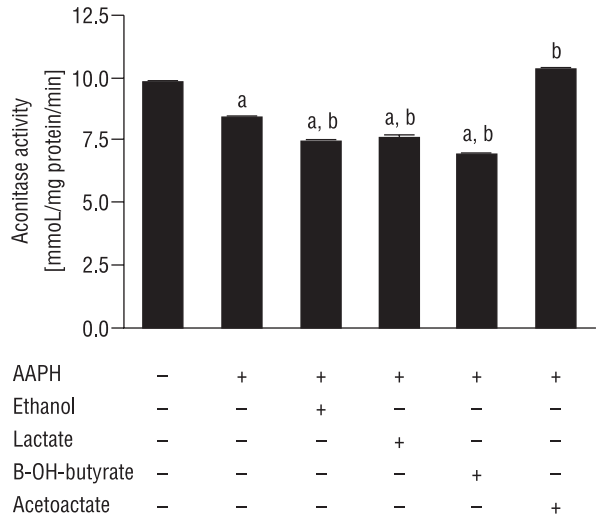

Figure 3. Acetoacetate protects AAPH-induced aconitase inactivation. Cells were treated for 5 hours with AAPH (6.0 mM) in the presence or absence of 2-hour pretreatment with ethanol (20 mM) beta-hydroxybutyrate (20 mM), lactate (20 mM), or acetoacetate (10 mM). Data are mean ± standard deviation (n = 5); [a]p < 0.05, significantly different compared to PBS-treated control; [b]p < 0.05, significantly different compared to AAPH-alone treatment group by one-way ANOVA followed by Bonferroni's multiple comparison test.

aconitase activity. The enzyme possesses an FeS cluster, and oxidation of Fe^{+2} by superoxide leads to the enzyme's inactivation [8]. Cells treated with AAPH for 30 minutes showed a decrease in aconitase activity. NADH-generating substrates enhanced AAPH aconitase inactivation. However, AAPH treated cells showed slightly higher aconitase activity compared to the control cells when AcA was present in the incubation medium (Fig. 3). Cells incubated only with the NADH-generating substrates had the same aconitase activity as the control cells (not shown).

NADH-generating substrates inhibit AAPH-induced lipid peroxidation

In this study, we also measured the amount of lipid peroxidation products generated in AAPH-treated cells. The level of malondialdehyde generated in the cells treated with AAPH (6.0 mM) for 24 hours was remarkably higher than that of the control cells. AcA (10 mM) had no effect on malondialdehyde formation in the AAPH-treated cells. On the other hand, the pretreatment of cells with ethanol (20 mM), βHB (20 mM) or lactate (20 mM) suppressed the AAPH-induced increases in the production of malondialdehyde although ethanol was the least effective of the three tested substrates (Fig. 4).

Distinct effects of NADH-generating and NADH-consuming substrates on AAPH-induced cell death

In the present study, PI was used to detect both apoptotic and necrotic changes of AAPH-treated cells. PI is able to pass only through the compromised plasma membrane of necrotic cells. Thus, the nuclei of necrotic cells are stained with PI. On the other hand, PI can enter the cytoplasm of both intact and apoptotic cells after a membrane permeabilization. The cells with sub-diploid DNA content had been counted as an apoptotic fraction. When the cells were cultured for 5 or 24 hours in the presence of 6.0 mM AAPH, 11.53% and 32.83% of the cells became apoptotic, respectively, while the pretreatment of cells with NADH-generating substrates, including ethanol, suppressed it (Fig. 5A, B). Population of apoptotic cells in the sample treated with

