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Aerobic Glycolysis by Proliferating Cells: Protection against Oxidative Stress at the Expense of Energy Yield

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Primary cultures of mitogen-activated rat thymocytes were used to study energy metabolism, gene expression of glycolytic enzymes, and production of reactive oxygen species during cell cycle progression. During transition from the resting to the proliferating state a 7- to 10-fold increase of glycolytic enzyme induction occurs which enables the cells to meet the enhanced energy demand by increased aerobic glycolysis. Cellular redox changes have been found to regulate gene expression of glycolytic enzymes by reversible oxidative inactivation of Sp1-binding to the cognate DNA-binding sites in the promoter region. In contrast to nonproliferating cells, production of phorbol 12-myristate 13-acetate (PMA)-primed reactive oxygen species (ROS) in proliferating rat thymocytes and HL-60 cells is nearly abolished. Pyruvate, a product of aerobic glycolysis, is an effective scavenger of ROS, which could be shown to be generated mainly at the site of complex III of the mitochondrial respiratory chain. Aerobic glycolysis by proliferating cells is discussed as a means to minimize oxidative stress during the phases of the cell cycle when maximally enhanced biosynthesis and cell division do occur.

KEY WORDS: Proliferating cells; energy supply; aerobic glycolysis; reactive oxygen species.

The phenomenon of aerobic glycolysis in tumor cells, first reported by Warburg *et al.* (1924), is one of the fundamental problems of tumor biochemistry not yet fully understood. Numerous consecutive studies confirmed the high rate of glucose degradation to lactate despite the presence of oxygen and mitochondria in a variety of fast growing tumor cells (Warburg, 1929, 1930; Crabtree, 1929; Aisenberg, 1961; Weinhouse, 1966, 1976; Burk *et al.*, 1967; Racker, 1976; Bustamante and Pedersen, 1977; Pedersen, 1978; Eigenbrodt and Glossmann, 1980; Brand *et al.*, 1986; Argiles and Lopez-Soriano, 1990; Bagetto, 1992; Mazurek *et al.*, 1997). However, aerobic glycolysis is not a unique feature of tumor cells as it is also found in nontransformed proliferating cells in the presence of sufficient glucose (Wang *et al.*, 1976; Reitzer *et al.*, 1980; McKeehan, 1982; Brand, 1985; Tollefsbol and Cohen, 1985; Dröge *et al.*, 1987; Brand *et al.*, 1988; Mujica *et al.*, 1991; Seshagiri and Bavister, 1991; Barron *et al.*, 1991; Greiner *et al.*, 1994). Alteration from oxidative to glycolytic glucose metabolism in tumor cells is discussed as a consequence of several causes: (i) Numerous papers report on the induction of isoenzymes with special features, in particular hexokinase type II which have been shown to be bound to the outer mitochondrial membrane (Rose and Warms, 1967; Bartley *et al.*, 1975; Ouchi and Ishibashi, 1975; Bustamante and Pedersen, 1977; Bustamante *et al.*, 1981; Arora and Pedersen, 1988), 6-phosphofructo-1-kinase (Dunaway and Karsten, 1985; Eigenbrodt *et al.*, 1985), 6-phosphofructo-2-kinase (Hue and Rider, 1987; Crepin *et al.*, 1989), and pyruvate kinase (Noguchi *et al.*, 1984; Eigenbrodt and Glossman, 1980; Ashizawa *et al.*, 1991; Oude-Weernink *et al.*, 1991). (ii) Racker (1976) reported an increased activity of Na^+-K^+-ATPase in tumor cells, thus increasing the ADP availability and consequently the flow through pyruvate kinase. (iii) LaNoue *et al.* (1977) discussed that the glycerol-phosphate and the malate-aspartate hydrogen shuttles somehow fail to transport hydrogen

