Pantothenic acid and pantothenol increase biosynthesis of glutathione by boosting cell energetics

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Abstract We have previously observed (summarized in BioFactors 17 (2003) 61) that pantothenic acid, pantothenol and other derivatives that are precursors of CoA protect cells and whole organs against peroxidative damage by increasing the content of cell glutathione. The present investigation was aimed to elucidate the mechanism of this increase in human lymphoblastoic (Jurkat) cells. It showed that incubation of the cells with pantothenic acid or pantothenol increased mainly the content of free glutathione, with little effect on protein-bound glutathione. Buthionine sulfoximine, an inhibitor of glutathione synthesis, prevented this increase. Increase of the content of free glutathione, as produced by pantothenic acid or pantothenol, was largely prevented by respiratory chain inhibitor rotenone, inhibitor of mitochondrial ATP synthesis oligomycin and uncoupler of oxidative phosphorylation of carbonyl cyanide 3-chlorophenylhydrazone. These treatments also decreased the cellular content of ATP. Preincubation with pantothenic acid or pantothenol also increased cell respiration with pyruvate as the exogenous substrate. Although no significant increase of total cell CoA content could be found, it is concluded that the increase of the glutathione level was due to increased production of ATP that was, in turn, a result of the increased content of mitochondrial CoA.

Keywords: Glutathione; Pantothenic acid; Pantothenol; CoA; Jurkat cell

1. Introduction

A protective effect of pantothenic acid and its derivatives against cell damage produced by oxygen free radicals has been reported from this laboratory during the last decade [1–6]. These findings are in line with the observations by others on radioprotective and antioxidative effects of pantothenic acid and pantothenol and their beneficial action in alleviating some pathological processes (for references, see [5,6]). However, pantothenic acid is not an antioxidant as such, as it did not prevent phospholipid peroxidation in liposomes and its effect on cells required preincubation at room temperature or at 37 °C, but not at 0 °C [1]. Pantothenic acid and its reduced derivative, pantothenol, are precursors of CoA and, in fact, they increased the levels of this coenzyme in Ehrlich ascites tumor cells [1] and rat liver [3]. On the other hand, homopantothenic acid, that is not CoA precursor, did not exert protective effect against reactive oxygen species [1].

We have also observed that pantothenic acid and pantothenol administered to cell culture [2,5] and to whole animals [3,4] greatly increased the content of glutathione and shifted its redox state towards the reduced form. Glutathione is the most abundant low molecular weight thiol-containing compound in the cell and is a well-recognized antioxidant [7–9]. Therefore, we have proposed that the observed protective effects of pantothenic acid and some of its derivatives against cell and tissue peroxidative injury may be due to stimulation of glutathione biosynthesis [2–6]. The aim of the present work was to elucidate the mechanism(s) of this stimulation.

2. Materials and methods

2.1. Chemicals

All chemicals and enzymes were from Sigma/Aldrich (Poznań, Poland).

2.2. Cell culture

Human lymphoblastoic T (Jurkat) cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine and penicillin plus streptomycin (Gibco-BRL, Warsaw, Poland) in the atmosphere of air supplemented with 5% CO_2 at 37 °C. The cells were seeded every 2 or 3 days. Aliquots of $5–10 \times 10^6$ cells (about 1–2 mg protein) were transferred to 5 ml of the same growth medium without serum containing, in addition, 1 mM pyruvate and supplemented, or not, with Na-pantothenate or pantothenol and other additions as indicated and the incubation was continued for 3 h. Thereafter, the cells were separated by brief centrifugation and used for chemical analyses and measurements of respiration.

2.3. Analytical procedures

For determination of glutathione, ATP and CoA the cells, separated as described above, were washed with PBS (ionic composition: 141 mM Na^+, 4.2 mM K^+, 140 mM Cl^- and 3.5 mM P_i; pH 7.2) and lysed in water by freezing and thawing. The lysate was deproteinized with 0.6 M (final) perchloric acid and centrifuged. The protein pellet was used for determination of protein-bound glutathione, whereas the supernatant was neutralized with KOH and briefly centrifuged to remove precipitated K-perchlorate. The resulting clear supernatant was used for determination of reduced (GSH) and oxidized (GSSG) glutathione, ATP and CoA. All these manipulations were performed at 0–4°.

