

Short Communication

Protective effect of pantothenic acid and related compounds against permeabilization of Ehrlich ascites tumour cells by digitonin

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Preincubation of Ehrlich ascites tumour cells with millimolar concentrations of pantothenic acid, pantothenol or pantethine, but not with homopantothenic acid, at 22°C or 32°C, but not at 0°C, makes the plasma membrane more resistant to the damaging effect of submillimolar concentrations of digitonin. It is proposed that this increased resistance is due to the increased rate of cholesterol biosynthesis. In fact, incorporation of [¹⁴C]acetate into cholesterol is by 45% increased in the cells preincubated with pantothenic acid; this probably reflects elevation of the content of CoA in such cells [Slyshenkov, V.S., Rakowska, M., Moiseenok, A.G. & Wojtczak, L. (1995) *Free Radical Biol. Med.* 19, 767-772].

It has been shown previously that pantothenic acid and some of its related compounds, pantothenol, 4'-phosphopantothenic acid and pantethine, protect Ehrlich ascites tumour cells against lipid peroxidation produced by oxygen free radicals and against the resulting damage of the plasma membrane [1] and lesions of energy-coupling processes in mitochondria [2]. This protective effect is most likely due to increased biosynthesis of CoA [1] and glutathione [2], both compounds being involved in membrane repair mechanisms and/or removal of peroxidation products. In fact, homopantothenic acid, which is not a precursor of CoA, was without effect. In the present article we demonstrate that pantothenic acid and its derivatives also protect Ehrlich ascites tumour cells against permeabilization of their plasma membrane by digitonin. We found that preincubation with pantothenic acid stimulates the synthesis of cholesterol in ascites cells and we

suggest that this process can be responsible for the increased resistance of the plasma membrane against the damaging effect of digitonin.

MATERIALS AND METHODS

Sodium D-pantothenate was from Koch-Light (Colnbrook, Slough, U.K.), D-pantothenol from Hoffmann-La Roche (Basel, Switzerland), pantethine from Maggioni (Torino, Italy) and homopantothenic acid (Ca salt) from N.P.O. Vitaminy (Moscow, Russia). [1-¹⁴C]Acetate, sodium salt, was obtained from the Institute for Nuclear Research (Świerk, Poland).

Ehrlich ascites tumour cells were cultivated in the peritoneal cavity of white Swiss female mice, harvested 7-9 days after inoculation and isolated as described previously [1]. The cells were suspended in the medium containing 150 mM NaCl, 10 mM Tris/HCl (pH 7.4) and 5 mM

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KCl (NTK medium) and kept on ice until they were used for the experiments.

Preincubation of the cells with pantothenic acid or related compounds was carried out in the NTK medium (for digitonin-permeabilization experiments) or in the complete saline medium containing 150 mM NaCl, 3 mM KCl, 1.5 mM Na-phosphate, 0.6 mM MgCl₂, 1 mM CaCl₂ and 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulphonic acid) (Hepes) neutralized with NaOH, pH 7.4 (for measurements of cholesterol biosynthesis).

Permeabilization of the plasma membrane was investigated by sedimenting the cells during 5 min at 3000 × *g* and measuring lactate dehydrogenase (EC 1.1.1.27) remaining in the supernatant [1]. The enzyme was determined spectrophotometrically with pyruvate and NADH as substrates [3].

For measuring cholesterol biosynthesis, the cells (about 15 mg protein/ml) were incubated at 22°C under gentle shaking in the complete saline medium supplemented with 0.1 mM [¹⁴C]acetate (about 1.4 μCi/ml). Aliquots of 1.0 ml were withdrawn and pipetted into centrifuge tubes containing perchloric acid (0.5 M final concentration). Total labelled cholesterol was separated as the digitonin complex [4]. This was performed as follows. The precipitated protein was sedimented by centrifugation, washed twice with water and suspended in 1 ml water to which 1 ml of 6% KOH in ethanol (w/v) was added. The samples were then heated at 90°C during 1 h to hydrolyze cholesterol esters. After cooling, about 0.1 mg of unlabelled cholesterol (dissolved in chloroform) was added to each sample and the samples were extracted with 2 ml hexane. Separation of the phases was facilitated by brief centrifugation at 3000 r.p.m. The hexane extracts were washed with water, followed by centrifugation as before, and evaporated to dryness. Then, 2 ml of the ethanol-acetone (1:1, v/v) mixture was added to each tube and left for 1 h for complete dissolution of the lipid content. Thereafter, the contents were passed through small paper filters which were then washed with 2 ml of the ethanol/acetone mixture. Two millilitres of digitonin solution (5 mg digitonin in 1.0 ml of 50% ethanol) was then added to the filtrate and the samples were left overnight in the dark. The precipitated chole-