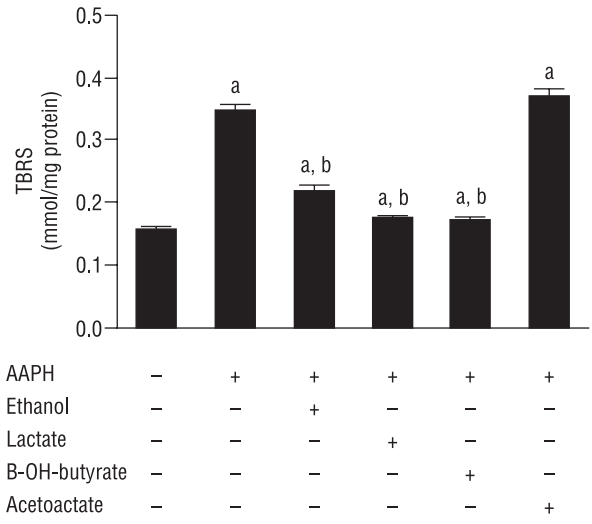

Figure 4. NADH-generating substrates reduced AAPH-induced lipid peroxidation. RL-34 cells were treated for 24 hours with AAPH (6.0 mM) in the presence or absence of beta-hydroxybutyrate (20 mM), ethanol (20 mM), lactate (20 mM), or acetoacetate (10 mM). Data are mean ± standard deviation (n = 5);
[a]$p < 0.05$, significantly different compared to PBS-treated control;
[b]$p < 0.05$, significantly different compared to AAPH-alone treatment group by one-way ANOVA followed by Bonferroni's multiple comparison test.

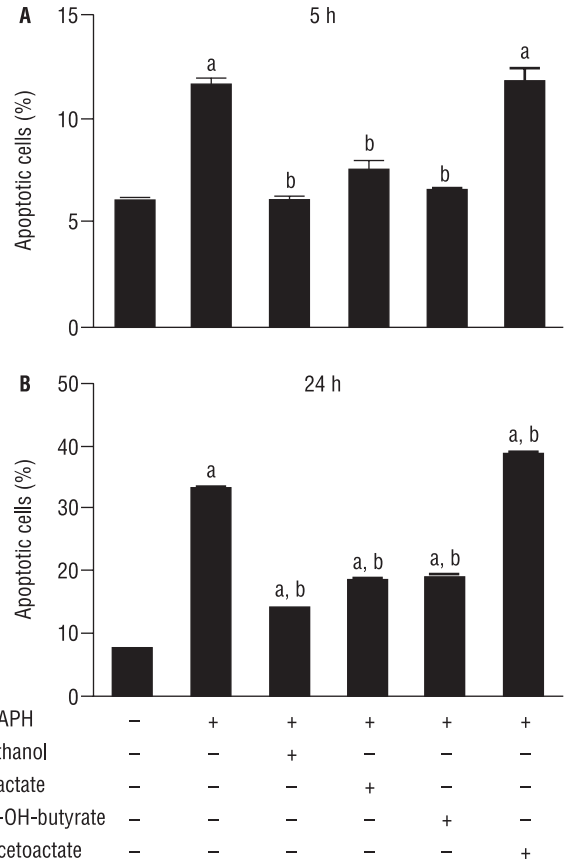

Figure 5. NADH-generating substrates reduced AAPH-induced apoptosis. Analysis of sub-diploid fraction in RL-34 cells following 6 mM AAPH treatment for 5 hours (**A**) and for 24 hours (**B**) in the presence or absence of ethanol (20 mM), lactate (20 mM), beta-hydroxybutyrate (20 mM), or acetoacetate (10 mM). Data are mean ± standard deviation (n = 3);
[a]$p < 0.05$, significantly different compared to PBS-treated control;
[b]$p < 0.05$, significantly different compared to AAPH-alone treatment group by one-way ANOVA followed by Bonferroni's multiple comparison test.

acetoacetate and AAPH was essentially the same as those treated with AAPH alone. Similar protective effects of NADH generating substrates were observed in AAPH-induced necrosis. When the cells were cultured in the presence of AAPH (6.0 mM), the population of necrotic cells significantly increased, reaching 11.4% and 24.5% after 5 and 24 hours, respectively. The pretreatment of cells with AcA and AAPH caused an enormous increase in necrotic cell death (35.4%). NADH-generating substrates, including ethanol, were effective in lowering the necrotic changes in AAPH-treated cells (Fig. 6A, B)

NADH concentration

Treatment of RL-34 cells with 6 mM AAPH for 24 hours led to a significant drop in NADH and NADPH concentrations (Table 1). Such an effect of AAPH was not observed when 20 mM ethanol was present (Table 1). Similar results were observed in the presence of other NADH-generating substrates (data not shown). In addition, NADH levels in the AAPH and ethanol treated group was higher than in the control group but lower than in the ethanol-only treated cells. The lowest concentration of NADH was observed in the cells exposed to AAPH and 10 mM AcA (Table 1).