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from glycolytically generated NADH into the mitochondria, thus forcing tumor cells to reoxidize NADH cytosolically by lactate dehydrogenase. (iv) Increases in glycolytic enzyme capacities have been shown to correlate well with cancerogenesis (Weber and Morris, 1963; Weber, 1977; Balinsky *et al.*, 1983; Baumann *et al.*, 1988; Board *et al.*, 1990) as well as with controlled proliferation (Ardawi and Newsholme, 1982; Diaz-Espada and Lopez-Alarcon, 1982; Brand, 1985; Tollefsbol and Cohen, 1985; Brand *et al.*, 1988; Marjanovic *et al.*, 1988; Netzker *et al.*, 1992; Marjanovic *et al.*, 1993; Greiner *et al.*, 1994; Netzker *et al.*, 1994).

The observed qualitative and quantitative changes in glycolytic enzyme activities, glucose transport, and hydrogen shuttles can account, at least in part, for aerobic glycolysis by proliferating cells, yet the reason for the transition from oxidative glucose metabolism to aerobic glycolysis remains to be elucidated. In particular, it is still unclear why tumor cells and normal proliferating cells meet their enhanced energy requirement from glycolysis even though this pathway is far less effective in ATP production than glucose oxidation.

Glycolytic glucose degradation to lactate is the only means for the cell to produce ATP without utilization of oxygen. Wherever oxygen reacts with iron-containing proteins, e.g., complexes of the mitochondrial respiratory chain, reactive oxygen species (ROS) such as superoxide anions ($^{\cdot}O_2^-$), peroxide anions ($^-O–O^-$), and hydroxyl radicals ($HO^{\cdot}$) can be generated. Interaction of ROS with cellular macromolecules (DNA, proteins) and lipids under steady-state conditions can lead to oxidative damage if the antioxidant defense is not fully efficient. It is therefore hypothesized that transition to aerobic glycolysis serves as a means to minimize the production of ROS in cells during the critical phases of enhanced biosynthesis and cell division.

A CELL CYCLE MODEL WITH PRIMARY CULTURES OF MITOGEN-ACTIVATED THYMOCYTES

Despite the immense work which has been carried out since the discovery of aerobic glycolysis in tumor cells by Warburg *et al.* (1924) I felt encouraged to enter this field more than 10 years ago because two major questions remained yet unresolved: (i) *How* do proliferating cells achieve the transition from partly oxidative to mainly glycolytic glucose degradation to lactate and are still able to supply sufficient amounts of ATP and (ii) *why*, despite lower energy yields, do tumor cells and other proliferating cells maintain a high glycolytic rate even under aerobic conditions?

To address these questions a cell cycle model with primary cultures of mitogen-activated rat thymocytes has been established (Brand, 1985). This system allows detailed studies on cell cycle-related metabolic and enzymatic events and on gene regulation in resting and proliferating cells. It turned out to be a particularly useful tool to study the changes in energy metabolism as cells undergo a transition from the resting to the proliferating state. Cell division of mitogen-activated thymocytes is completed between 72 and 84 h of culture with the S-phase of the cell cycle peaking between 44 and 48 h as judged from maximal [3H]thymidine incorporation into DNA, maximal cellular DNA and protein content, cell volume and cell number measurements (Brand *et al.*, 1988; Schöbitz *et al.*, 1991; Guse *et al.*, 1993), and electron microscopy (Brand, 1987).

GLUCOSE IS AN ESSENTIAL ENERGY SOURCE FOR PROLIFERATING CELLS

One major question in the last two decades concerned the nature of energetic fuels for tumor and controlled proliferating cells. Glutamine has been suggested by Reitzer *et al.* (1979) and by Zielke *et al.* (1984) to be a major energy source for cultured mammalian tumor cells and for rapidly dividing cells (Krebs, 1981). We repeated these studies with resting and proliferating rat thymocytes (Brand, 1985) and found that these cells indeed utilize glutamine to a significant extent when incubated for a few hours. However, when the glucose in the culture medium was replaced by glutamine, by a combination of glutamine and ribose, or by glutamine and uridine, the mitogen-activated rat thymocytes failed to induce their glycolytic enzymes and as a consequence ceased cell cycle progression and cell division (Greiner *et al.*, 1994). From this result we conclude that glucose is an essential energy source for proliferating cells.

