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GLYOXYLIC ACID PREVENTS NAD^+ AND NADH DEPLETION IN K562 CELLS CULTURED AT LIMITING DILUTION

Elena Zocchi, Carolina Polvani and Lucrezia Guida

Department of Biochemistry, University of Genoa, Viale Benedetto XV/1, 16132 Genoa, Italy

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Summary: K562 erythroleukemic cells cultured at low population density in the absence of serum die within 12-24 hours, unless 0.1 mM glyoxylic acid is added to the culture medium. Earlier events, preceding cell death and occurring within 2 hours culture, are: a) a marked drop of both the NAD^+/NADH ratio and the NAD^+ concentration, which is prevented by 10 mM benzamide, b) an increased biosynthesis of NAD^+, leading to extensive depletion of cellular ATP. In the presence of 0.1 mM glyoxylic acid the NAD^+/NADH ratio as well as their absolute concentrations remain unchanged, while NAD^+ biosynthesis is absent. A NAD^+/NADH glycohydrolase activity is present in the cell extract, inhibited by 10 mM benzamide and with a higher affinity for NADH than for NAD^+. Preservation of a high NAD^+/NADH ratio by glyoxylic acid apparently prevents enzyme activity and the related loss of pyridine nucleotides.

Ketoacids are essential for growth of cells, both untransformed and neoplastic (1-3), cultured at low population density in the absence as well as in the presence of serum. In a recent study we extended this observation to glyoxylic acid, which proved to be active on K562 human erythroleukemic cells at a concentration 20 times lower than required for pyruvate or oxaloacetate (4). Moreover, metabolism of glyoxylic acid was limited to its stoichiometric reduction to glycolic acid, an excess of which would inhibit cell growth. These findings suggest an effect of glyoxylic acid on the NAD^+/NADH ratio, which in turn would affect cell survival and proliferation.

In order to better understand the mechanism(s) of this growth promoting effect we investigated the NAD^+ and NADH content of K562 cells cultured at low density in the absence or in the presence of glyoxylic acid. The results reported in this paper indicate that the mechanism of glyoxylic acid (and possibly ketoacid)-induced cell survival and proliferation is related to the preservation of the pyridine nucleotide pool.

Abbreviations: FCS, fetal calf serum; NEAA, non essential aminoacids; ADPR, ADPribose; LDH, lactate dehydrogenase; GOX, glyoxylic acid; MTT, thiazolyl blue; PES, phenazine ethosulfate.