sterol-digitonin complex was sedimented by centrifugation, washed twice with ethyl ether and dissolved in 1.0 ml methanol. Therefrom aliquots of 0.8 ml were taken for scintillation counting.

Protein was determined by the biuret method.

RESULTS

Membrane damage by digitonin

Ehrlich ascites tumour cells incubated for 5 min at 0°C in the presence of submillimolar concentrations of digitonin (added as 10% solution in dimethylsulphoxide) became leaky to cytoplasmic proteins [5]. This was determined by measuring leakage of lactate dehydrogenase. It was found that preincubation of the cells with pantothenate partly protected the cell membrane against permeabilization by digitonin. This protective effect was most pronounced at digitonin concentrations of 0.02–0.04% (0.15–0.30 mM) producing 20–40% permeabilization of the membrane. No protection could be obtained at 0.06% and higher digitonin concentrations (above 70% permeabilization) (Fig. 1).

A similar protective effect was observed with pantothenol and pantethine but not with homopantothenate (Table 1).

The protective effect was dependent on both the time and the temperature of preincubation. For example, at 0°C the preincubation with 1 mM pantothenic acid for as long as 60 min had practically no protective effect; at 22°C pantothenic acid exerted its maximum protective effect after 40 min preincubation and at 32°C after 20 min preincubation (not shown). Similar results were obtained with pantothenol and pantethine.

Cholesterol biosynthesis

The rate of cholesterol synthesis in Ehrlich ascites tumour cells was investigated by measuring incorporation of [¹⁴C]acetate into cholesterol. As shown in a preliminary experiment (Fig. 2), this incorporation was approximately linear for 30–40 min. Preincubation of the cells with pantothenic acid considerably increased the rate of [¹⁴C]acetate incorporation. This effect was fairly reproducible and statistically significant as calculated by Student's *t*-test. Mean values ±SD for three different cell preparations were 0.67 ± 0.04 and 0.98 ± 0.05

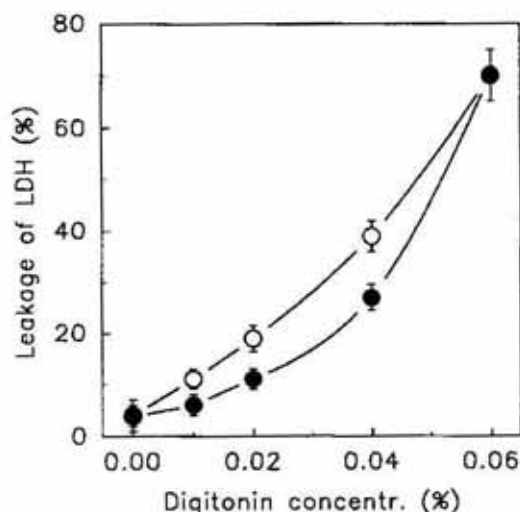


Fig. 1. Protective effect of pantothenic acid against plasma membrane permeabilization by digitonin.

Ehrlich ascites tumour cells (6 mg protein/ml) were preincubated for 40 min at 22°C in the NTK medium without (○) or with (●) 1 mM pantothenic acid. Thereafter, the suspensions were transferred to 0°C for 5 min and then incubated at this temperature for 5 min with digitonin at concentrations indicated at the abscissa. Leakage of lactate dehydrogenase (LDH) was measured as described under Materials and Methods and expressed as percentage of the total activity of this enzyme liberated after complete permeabilization of the cells with 0.1% digitonin. The points indicate mean values \pm SD for 3 experiments.

pmol/h per mg protein ($P < 0.005$) for the control cells and the cells preincubated with pantothenic acid, respectively.