ALTERATIONS OF GLUCOSE METABOLISM DURING TRANSITION OF THYMOCYTES FROM THE RESTING TO THE PROLIFERATING STATE

Thymocytes provide a model system for the study of the energy metabolism of a nontransformed cell

since upon stimulation with Concanavalin A it enters the cell cycle, and in some respect the model mimics the transformation of a noncancer cell to a cancer cell. During cell cycle progression the rate of glucose conversion to lactate increases 19-fold, peaking in the S-phase between 44 and 48 h of culture. At this time the proliferating thymocytes exhibit lactate production, glucose utilization, and glycolytic enzyme activities which are characteristic of a variety of tumor tissues (Baumann *et al.*, 1988) and lymphoblastoid cell lines (Brand *et al.*, 1986).

Alteration of partly oxidative to almost complete glycolytic degradation to pyruvate and mainly lactate occurs during transition from the resting to the proliferating state (Table I, from Brand and Hermfisse, 1997). The amount of [U-^{14}C]glucose recovered in $^{14}CO_2$ during incubation by stimulated and nonstimulated thymocytes—indicative of the rate of complete glucose oxidation—is almost the same (6.6 vs 8.4 μmol). This finding suggests that the *enhanced* energy demand for increased biosynthesis and cell division during proliferation is met solely by glycolysis. This conclusion is supported by our finding that the rate of oxygen consumption by proliferating thymocytes determined with glutamine in the incubation decreased markedly upon addition of glucose, whereas that of resting cells is not affected. Moreover, suppression of oxygen consumption by glucose in proliferating cells (Crabtree effect) leads to a basal oxygen consumption equal to that of resting cells (Guppy *et al.*, 1993). Using [3,4-^{14}C]-labeled glucose, the flux of glucose carbons through the pyruvate dehydrogenase reaction has been determined by $^{14}CO_2$ release to remain constant in resting and proliferating thymocytes despite the 19-fold higher glucose consumption rate in the stimulated cells (Greiner *et al.*, 1994). This result and the equal release of $^{14}CO_2$ from [U-^{14}C]-labeled glucose indicate that pyruvate oxidation in the mitochondria is not increased in proliferating cells.

Calculations of the ATP yields reveal that 86% of total ATP in the proliferating cells are produced by glycolytic glucose breakdown to pyruvate and lactate and only 14% by oxidation of pyruvate derived from glucose to CO_2 and H_2O in the citric acid cycle and the respiratory chain. In resting thymocytes the situation is reversed (Table I, from Brand and Hermfisse, 1997).

Aerobic glycolysis renders growing cells less dependent on oxygen and thereby improves their survival in a hypoxic environment (Eigenbrodt and Glossmann, 1980). Furthermore, acidification of the surrounding tissue by aerobic glycolysis facilitates penetration of blood vessels and migration into inflammatory areas by T-lymphocytes through activation of enzymes such as endo-beta-D-glucuronidase (heparanase) as reported by Gilat *et al.* (1995).

Table I. ATP Yield in Resting and Proliferating Rat Thymocytes from Glycolytic and Oxidative Glucose Metabolism[a]

[U-^{14}C]Glucose recovered in	Resting thymocytes		Proliferating thymocytes	
Lactate	19 ± 2.8		700 ± 46.7	
Pyruvate	1.5 ± 0.15		20.8 ± 2.2	
$^{14}CO_2$[b]	8.4 ± 0.8		6.6 ± 0.7	
ATP produced from		%		%
Glycolysis	41	12	1442	86
Oxidation	302	88	238	14
Total ATP yield	344	100	1680	100