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MATERIALS AND METHODS

Materials. NAD^+ and NADH (grade 1) were purchased from Boehringer, Milan, Italy. Concentrations of the stock solutions (prepared fresh for every experiment) were determined spectrophotometrically with yeast alcohol dehydrogenase (ADH) in 0.25M Tris-HCl pH 8.0. All other chemicals, including ADH, were obtained from Sigma Chemical Co., St.Louis, MO, USA. ADH was dissolved in water (10mg/ml) and dialyzed against charcoal (1g/l) for 24 hours. The dialyzed enzyme was stored at -20 °C.
[2,8-^{3}H]Adenosine (30 Ci/mmol) was purchased from NEN, Milan, Italy
Cell culture. K562 human erythroleukemic cells were maintained as described elsewhere (4). Exponentially growing cells (at 3-6x10^5 cells/ml) were used for the experiments.
For determination of pyridine nucleotide content, cells were washed twice in PBS at room temperature, diluted to 1.5x10^4/ml in warm serum-free medium (RPMI 1640 supplemented with NEAA, glutamine and antibiotics) and 4 ml suspension (6x10^5 cells) was either centrifuged and extracted in 1 ml cycling buffer (zero time) or seeded in tissue-culture Petri dishes. Serum-free medium, with or without (control) glyoxylic acid or benzamide, was added to a final volume of 30 ml per dish (2x10^4 cells/ml) and the dishes were incubated at 37°C in 5% CO_2 . After 2 hours the cells were recovered, centrifuged and the pellet from each dish extracted with 1 ml cycling buffer. Pyridine nucleotide content and protein concentration were determined as described below. For labeling with [^{3}H]adenosine, cells were cultured at 3x10^5/ml in complete medium,containing 10% FCS, in the presence of [^{3}H]adenosine(4x10^6 cpm/10^6 cells). After 2 hours incubation cells were recovered , washed twice in PBS and processed as described above for further culture at limiting dilution without serum.
Cycling assay. For determination of NAD^+ and NADH content a method originally developed for erythrocytes was utilized (5), with minor modifications. Pelleted cells (6x10^5)were extracted with 1 ml ice-cold cycling buffer (100 mM sodium carbonate, 20 mM sodium bicarbonate, 10 mM nicotinamide,pH 10.0) and immediately frozen in a dry ice-acetone bath. After thawing in a room temperature water bath aliquots of the extracts (600 μl) were heated at 60°C for 30' in a water bath and then chilled at 0°C. The pyridine nucleotide content of the heated (containing NADH only) and unheated (containing both NAD^+ and NADH) extracts was determined on 100 μl aliquots as described in (5) except for the final concentrations of ADH and ethanol. Thus, 1 ml reaction mixture contained 100 μM Tris-HCl pH 8.0 , 2 μM PES, 0.5 μM MTT, 0.05 mg/ml ADH and 400 μM ethanol. The absorbance at 570 nm was determined before and 30 min after addition of standards or sample solutions (cuvettes were kept in the dark at 37° C). A blank (without NAD^+ or NADH) and at least 3 standards were run with each assay.
NAD^+ turnover. To remove proteins, 200 μl aliquots of heated and unheated extracts of labeled cells (at time zero and after 2 hours culture at limiting dilution) were filtered by brief centrifugation through a nitrocellulose filter (0.2 μ) in Eppendorf tubes. Fifty μl of deproteinized (heated and unheated) samples of each extract were subjected to the cycling assay to determine the concentration of NAD^+ , while 20 μl of the unheated sample (containing 2 μl standard NAD^+ and ATP solutions) were analyzed through HPLC as described below and the radioactivity of the NAD^+ peak was determined. The specific activity of NAD^+ in the extract was then calculated as the ratio between cpm recovered in the NAD^+-containing fractions and picomoles determined with the cycling assay in the same volume.
HPLC analysis. Separation of nucleotides and nucleosides in labeled K562 cell extracts was performed on an HP1090 workstation, equipped with diode array detector and automated injector, with a 100x2.1 mm ODS-Hypersil column (Hewlett Packard) at a flow rate of 0.8 ml/min with a gradient composed of buffer A:0.1 M potassium biphosphate, 15 ml/l Pic A (Waters) at pH 6.3 and B:70% buffer A, 30% methanol at pH 7.5 ,with the following timetable:0-2.5min:100% A; 2.5- 6min:0-2% B; 6-22min:2-25% B. Post-time:10min.
Retention times (min) of standard compounds (detection at 254 and 340 nm) were as follows: nicotinamide 0.8; NAD^+ 1.23; AMP 1.92; adenosine 2.32; ADPR 4.05; ADP 5.98; ATP 12.9; NADH 14.2.
Fractions of 0.2 min were collected, Pico Fluor 40 (Packard, Milan,Italy) was added to the vials and radioactivity was measured with a β -counter (Beckman). Recovered radioactivity was always > 85% of the injected radioactivity (1.5-3.0x10^4cpm).

Determination of NAD^+/NADHase activity. For determination of the enzyme activity, $8x10^6$ washed pelleted cells were suspended in 1 ml 0.05 M Tris-HCl, pH 7.5, or 6.5 and briefly sonicated. The extract was incubated 30 min at 37°C and chilled at 0°C, while the protein concentration was determined and a cycling assay was performed on an aliquot to check depletion of endogenous pyridine nucleotides. The depleted extract was used for determination of enzyme activity. The 70 μl incubation mixture contained 0.05 M Tris-HCl, pH 7.5, or 6.5, 2.0 mg cell extract/ml, NAD^+ or NADH at the indicated final concentrations with or without addition of inhibitors. The reaction was started by addition of the substrate. At time zero and after 15 min incubation 20 μl aliquots were removed, promptly diluted ten-fold in ice-cold cycling buffer to stop the reaction and subjected to cycling assay.
K_m and V_{max} values for NAD^+ and NADH were obtained with linear regression analysis of Lineweaver-Burk plots.
Protein concentration was determined with the method of Lowry et al.(6) using BSA as standard.