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Abbreviations: BSO, buthionine sulfoximine; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone

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Determination of glutathione. GSSG was measured with glutathione reductase and NADPH [10]. Thereafter, total free glutathione, i.e., original GSH plus GSH formed by reduction of GSSG with NADPH, was assayed enzymatically using the recycling procedure with glutathione reductase and 5,5′-dithio-bis(2-nitrobenzoic) acid [11]. In samples in which *N*-acetylcysteine was present, total glutathione was estimated with glutathione-*S*-transferase and 1-chloro-2, 4-dinitrobenzene [12]. Original GSH was calculated as difference between total glutathione and GSSG, remembering that reduction of one molecule of GSSG results in two molecules of GSH. For measuring protein-bound glutathione, the protein pellet was suspended in 0.2 ml of 0.1 M phosphate buffer adjusted to pH 11 and incubated for 1 h at 37 °C to split protein–glutathione disulfide bonds by reduction with endogenous protein thiol groups [13]. Thereafter, protein was precipitated with perchloric acid, the supernatant was neutralized and glutathione was determined by a modification of the recycling procedure with glutathione reductase. The protein pellet was dissolved in 0.5 M NaOH and used for protein determination by the method of Lowry et al. [14].

CoA. CoA was determined fluorimetrically by measuring reduction of NAD^+ in the presence of 2-oxoglutarate and oxoglutarate dehydrogenase [15] after a mild alkaline hydrolysis to secure the estimation of total CoA [16].

ATP. ATP was determined by reduction of $NADP^+$ in the presence of glucose, hexokinase and glucose-6-phosphate dehydrogenase [15].

2.4. Cell respiration

This was measured at 37 °C using Clark type oxygen electrode (YSI, Yellow Springs, OH, USA) in PBS medium supplemented with 5 mM pyruvate. The mixture contained 20×10^6 cells (about 4 mg protein) in 1.0 ml.

2.5. Statistical evaluation

Mean values for at least three independent experiments ± S.D. are shown throughout. Statistical significance was evaluated using paired Student's *t* test.

3. Results and discussion

Most of cell glutathione is present in the free form within the cytosol and mitochondria. However, a variable amount can be bound to proteins, forming protein–glutathione disulfides (GSSP) [17]. The proportion of protein-bound glutathione may depend on physiological or pathological conditions of the cell [18]. In particular, oxidative stress and a shift of the cell redox status towards oxidative conditions increase protein glutathionylation [19–21]. Our first idea was therefore that an increase of total glutathione observed after treatment of the cells [2,5] or whole animals [3,4] with pantothenic acid or pantothenol might arise from reductive breakage of disulfide bonds in GSSP and liberation of GSH. Such an assumption could be supported by our observation [2–5] that an increase of total free glutathione was always accompanied by a shift to its reduced form.

To check this hypothesis, we examined the effect of pantothenic acid and pantothenol on the amount of free and protein-bound glutathione in Jurkat cells. The cells were incubated without or with pantothenate or pantothenol as in our previous study [5] and analyzed for the content of both free and protein-bound glutathione. It was found that the content of GSSP in Jurkat cells was small, not exceeding 10% of the free glutathione (Table 1). Therefore, splitting of GSSP could not account for a large increase of free glutathione, sometimes reaching 160%, after incubation of the cells with pantothenic acid [5]. This conclusion was confirmed by direct measurements of both free and protein-bound glutathione in cells preincubated for 3 h with pantothenic acid or pantothenol. It was found that both fractions increased significantly (Table 2). It can be therefore concluded that incubation with pantothenic derivatives does not produce a significant shift from bound to free glutathione but, most likely, boosts a net production of glutathione. This conclusion is corroborated by comparison with the effect of *N*-acetylcysteine, a known precursor of intracellular glutathione synthesis (Table 2). It is also noteworthy that pantothenic acid, pantothenol and *N*-acetylcysteine resulted in a shift towards the reduced form, as depicted by an increase of the GSH/GSSG ratio (Table 2).

To verify the assumption that pantothenic derivatives increase biosynthesis of glutathione, we first examined the effect of buthionine sulfoximine (BSO), a known inhibitor of glutathione biosynthesis [22]. As a result of 3 h incubation with this inhibitor the amount of glutathione in Jurkat cells was decreased by about 30% and, interestingly, the increase of both free and protein-bound glutathione by pantothenic acid, pantothenol and *N*-acetylcysteine was almost completely blocked (Table 2). From this observation, it can be concluded that the increase of glutathione as a result of incubation with pantothenic derivatives comes from a net synthesis of this compound.

Table 1
The content of various forms of glutathione in Jurkat cells

Glutathione	Amount (nmol/mg protein) or ratio
Reduced (GSH)	14.5 ± 3.9
Oxidized (GSSG)	0.70 ± 0.23
Total free (GSH + 2 × GSSG)	**15.9 ± 4.0**
GSH/GSSG	22 ± 6
Protein-bound (GSSP)	1.13 ± 0.13
Total (GSH + 2 × GSSG + GSSP)	**17.0 ± 3.9**

Mean values ± S.D. for eight cell preparations.