[a] Proliferation of cultured thymocytes was achieved by concanavalin A (10 μg/ml) and interleukin 2 (10 U/ml). Nonstimulated resting and ConA-stimulated proliferating cells, harvested after 48 h of culture, were incubated at a cell density between 1 and 5 × 10^8 with 4 mM [U-^{14}C]-labeled glucose for 60–120 min at 37°C. Mean values ± SEM are given in μmol × h^{-1} × $(10^{10}$ cells$)^{-1}$ for 14 separate experiments. The calculation of ATP is based on the production of 2 moles ATP per mole of glycolytically and 36 moles ATP per mole of oxidatively degraded glucose (3 moles of ATP per 1 mole of NADH and 2 moles ATP per 1 mole of $FADH_2$ oxidized; a proton leak (Brand, 1990) decreasing these ratios is not considered in this calculation).

[b] The amount of $^{14}CO_2$ released in the pentose phosphate pathway has been subtracted in order to obtain the amount of [U-^{14}C]glucose oxidized to $^{14}CO_2$ in the mitochondria.

KINETIC CAPACITIES OF GLYCOLYTIC ENZYMES IN RESTING AND PROLIFERATING THYMOCYTES

Our finding of a 19-fold increase in the rate of glycolytic glucose degradation to lactate during transition of rat thymocytes from the resting to the proliferating state raises the question *how* this increase in glycolytic activity can be achieved. Rat thymocytes cultured in a glucose-containing medium and stimulated by Concanavalin A and Interleukin 2 increase their maximum activities of glycolytic enzymes 7- to 10-fold in the S-phase of the cell cycle (Brand *et al.*, 1988; Netzker *et al.*, 1994; Greiner *et al.*, 1994). In the case of aldolase A, glyceraldehyde-3-phosphate dehydrogenase, hexokinase type I and II, and pyruvate kinase type M2, a corresponding increase in the mRNA

levels has been measured as well (Netzker *et al.*, 1992), pointing to an enhanced transcription of the genes of these enzymes or to a decreased mRNA degradation. No changes of the isozyme forms were observed during cell cycle progression. Brand (1987) reported that putrescine, a product of the ornithine decarboxylase reaction, is essential for glycolytic enzyme induction in rat thymocytes.

V_{max} values of enzymes measured in cell extracts are not indicative for actual flux rates in intact cells because these are subject to cellular substrate concentrations and metabolic control mechanisms. However, V_{max} values are indicative for the cellular enzyme concentrations and thus for the catalytic capacity of an individual enzyme inside the cell. The actual glycolytic flux rate in proliferating thymocytes was determined to be 727 $\mu mol \times h^{-1} \times (10^{10}\ cells)^{-1}$ (Table I). This rate is far lower than the kinetic capacity of the rate-limiting enzyme, 6-phosphofructo-1-kinase (PFK), which was found to be 4550 $\mu mol \times h^{-1} \times (10^{10}\ cells)^{-1}$ (Table II). In consideration of the K_m of a specific enzyme, the V_{max} value allows for a rough estimation of the substrate binding capacity of this enzyme. The large cellular contents of the enzymes phosphoglycerate kinase (PGK), pyruvate kinase (PK), and lactate dehydrogenase (LDH) provide high capacities for substrate binding and can thus secure sufficient supply of ADP and NAD^+ for increased glycolytic flux rates to meet the enhanced ATP demand of the proliferating thymocytes solely via aerobic glycolysis. When compared to the ADP-producing kinases (hexokinase and PFK), the maximum activities of the ADP-utilizing kinases (PGK and PK) are remarkably higher both in resting as well as in proliferating rat thymocytes. This implies that the high binding capacity for cytosolic ADP could limit ADP transport into mitochondria by competition between PGK and PK and the mitochondrial ADP/ATP antiport system. As a consequence of the reduced availability of ADP in the mitochondria the rate of glycolytic degradation of glucose to lactate in the cytosol would increase at the expense of glucose oxidation in the mitochondria. We conclude that the 7- to 10-fold induction of glycolytic enzymes during the S-phase of the cell cycle and the discussed limitation of ADP transport into the mitochondria enables proliferating thymocytes to provide sufficient ATP via aerobic glycolysis.