RESULTS

Pyridine nucleotide cell content. The sum of NAD^+ and NADH content of K562 cells grown under optimal culture conditions (10% FCS, $1.5\text{-}3.0x10^5$ cells/ml) was 5.0 ± 0.31 nanomoles/mg protein (mean $\pm$ SD of 8 determinations). Washing of the cells did not modify this value (Table 1, zero time). On the other hand, a severe depletion of NAD^+ and, to a lower extent, of NADH occurred when washed cells were subsequently cultured at low density in the absence of serum: after 2 hours, the content of NAD^+ and NADH and their ratio dropped to 12%, 63% and 19%, respectively, of the normal values, while the ATP concentration was below detectability, i.e. < 2.5 nanomoles/mg protein (Table 1). These modifications occurred also in the presence of serum.

Addition of 100 μM glyoxylic acid to the culture medium preserved the NAD^+, NADH and ATP content of the cells. Ten millimolar benzamide also prevented nucleotide pool depletion, while micromolar concentrations were ineffective (Table 1).

NAD^+ turnover. Incorporation of [^{3}H]adenosine into K562 cells was near maximal after 2 hours exposure to the labeled nucleoside. By that time, more than 80% of the incorporated radioactivity was in the form of ATP and 3% only in the form of NAD^+ (Table 2, zero time). These conditions were considered suitable to detect any subsequent NAD^+ biosynthesis from

TABLE 1. NAD^+, NADH and ATP content of K562 cells

	NAD^+	NADH	ATP	NAD^+/NADH
	(nanomoles/mg protein)			ratio
Zero time	4.53	0.41	29.6	11.0
C	0.54	0.26	ND	2.1
C + 10 μM B	0.47	0.18	ND	2.6
C + 10 mM B	3.72	0.36	24.5	9.3
GOX	4.24	0.45	23.6	9.4

K562 cells washed free of FCS (zero time) were cultured for 2 hours in RPMI at $2x10^4$ cells/ml with or without (control) 100 μM glyoxylic acid or in the presence of benzamide. Cell extracts prepared as described were subjected to cycling assay for determination of pyridine nucleotide content. Data are mean of 4 experiments (SD $\leq$ 11%). ND = not detected C = control , C + 10 μM B = control + 10 μM benzamide , C + 10 mM B = control + 10 mM benzamide , GOX = Glyoxylic acid.

TABLE 2. NAD^+ turnover: incorporation of labeled adenosine into NAD^+

	% of radioactivity incorporated as		NAD^+ specific activity (cpm/picomole)
	A	NAD^+	ATP
zero time	2.2	2.7	81.5	105
C	30.2	23.4	15.6	3605
C + 10 μM B	31.8	27.5	16.4	3085
C + 10 mM B	3.6	3.4	67.3	129
GOX	1.2	5.6	74.5	95

After labeling with [^{3}H]adenosine (see Materials and Methods) cells were washed and either extracted in cycling buffer (time zero) or cultured as described in Table 1. Cell extracts were subjected to cycling assay for determination of NAD^+ content and to HPLC analysis for separation and evaluation of labeled compounds. A = adenosine. Results are the mean of 3 experiments (SD $\leq$ 13%).

labeled ATP. After 2 hours culture of the labeled cells at low density ($2x10^4$/ml) and in the absence of serum, the specific activity of NAD^+ increased to more than 30 times the initial value (Table 2) and most of the incorporated radioactivity was in the form of NAD^+ and adenosine. The same extent of labeled ATP incorporation into NAD^+ occurred also in the presence of 10 μM benzamide (Table 2).

On the other hand, the specific activity of NAD^+ and the percentage of radioactivity incorporated as ATP remained unchanged with respect to zero time values if 100 μM glyoxylic acid or 10 mM benzamide were added to the culture medium (Table 2).

NAD^+/NADH ase activity of K562 cell extracts. "In vitro" consumption of endogenous NAD^+ and NADH was observed upon incubation of a K562 cell extract ($8x10^6$cells/ml hypotonic Tris-HCl pH7.5) prepared by brief sonication. After 30 min incubation the sum of NAD^+ and NADH content was reduced to less than 10% of the initial value. Intact cells suspended at $8x10^6$ cells/ml in isotonic Tris-HCl, pH7.5, did not exhibit NAD^+/NADH ase activity. Upon centrifugation of the extract at 400 x g for 3 min the enzyme activity could be demonstrated in the pellet only. Addition of protease inhibitors (pepstatin, leupeptin and PMSF) did not modify the rate of NAD^+/NADH consumption of the extract, thus they were omitted.