Structural changes of mitochondria in AAPH-treated cells

Because cell death is often preceded by mitochondria structural and functional changes, our next goal was to find out if AAPH toxicity leads to mitochondrial structure changes and if NADH substrates will ameliorate these changes. At a concentration of 1.5 mM, AAPH induced swelling of mitochondria in some cells after 24 hours (Fig. 7B), compared to the control. When the concentration of AAPH was raised to 6.0 mM, the mitochondria in almost every cell became extremely enlarged (Fig. 7C), and necrotic and apoptotic cells were often encountered (Fig. 7D). In the cells treated with AAPH in the presence of acetoacetate, the number of necrotic cells further in-

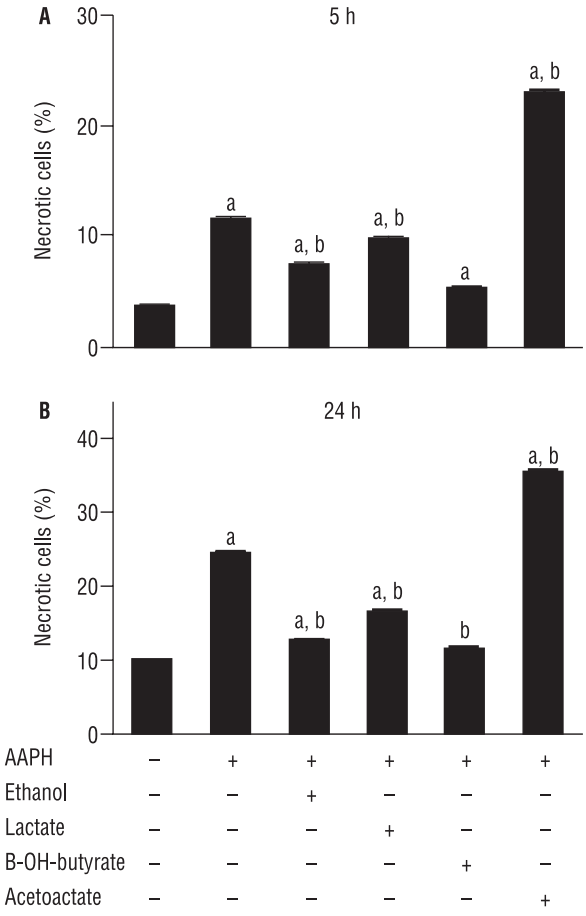

Figure 6. NADH-generating substrates reduced AAPH-induced necrosis. Analysis of RL-34 cells fraction which stained with propidium iodide without permeabilization, following a 6 mM AAPH treatment for 5 hours (**A**) and for 24 hours (**B**) in the presence or absence of ethanol (20 mM), lactate (20 mM), beta-hydroxybutyrate (20 mM) or acetoacetate (10 mM). Data are mean ± ± standard deviation (n = 3);
[a]$p < 0.05$, significantly different compared to PBS-treated control;
[b]$p < 0.05$, significantly different compared to AAPH-alone treatment group by one-way ANOVA followed by Bonferroni's multiple comparison test.

creased, compared to those treated with AAPH alone, corresponding to the results obtained by flow cytometric analysis of these cells (Fig. 7E). The pretreatment of cells with ethanol protected against the toxic effect of AAPH to a remarkable degree (Fig. 7F). However, in some cells swollen mitochondria were still observed (Fig. 7G). Co-treatment of the cells with βHB had protective effect on AAPH-induced structural changes similar to ethanol, as described above (Fig. 7H).

DISCUSSION

Only recently it has been suggested that NAD(P)H should be considered a decisive, directly operating antioxidant especially in mitochondrial compartments of the cell [12, 13, 21]. In our study, we present data supporting the mention that NADH, besides being a hydrogen anion donor in a variety of enzymatic processes, may directly react with peroxyl radical inside the cell.