MECHANISMS INVOLVED IN GENE ACTIVATION OF GLYCOLYTIC ENZYMES IN PROLIFERATING RAT THYMOCYTES

We studied the regulation of cell cycle-associated gene expression of aldolase A and pyruvate kinase M, representative of glycolytic enzymes. By DNase I footprinting and mobility shift competition assays five binding sites for the activating transcription factor Sp1 and one site for an AP-1-like nuclear factor could be identified in the activating region of the proximal aldolase A AH1 promoter downstream of −400 (Hermfisse *et al.*, 1996). Site-directed mutagenesis of the five GC-boxes and the AP-1 binding site and transfection assays revealed that all six *cis*-elements are involved in maximal stimulation of the promoter. Sp1, present in nuclear extracts from both resting and proliferating thymocytes in equal amounts as analyzed by Western blotting, binds to its cognate DNA sequences (GC-boxes) with a decreased efficiency in resting com-

Table II. Kinetic Capacities of ADP- and NADH-Metabolizing Glycolytic Enzymes in Resting and Proliferating Rat Thymocytes[a]

	ADP producing		ADP utilizing		NADH producing	NADH utilizing
Culture time h	HK	PFK	PGK	PK	GAPDH	LDH
0	1.08 ± 0.19 (21)	0.50 ± 0.01 (11)	21.92 ± 0.18 (9)	7.23 ± 0.23 (24)	1.46 ± 0.19 (7)	13.83 ± 1.7 (23)
44–48	7.16 ± 1.71 (23)	4.55 ± 0.20 (16)	161.36 ± 7.47 (8)	87.48 ± 4.80 (26)	8.15 ± 0.42 (8)	112.89 ± 6.4 (26)
72	5.02 ± 1.27 (19)	3.86 ± 0.22 (13)	142.45 ± 12.42 (7)	61.25 ± 2.90 (25)	3.04 ± 0.43 (10)	82.09 ± 7.83 (25)

[a] Enzyme activities (V_{max} values) were determined in extracts prepared by ultrasonication from cells harvested after the indicated culture times. Mean values ± SEM from separate experiments (numbers of individual experiments in brackets) are expressed as $mmol \times h^{-1} \times (10^{10}\ cells)^{-1}$.

pared to proliferating cells (Fig. 1A, lanes 1 and 3, from Schäfer *et al.*, 1996). From these results we conclude that Sp1 is involved in the mechanism by which the promoter of the aldolase A gene achieves high-level transcription during the S-phase of the cell cycle.

The promoter of the pyruvate kinase M gene contains three GC-boxes in the 5'-flanking region. Site-directed mutagenesis of these *cis*-regulatory elements revealed that two of them are involved in the stimulation of the core promoter. These two regions were also protected in DNase I footprinting assays (Netzker *et al.*, 1997). As in the case of the aldolase promoter, Sp1 in nuclear extracts from resting thymocytes has a lower binding efficiency to these GC-boxes than Sp1 in nuclear extracts from proliferating cells (Fig. 1B, lanes 6 and 8, from Schäfer *et al.*, 1996). These data indicate that binding of Sp1 at two proximal GC-boxes is required for promoter activation of the pyruvate kinase M gene and thus contributes to the observed cell cycle-dependent expression of this enzyme in rat thymocytes during the late G1-and S-phase.