TABLE 3. NAD^+ and NADH consumption in K562 cell extracts

	NAD^+	NADH
	(nanomoles/mg protein/15min)	
C	1.26 (0.87)[a]	1.24(1.06)[a]
C + 10 μM B	1.12	1.15
C + 10 mM B	0.02	0.07

A K562 cell extract (pH 7.5), prepared as described, was incubated at 37°C with either NAD^+ or NADH (5 nanomoles/mg protein initial concentration) with or without (control) added benzamide. After 15 minutes, aliquots of the incubations were diluted ten-fold in carbonate buffer and the pyridine nucleotide content was determined through cycling assay.
([a]) Values obtained with cell extract prepared at pH 6.5.
Results from a representative experiment are shown.

When 5 nanomoles/mg NAD^+ or NADH were added to a cell extract depleted in endogenous pyridine nucleotides, the initial rates of NAD^+ and NADH consumption were almost identical i.e. 1.26 and 1.24 nanomoles/mg/15min at pH7.5 and 37°C (Table 3). The same NADH consumption was observed under conditions designed to prevent NADH oxidation by the cell extract, i.e. excess glutamic acid (50 nanomoles/mg) and glutamic dehydrogenase (not shown). The rates of consumption of NAD^+ and NADH at pH 6.5 were 69% and 85%, respectively, of those observed at pH 7.5 . The NAD^+/NADH-cleaving activity was almost completely inhibited by 10 mM benzamide, while 11% and 8% inhibition only was exerted on NAD^+ and NADH consumption, respectively, by 10 μM benzamide .

K_m and V_{max} values of NAD^+/NADHase activity were determined on aliquots of cell extract depleted of endogenous pyridine nucleotides (Table 4). The Km value for NAD^+ was 6 times higher than for NADH (92.4 vs. 15.4 μM), while the apparent V_{max} with NAD^+ was 4 times higher than with NADH (0.88 vs. 0.20 nanomoles/mg cell protein/min).

Incubations containing the highest substrate concentrations (0.18 mM) were analyzed by HPLC to determine the reaction products. After 15 min incubation, nicotinamide and ADPribose were the only products detected. NAD^+/NADH glycohydrolase activity was confirmed by incubation of [^{14}C]carbonylnicotinamide-labeled NAD^+ with K562 cell extract and subsequent HPLC separation of free [^{14}C]nicotinamide : the amounts of [^{14}C]nicotinamide produced and [^{14}C]NAD^+ consumed were stoichiometric.

DISCUSSION

Within 2 hours culture of K562 erythroleukemic cells at low population density a severe (c.a.90%) NAD^+ and NADH depletion takes place (Table 1) despite an accelerated NAD^+ biosynthesis from ATP (Table 2). Although ineffective in restoring the NAD^+ content, this increased NAD^+ synthesis eventually results in depletion of cellular ATP also (Table 1). Addition of 100 μM glyoxylic acid to the culture medium preserves the pyridine nucleotide pool (Table 1). As the biosynthesis of NAD^+ from ATP is not active in glyoxylic acid- treated cells (Table 2), preservation of the NAD^+/NADH content is the result of absent drainage.

TABLE 4. K_m and V_{max} values of NAD^+/NADH ase activity for NAD^+ and NADH

Substrate	K_m (nanomoles/mg)	V_{max} (nanomoles/mg/min)
NAD^+	49.50	0.88
NADH	8.03	0.20

K_m and V_{max} values were obtained as described under Materials and Methods on a K562 cell extract at pH 7.5 . The K_m values are expressed in nanomoles/mg to allow comparison with the intracellular concentrations of NAD^+ and NADH reported in Table 1. The μM values of the affinity constants for NAD^+ and NADH are 92.4 and 15.4, respectively.

On the one hand these observations shed light on the mechanism of glyoxylic acid-induced cell survival and proliferation at low population density (4), since an extensive depletion of NAD^+, NADH and ATP content is incompatible with cell survival (7,8). On the other hand, the question arises as to the cause of NAD^+ and NADH consumption in control cells.