Table 1. Ethanol protects against 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced drop in NAD(P)H. RL-34 cell were treated with 6 mM AAPH for 24 hours with and without co-treatment with ethanol 20 mM or acetoacetate (AcA) 10 mM for 24 hours

	NADH [nmoL/mg protein]	NADPH [nmoL/mg protein]
Control	4.16 ± 0.18[a]	0.86 ± 0.033[a]
AAPH	3.46 ± 0.12[b]	0.65 ± 0.07[b]
AAPH + Ethanol	5.2 ± 0.61[c]	0.82 ± 0.054[a]
AAPH + AcA	2.67 ± 0.31[d]	0.78 ± 0.036[a]
Ethanol	5.98 ± 0.65[c]	0.95 ± 0.12[a]
AcA	2.96 ± 0.27[d]	0.81 ± 0.08[a]

*Results are expressed as mean ± standard deviation for n = 5; values bearing different letters are significantly different at $p < 0.05$

AAPH, known to generate the peroxyl radical, induces apoptotic and necrotic changes in cultured RL-34 cells. However, NADH-generating substances, including ethanol, reduce these changes whereas acetoacetate, which consumes NADH, enhances necrosis. These data are in agreement with some recent reports showing that rising intracellular NADH concentration renders better protection against toxic compounds. For instance, cells over-expressing G6PD are more resistant to tBOOH toxicity, and this has been linked to elevated concentrations of NADPH and glutathione [14]. On the other hand, some data show that the increased NADH/NAD^+ ratio by NADH-generating substrates augments cells damage during ischaemia [11].

Intracellular levels of free radicals in the cells treated with AAPH at 6mM concentrations increase when they are judged by the fluorescence intensity of DCF (Fig. 2). This is accompanied by a significant decrease in NAD(P)H concentration (Table 1). Additional treatment of these cells with AcA enhances AAPH-induced ROS formation. In contrast, ethanol (and other NADH-generating substrates) significantly raised the level of NADH and reduced free radical formation in AAPH-treated cells (Figs. 2, 6). Interestingly, none of the substrates alone modulated ROS formation in the cells. AAPH treatment of RL-34