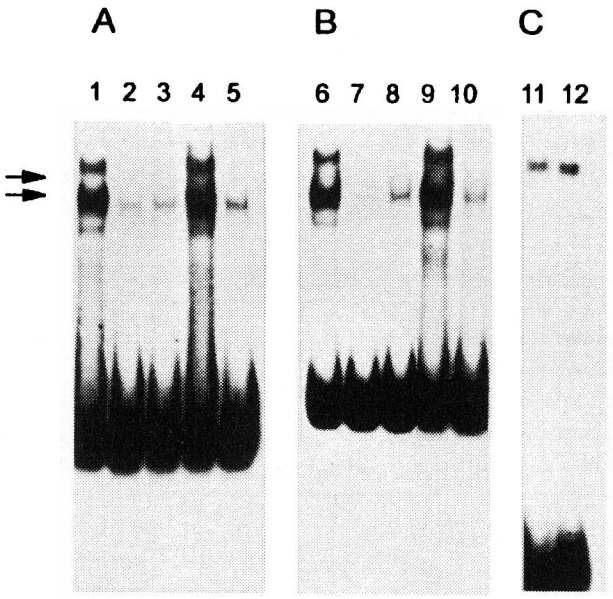


Fig. 1. Hydrogen peroxide inhibits and dithioerythritol restores the Sp1 DNA-binding activity. Electrophoretic mobility shift assays were performed using (A) a fragment of the aldolase A AH1 promoter spanning from position +14 to −84 containing two GC-boxes, (B) a fragment of the pyruvate kinase M promoter spanning from +15 to −86 containing two GC-boxes, (C) a C/EBP oligonucleotide. The following nuclear extracts were used: from proliferating thymocytes (lanes 1, 6, and 11); from proliferating thymocytes incubated with 5 mM H_2O_2 (lanes 2, 7, and 12); from resting thymocytes (lanes 3 and 8); from resting thymocytes incubated with 7.5 mM dithioerythritol (lanes 4 and 9); from proliferating thymocytes incubated with anti-Sp1 antibody (lanes 5 and 10).

REDOX REGULATED BINDING EFFICIENCY OF THE ACTIVATING TRANSCRIPTION FACTOR Sp1

Ammendola *et al.* (1994) have shown that oxidation of Sp1 affects its DNA binding efficiency. We therefore investigated whether the different band intensities observed in the gel shift assays are a result of different redox states of the Sp1 protein in the nuclear extracts from resting and proliferating thymocytes. In order to test the sensitivity of Sp1 to oxidizing agents, nuclear extracts from proliferating thymocytes—which have been shown to have a high Sp1 binding efficiency—were incubated with 5 mM hydrogen peroxide. DNA binding ability of Sp1 to the promoter fragments from both the aldolase A and the pyruvate kinase M gene were markedly decreased (Fig. 2A, lane 2 and Fig. 2B, lane 7). Incubation of nuclear extracts from resting thymocytes—which has been shown to have a low Sp1 binding efficiency—with 7.5 mM dithioerythritol (DTE) resulted in an increase of binding intensity similar to that observed with nuclear extracts from proliferating cells (Fig. 1A, lane 4 and Fig. 1B, lane 9). This result suggests that the low DNA binding efficiency of Sp1 observed with nuclear extracts from resting thymocytes may reflect the sensitivity of Sp1 to peroxide anions. The Sp1 protein contains zinc finger motifs in the DNA binding domains, where the zinc ion (Zn^{2+}) is coordinated to two cysteine and two histidine residues. Oxidation of the cysteine residues in Sp1 causes loss of binding to the cognate DNA element. In a control experiment nuclear extracts from proliferating thymocytes were incubated with the consensus sequence of the transcriptional factor C/EBP, which is known to be unaffected by the redox state (Poli *et al.*, 1990). As expected, C/EBP-DNA-binding efficiency was not changed upon treatment with 5 mM hydrogen peroxide (Fig. 1C, lanes 11 and 12). From these results we conclude that the Sp1 protein in the nuclear extracts from proliferating thymocytes is predominantly present in the reduced form and therefore able to bind to the GC-boxes, thus provoking enhanced gene expression of glycolytic enzymes during proliferation. In contrast, Sp1 in nuclear extracts from resting cells appears to be partly oxidized leading to decreased DNA-binding efficiency and subse-