The following enzyme activities are known to break down NAD^+ (NADH): a) poly ADPribose polymerase (ADPRP) (E.C. 2.4.2.30) b) mono ADPribose transferase (ADPRT) c) NAD^+ (NADH) glycohydrolase (NADase) (E.C. 3.2.2.5) and d) NAD^+ pyrophosphatase (E.C. 3.6.1.22). As the source of enzyme activity in this study was a crude cell extract, possibly containing hydrolase(s) capable of degrading poly ADPribose to monomers or cleaving ADPribose from acceptor proteins, the finding that reaction products of NAD^+/NADH degradation are nicotinamide and ADPribose (Results) rules out the pyrophosphatase only. The involvement of ADPRP in NAD^+/NADH consumption can be excluded because a) micromolar concentrations of benzamide known to inhibit polymerase activity (9) fail to prevent pyridine nucleotide depletion in intact K562 cells (Table 1) as well as in cell extracts (Table 3); b) no poly ADPribose has been detected in K562 cells within 2 hours culture at low population density (4), probably as a consequence of extensive NAD^+ depletion (Table 1). Finally, ADPRT also cannot be responsible for NAD^+ and NADH depletion, because this enzyme activity selectively utilizes NAD^+ as donor of the ADPribose moiety, while the pyridine nucleotide consumption in the K562 cell extracts proceeds at the same rate with 5 nanomoles/mg starting concentration of either NAD^+ or NADH (Table 3). The possibility of NADH oxidation by enzymes in the cell extract and subsequent consumption of NAD^+ was considered, but the following observations point to NADH as a real substrate for NAD^+ase activity: a) the same rate of NADH consumption is observed under conditions preventing NADH oxidation (excess glutamic acid and glutamic dehydrogenase, see Results) and ,b) if NADH oxidation would precede breakdown, a K_m value for NADH equal to or higher than that for NAD^+ would be expected, while the opposite was observed (Table 4).

On the other hand, the following observations point to a NAD^+/NADH glycohydrolase activity as being responsible for the observed pyridine nucleotide pool depletion: a) millimolar benzamide completely inhibits NAD^+ and NADH consumption in intact cells (Table 1) and in cell extracts (Table 3) (9); b) enzyme activity in cell extracts occurs over a wide pH range (Table 3) (10); c) a NAD^+/NADHase activity exists on the outer side of the erythrocyte membranes (11), possibly as a result of enzyme extrusion during erythroblast differentiation; d) the apparent K_m value for NAD^+, though obtained with a crude cell extract as enzyme source, is similar to the reported K_m value of purified rat liver mitochondrial NAD^+ glycohydrolase (12).

Since this enzyme is apparently inactive in cells cultured in the presence of glyoxylic acid, a clue to the mechanism of its activation could be the NAD^+/NADH ratio (or content) of the cells. Metabolism of glyoxylic acid in K562 cells is namely limited to LDH-catalyzed reduction to glycolic acid (4). Although the affinity of NAD^+ase for NADH is 6 times higher than for

NAD^+ (Table 4), initial rates of NAD^+ or NADH consumption would be the same if the pyridine nucleotide pool of K562 cells (5 nanomoles/mg protein) were composed of NAD^+ only or NADH only (Table 3). Thus, a shift in the NAD^+/NADH ratio (which actually occurs in control cells but not in glyoxylic acid- treated cells, Table 1) seems not to be directly involved in enzyme activation.

A possible mechanism of reversible modulation of NAD^+/NADHase activity could be (auto)mono ADPribosylation of the enzyme (13). A growing number of observations involves this modification in regulation of enzyme activity (14,15). Moreover, ADPribosylating activities are dependent on availability of oxidized NAD^+. Positive as well as negative changes of NAD^+ levels appear to be correlated with corresponding modifications of mono(ADPribose)protein conjugates in many tissues (15) and defective ADPribosylation was described in fibroblasts, where the NAD^+/NADH ratio was decreased by culture in the presence of high concentrations of lactate (16). Since K562 cells depend on anaerobic glycolysis for energy production, NADH accumulation would result in glycolysis arrest and cell death. As a working hypothesis concerning the physiological role of this NAD^+/NADHase activity, a mechanism detecting and preventing NADH accumulation inside the cell could be envisaged, whereby a fall in the NAD^+/NADH ratio would result in defective ADPribosylation, NADH glycohydrolase activation and stimulation of NAD^+ biosynthesis.

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