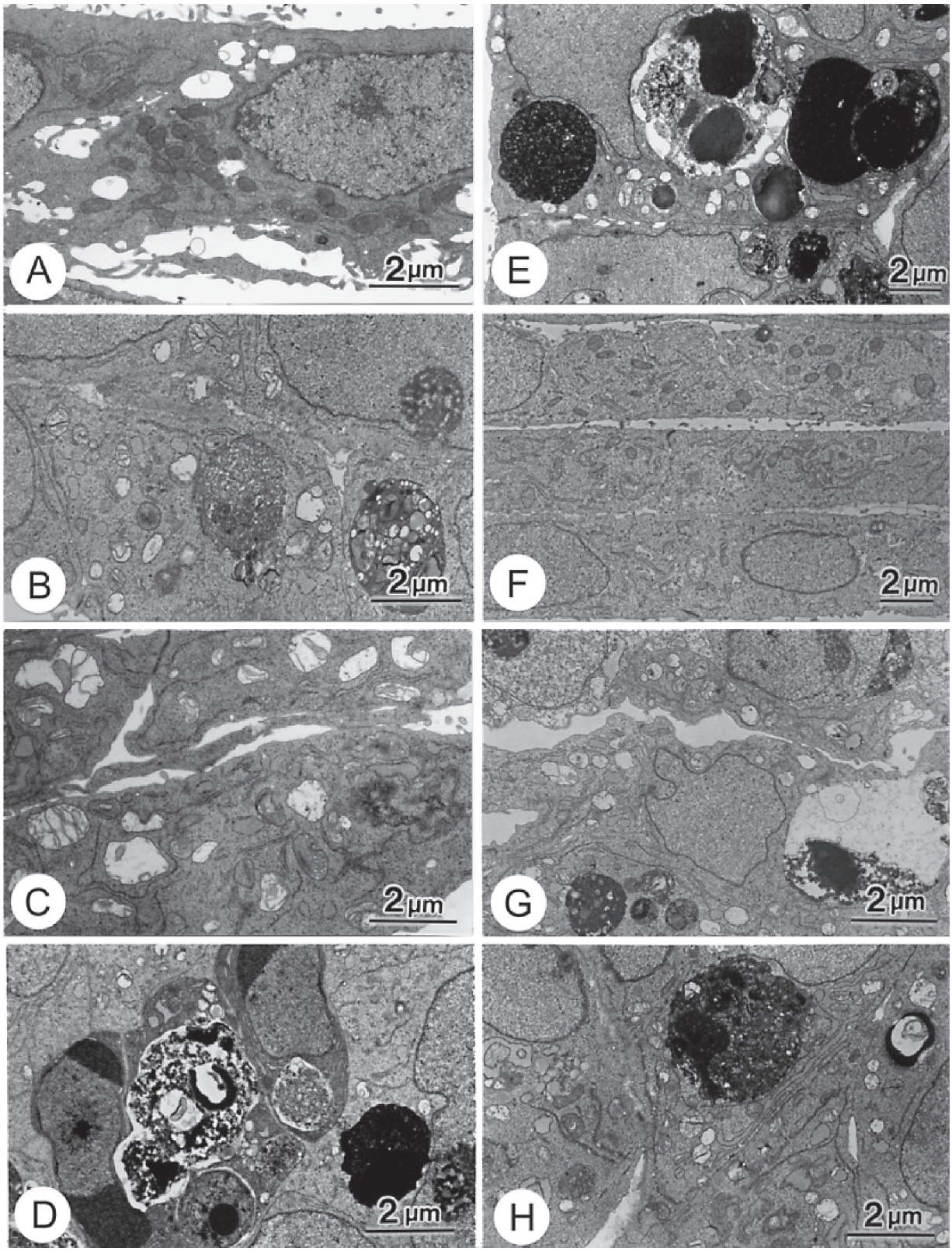

Figure 7. Beta-hydroxybutyrate and ethanol reduced AAPH-induced changes in mitochondrial structure. Electron micrographs of RL-34 cells, control (**A**) treated for 24 hours with AAPH at a concentration of 1.5 mM (**B**), 6.0 mM (**C, D**), with AAPH (6.0 mM) in the presence of acetoacetate (10 mM) (**E**), ethanol (20 mM) (**F, G**), or beta-hydroxybutyrate (20 mM) (**H**). Magnifications of electron micrographs: 10,000 (A, B–D, G, H); 6,000 (E, F).

cells also induced a lipid peroxidation process, and all NADH-generating substrates exerted a protective effect, while AcA had no effect (Fig. 6). These results are in agreement with our recent report in which we demonstrated that NADH inhibited egg yolk lecithin liposome peroxidation induced by AAPH [20]. Overall, increased NADH concentration inside the cell may influence many different processes; however, the results obtained by others and presented in this study suggest that direct reaction of NADH with free radicals inside the living cell could be of physiological importance. Ethanol enhanced AAPH-induced superoxide generation by RL-34 cells, while AcA reduced the production to the control level (Fig. 3). Increased superoxide formation correlates with a drop in aconitase activity, which has been reported to be inactivated by a superoxide [8].

Collectively, these data suggest that NADH generated via the metabolism of ethanol and other substrates may react with peroxyl radicals generated from AAPH, yielding $NAD^{\cdot}$, which reacts with molecular oxygen. This results in the elevation of intracellular levels of superoxide (Fig. 1). Peroxyl and carbon-centred radicals are intermediates during lipid and protein peroxidation processes. In addition, cells can be exposed to lipid hydroperoxides coming from the diet [22], which can decompose to alkoxyl and peroxyl radicals. Moreover, it has been shown that the rate constant for the reaction between NADH and peroxyl radical is much faster compared to the reaction with unsaturated fatty acids, reaching $1 \times \times 10^{8} M^{-1}S^{1}$ [13]. Thus, it is quite possible that in our setting, NADH can react with peroxyl radical but also with other radical species generated during the AAPH exposition. There could be several advantages for the cell from such a reaction. Firstly, a strong radical such as the peroxyl one is removed and converted to a weaker superoxide. Thus, the toxic process induced by this kind of radical is ameliorated. Reactive oxygen species may influence many different signalling pathways. For example, stress-activated protein kinases are activated by ROS. Interestingly, activation of such pathways in some conditions leads to further enhancement in ROS formation, e.g. by activation of NADPH oxidase, an increase of labile iron pool, and an increase of ROS formation by mitochondria [1, 23]. Therefore, the protective effect of NADH-generating substrates against AAPH-induced cell death on the one hand, may be related to direct NADH reaction with some ROS, and on the other hand, it may have indirect effect by modulating signalling pathways. In addition, raising the level of superoxide anion inside the cell can also be beneficial in this condition. As has been postulated before, superoxide radical is an important agent regulating cell metabolism. Inhibition of aconitase activity by superoxide will lead to tricarboxylic acid cycle impairment and growth inhibition which consequently increases cell survival (Fig. 1).

In this study we have shown data which suggest that the intracellular level of NADH has an influence on AAPH-induced cell death; namely, the present data suggest a possibility that NADH may serve as a directly-operating antioxidant in the living cell.

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