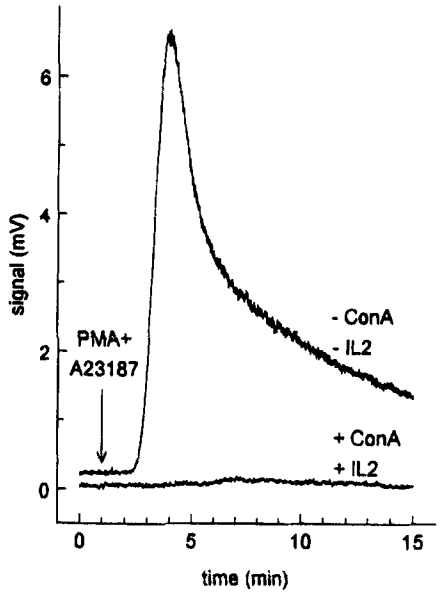


Fig. 2. PMA-initiated chemiluminescence response of nonstimulated and concanavalin A (ConA) plus interleukin 2 (IL-2)-stimulated rat thymocytes. The incubation medium in a total volume of 1 ml contained 5×10^7 cells each, 4 mM glucose, and 170 μM luminol in phosphate buffered saline, pH 7.4, with 0.02% EDTA. Luminol chemiluminescence as a measure of peroxide anion production was initiated by the addition of 2 μg PMA and 1 μmol A23187 calcium ionophore per 1 ml. Photon emission rate at 37°C from one assay vial is plotted on the vertical axis in mV and traced for 15 min by continuous recording with a chromato-integrator, model 2500 Hitachi. One representative experiment out of seven is shown.

quently to reduced gene expression. These studies imply a role of reactive oxygen species in the regulation of Sp1-binding affinity (Schäfer *et al.*, 1996.)

In subsequent studies (Hamm-Künzelmann *et al.*, 1997) we could show that under conditions of oxidative stress—transient exposure of mitogen-activated rat thymocytes to 50 μM hydrogen peroxide between 16 and 28 h of culture (late G1-phase)—results in a more than 60% decrease in the induction of glycolytic enzymes. Furthermore, cotransfection assays with a rat hepatoma cell line and Drosophila Schneider cells, which are completely devoid of endogenous Sp1, revealed that peroxide anions dramatically decrease the transcriptional activities of the Sp1-dependent aldolase A and pyruvate kinase M promoters leading to reduced reporter gene expression. These results indicate that cellular redox changes can regulate gene expression by reversible oxidative inactivation of Sp1-binding.

FORMATION AND ORIGIN OF REACTIVE OXYGEN SPECIES IN NONPROLIFERATING AND PROLIFERATING CELLS

The finding that increased aerobic glycolysis solely meets the enhanced ATP demand of proliferating cells leads to the yet unresolved question *why,* despite lower efficiency of ATP production, proliferating cells maintain a high glycolytic rate even under aerobic conditions and in the presence of intact mitochondria. Potent oxidizing agents such as peroxide and superoxide anions are generated in the cell in a variety of metabolic reactions utilizing molecular oxygen. I propose the hypothesis that transition to aerobic glycolysis and thus reduction of mitochondrial glucose oxidation is a means to protect cells against oxidative stress during the critical phases of enhanced biosynthesis and cell division. To test this hypothesis we measured the production of reactive oxygen species (ROS) in glucose-containing incubations of nonproliferating and proliferating thymocytes and HL-60 cells, which can be induced to differentiate to nonproliferating cells with the characteristics of granulocytes in the presence of dimethyl sulfoxide (DMSO) (Collins *et al.*, 1978). Measurements of peroxide anions revealed striking differences between the nonproliferation and the proliferating cells. Nonstimulated resting thymocytes showed a marked PMA-initiated luminol chemiluminescence signal, indicative for the formation of peroxide anions, whereas ConA-stimulated thymocytes cultured for 44 h (S-phase) failed to produce detectable amounts of peroxide anions (Fig. 2, from Schäfer *et al.*, 1996). This might be explained either by a reduction of metabolic reactions producing reactive oxygen intermediates and/or by the presence of an effective scavenging system inside the proliferating cells. The same result has been obtained with the HL-60 cells. The nonproliferating, DMSO-differentiated cells revealed a pronounced chemiluminescence response upon addition of PMA whereas the proliferating HL-60 cells did not respond at all (Brand and Hermfisse, 1997; data not shown here). For analysis of superoxide radical generation by these cells, superoxide dismutase-sensitive reduction of cytochrome *c* was measured (Fig. 3, from Brand and Hermfisse, 1997). The results of these experiments suggest that: (i) neither proliferating thymocytes nor proliferating HL-60 cells generate superoxide dismutase-sensitive superoxide anions; (ii) resting thymocytes, which extensively produce peroxide anions (Fig. 2), do not generate superoxide dismu-