Table 2
Effect of pantothenic acid, pantothenol and *N*-acetylcysteine on the content of various forms of glutathione in Jurkat cells

Treatment	Total free	GSH	GSSG	GSH/GSSG ratio	Protein-bound
Pantothenate	130 ± 5*	129 ± 6*	72 ± 5*	182 ± 6*	112 ± 3*
Pantothenol	131 ± 3*	132 ± 5*	69 ± 5*	188 ± 5*	113 ± 4*
N-Acetylcysteine	176 ± 3*	183 ± 5*	77 ± 3*	237 ± 4*	116 ± 2*
BSO	72 ± 3*	67 ± 5*	161 ± 5*	41 ± 4*	67 ± 7*
Pantothenate + BSO	82 ± 2#	80 ± 2#	124 ± 3#	63 ± 2#	97 ± 3#
Pantothenol + BSO	85 ± 2#	81 ± 2#	98 ± 2#	81 ± 2#	100 ± 4#
N-Acetylcysteine + BSO	90 ± 3#	89 ± 2#	102 ± 5#	88 ± 3#	85 ± 3#

The cells were incubated with 1 mM pantothenate, 1 mM pantothenol or 5 mM *N*-acetylcysteine for 3 h without or with 0.2 mM BSO. The numbers are percentages with respect to untreated cells. Values marked with * are significantly different from those for control, untreated, cells (assumed as 100%) at the level of $P < 0.05$. Values marked with # are significantly different from those for cells treated with BSO only.

Table 3
Effect of oligomycin, CCCP and rotenone on the content of ATP and glutathione and the influence of incubation with pantothenol

Treatment	ATP	Glutathione		
		Total free	GSH/GSSG ratio	Protein-bound
Oligomycin	80 ± 6*	89 ± 1*	71 ± 3*	96 ± 9
CCCP	68 ± 9*	53 ± 2*	39 ± 2*	85 ± 7
CCCP + oligomycin	82 ± 9	83 ± 2*	62 ± 4*	100 ± 7
Rotenone	85 ± 3*	96 ± 1	58 ± 3*	97 ± 3
Pantothenol	127 ± 3*	131 ± 3*	188 ± 5*	113 ± 4*
Pantothenol + oligomycin	111 ± 4	97 ± 5#	83 ± 1#	89 ± 3
Pantothenol + CCCP	73 ± 7#	78 ± 6#	63 ± 3#	89 ± 5
Pantothenol + CCCP + oligomycin	105 ± 3#	100 ± 2#	79 ± 3#	98 ± 2
Pantothenol + rotenone	82 ± 1#	95 ± 4#	85 ± 5#	100 ± 4

The cells were incubated with 1 mM pantothenol as in Table 1 with the following additions as indicated: 1.2 μM oligomycin, 5 μM CCCP or 1 μM rotenone. The numbers are percentage values with respect to untreated cells, in which ATP content was 10.5 ± 1.5 nmol/mg protein ($n = 6$). For the content of glutathione in control cells see, Table 1. Statistical significance at the level of $P < 0.05$ is indicated by * with respect to the untreated control and by # with respect to the cells treated with pantothenol only ($n = 3$–5).

A mechanism by which pantothenic acid and some of its derivatives promote biosynthesis of glutathione could be by increasing cellular energy production. To verify this assumption, the cells were treated with inhibitors of mitochondrial ATP production such as oligomycin, blocker of F_0F_1-ATP synthase; carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a protonophore; and rotenone, blocker of the respiratory chain at the complex I level [23]. Oligomycin, rotenone and CCCP + oligomycin, during 3 h treatment, decreased intracellular level of ATP by 15–20% due to a complete blockage of mitochondrial ATP synthesis (Table 3, upper part). CCCP alone resulted in a more profound depletion of ATP, because in this case glycolytically generated ATP was partially hydrolyzed by mitochondrial ATP synthase operating in the reverse direction, the process that could be prevented by oligomycin [23]. Parallel to a decrease in ATP level, we observed a decrease in free glutathione. The effect of ATP depletion on protein-bound glutathione was less evident, most likely because this glutathione fraction was less metabolically active.

Incubation of the cells with pantothenol (Table 3, lower part) or pantothenic acid (not shown) increased the level of ATP by about 30%, whereas oligomycin, CCCP and rotenone prevented this increase to a large extent. The correlation between the content of ATP and the level of free glutathione

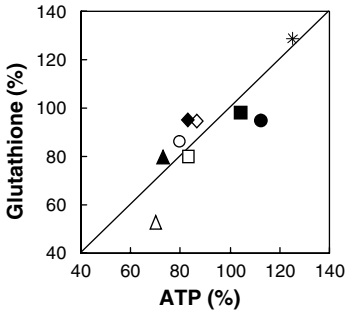

Fig. 1. Correlation between the levels of ATP and total free glutathione in Jurkat cells. The data are from Table 3. Open symbols, cells incubated without pantothenol; closed symbols, cells incubated with 1 mM pantothenol. Other additions: *, none, cells incubated with pantothenol only; ○●, oligomycin; △▲, CCCP; ■□, CCCP + oligomycin; ◊◆, rotenone.