Table 1

Effect of pantothenate and related compounds on digitonin-induced permeabilization of the plasma membrane in Ehrlich ascites tumour cells.

The cells were preincubated with pantothenate or its derivatives for 40 min at 22°C and thereafter treated with 0.04% digitonin as described in the legend to Fig. 1. The values for leakage of lactate dehydrogenase are means \pm SD for 3 experiments. Statistical significance between the cells preincubated with and without pantothenate or its related compounds, calculated using Student's *t*-test, is indicated by asterisk (*); $P < 0.02$.

Compound present during preincubation	Leakage of lactate dehydrogenase (%)
None	39 \pm 3
Pantothenate, 1.0 mM	28 \pm 2*
Pantothenol, 1.0 mM	27 \pm 3*
Pantethine, 0.5 mM	25 \pm 2*
Homopantothenate, 1.0 mM	37 \pm 2

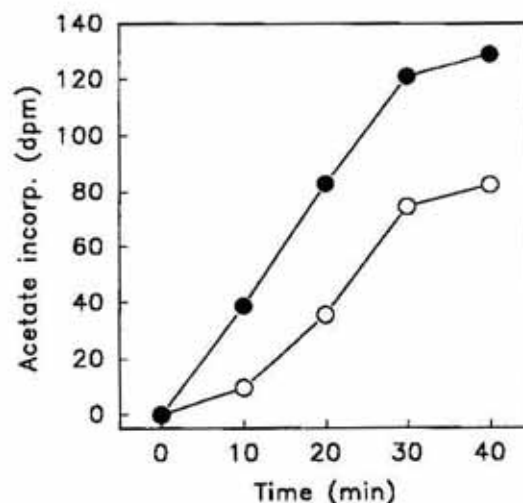


Fig. 2. Effect of preincubation with pantothenic acid on the incorporation of [14 C]acetate into cholesterol.

Ehrlich ascites tumour cells (15 mg protein/ml) were preincubated for 40 min at 22°C in the complete saline medium without (○) or with (●) 1 mM pantothenic acid. Thereafter, [14 C]acetate was added and the incubation was continued for next 40 min. Incorporation of the label into cholesterol was measured as described under Materials and Methods. Specific radioactivity of [14 C]acetate was 16 d.p.m./pmol.

DISCUSSION

Digitonin is known to disturb the integrity of biological membranes by complexing membrane cholesterol [6]. Therefore, the plasma membrane and the mitochondrial outer membrane in which the cholesterol content is relatively high [7, 8] are particularly sensitive to disruption by digitonin. The mechanism of the observed effect of pantothenic acid increasing the resistance of the plasma membrane to digitonin is not clear. The present results indicate that a metabolite of pantothenic acid rather than pantothenic acid as such is the active agent because the protective effect required a preincubation of the cells with this compound, similarly as it was the case with protection against lipid peroxidation [1] and mitochondrial damage [2]. It seems likely that the observed effect could be due to the increased intracellular content of CoA, as found previously [1]. This can be concluded from the observation that a similar effect was exerted by pantothenol and pantethine but not by homopantothenic acid which is not a precursor of CoA (Table 1).

It has been speculated previously [1] that the increased content of CoA may help Ehrlich ascites tumour cells to counteract the deleterious effects of oxygen free radicals by stimulating removal of lipid peroxides and potentiating membrane repair mechanisms, e.g. due to increased phospholipid biosynthesis. The latter process may also contribute to protection against the damaging effect of digitonin. Another possibility, suggested by the results of the present investigation, is the increased biosynthesis of cholesterol. Although the amount of cholesterol synthesized during 40 min of preincubation with pantothenic acid was negligible compared to the total cholesterol content of the cell which amounts to 15 µg/mg protein (about 40 nmol/mg protein) [9], it can be speculated that the newly synthesized cholesterol is more readily available to replace plasma membrane cholesterol complexed by digitonin, thus helping to prevent permeabilization of the membrane by digitonin. The role of CoA in cholesterol biosynthesis is obvious, as acetyl-CoA is the main precursor of this process.

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