(Fig. 1) supports our assumption that the observed changes of glutathione content are mostly due to its biosynthesis, the process that requires energy in the form of ATP.

The role of pantothenic acid and pantothenol in promoting mitochondrial ATP synthesis must be due to an increase of CoA content. In this context, it is worthy to mention that an increase of fatty acid oxidation and increase of biosynthesis of phospholipids and cholesterol in Ehrlich ascites cells preincubated with pantothenic derivatives have been observed by us previously [1,24]. All these processes require CoA and therefore it was interesting to see whether the level of this coenzyme changed. Our analyses showed, however, that the content of total (free and thioester-bound) CoA in Jurkat cells remained practically unaffected by incubation with pantothenic derivatives. It amounted to 0.46 ± 0.13, 0.46 ± 0.27 and 0.50 ± 0.19 nmol/mg protein for control cells and those preincubated with pantothenic acid and pantothenol, respectively ($n = 4$). However, due to limitation in the amount of available cells, only the content of CoA in whole cells could be estimated. Yet, the content of the mitochondrial pool of CoA may be important for the energy production of cells respiring with pyruvate as the external substrate. An increase of this fraction was, however, too small to contribute in an evident way to the total content of this coenzyme.

Therefore, we measured respiration of Jurkat cells in the PBS medium supplemented with pyruvate. Firstly, it was observed that the cells were in an intermediate state (termed here as 'steady state') between resting state, obtained after addition of oligomycin, and fully active state, recorded in the presence of the protonophore carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP). Secondly, it was found that oxygen uptake in the 'steady state' was by some 30% higher in cell preincubated with pantothenol or pantothenic acid than in control cells (Table 4). This can be again interpreted as activation of pyruvate oxidation due to an increase of the cellular level of CoA.

It can be thus concluded that pantothenic acid and pantothenol increase the content of glutathione in Jurkat cells, and presumably in other kinds of cells as observed previously [2–4], by boosting cell energetics due to increasing cell respiration and ATP synthesis. This, in turn, occurs due to an increased content of CoA for which pantothenic acid and pantothenol are known precursors. Expected consequences of an increased content of CoA in mitochondria are stimulation

Table 4
Effect of pantothenic acid and pantothenol on respiration of Jurkat cells

Preincubation	"Steady state" respiration	"Resting state" respiration	Uncoupled respiration
Without additions	9.1 ± 3.7	3.0 ± 1.3	28.0 ± 14.2
With pantothenate	11.7 ± 3.6*	3.8 ± 1.0	28.9 ± 13.2
With pantothenol	12.2 ± 3.8*	3.8 ± 1.0	30.1 ± 14.2

The cells were preincubated with 1 mM pantothenate or 1 mM pantothenol for 3 h and their respiration was measured in PBS supplemented with 5 mM pyruvate as substrate. The electrode measuring chamber contained cells corresponding to 2.5–3.0 mg protein in 1.0 ml total volume. "Resting state" respiration was measured in the presence of 1.2 μM oligomycin and uncoupled respiration in the presence of 1 μM FCCP. Oxygen uptake is expressed in nmol O/min per mg protein. Statistical significance at the level of $P < 0.05$ ($n = 5$) is indicated by asterisks (*).

of pyruvate oxidation, operation of the tricarboxylic acid cycle and oxidation of fatty acids. Thus, the following sequence of events occurs:

[loading the cells with pantothenic derivatives]
⇒ [increase in cell CoA content,
especially in mitochondrial CoA]
⇒ [increase in energy production and ATP synthesis]
⇒ [increase in glutathione synthesis].

As it is well known, two molecules of ATP are utilized for the synthesis of one molecule of GSH [9]. The requirement for energy in glutathione biosynthesis has already been observed by Konrad Bloch and colleagues half a century ago [25]. These authors also found a regulatory (inhibitory) effect of ADP [25]. Thus, the energy level of the cell must be an important factor in controlling glutathione biosynthesis, though it has been mostly neglected or overlooked in the present overviews [7–9]. In addition, both ATP and CoA are required for the synthesis of phospholipids and cholesterol and in this way participate in repair of cell membranes. This constituted an additional protecting mechanism by pantothenic derivatives against cell damage as observed previously [1,24].

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