tase-sensitive superoxide radicals either; and (iii) nonproliferating HL-60 cells, however, release large amounts of PMA-primed superoxide dismutase-sensitive oxygen radicals. These cells were differentiated by DMSO treatment into neutrophil-like granulocytes displaying an activity of NADPH oxidase exceeding that of superoxide dismutase.

In order to study the origin of the ROS, several experiments were performed using inhibitors of the mitochondrial electron transport system. Preincubation of resting thymocytes for 60 min either with rotenone, antimycin A, or, most pronounced by myxothiazol, depressed the PMA-primed luminol chemiluminescence response significantly (Brand and Hermfisse, 1997; data not shown here). This inhibition suggests that the peroxides are generated at the ubiquinol-cytochrome *b* site of the respiratory chain, confirming the results reported by Schultze-Osthoff *et al.* (1992).

PYRUVATE GENERATED BY AEROBIC GLYCOLYSIS IS AN EFFECTIVE ROS SCAVENGER

The results presented in Figs. 2 and 3 suggest that either generation of ROS is strongly reduced in cells during proliferation or ROS are effectively scavenged by antioxidants. Since in the mitochondria almost equal amounts of glucose are oxidized to CO_2 both in resting as well as in proliferating thymocytes (Table I), the potential for ROS production is nearly identical for both cells. The main end product of enhanced aerobic glycolysis by proliferating thymocytes is lactate; however, according to the thermodynamic equilibrium constant, pyruvate accumulates as well (Table I). Since an antioxidative function of pyruvate is well documented in the literature (Andrae *et al.*, 1985; O'Donnell-Tormey *et al.*, 1987; Dröge *et al.*, 1987), the lack of ROS production by proliferating thymocytes with enhanced glycolytic activity is likely to be due to effective scavenging of ROS by pyruvate. We could indeed show that pyruvate suppresses the PMA-induced chemiluminescence of nonproliferating cells quite effectively upon 15-min preincubation with physiologically relevant concentrations of pyruvate (Fig. 4). From these results it appears that the transition to aerobic glycolysis by proliferating thymocytes results in two cooperative effects: (i) generation of the antioxidant pyruvate and (ii) prevention of excessive ROS production by keeping the rate of mitochondrial glucose oxidation at basal levels (Table I). The poor ATP yield via glycolysis in proliferating thymocytes must be compensated by increased glycolytic rates which in turn demand increased glycolytic enzyme activities.

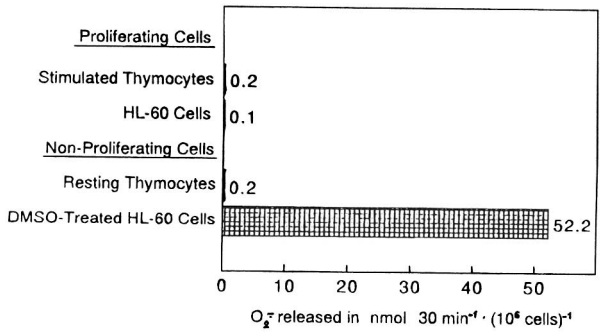
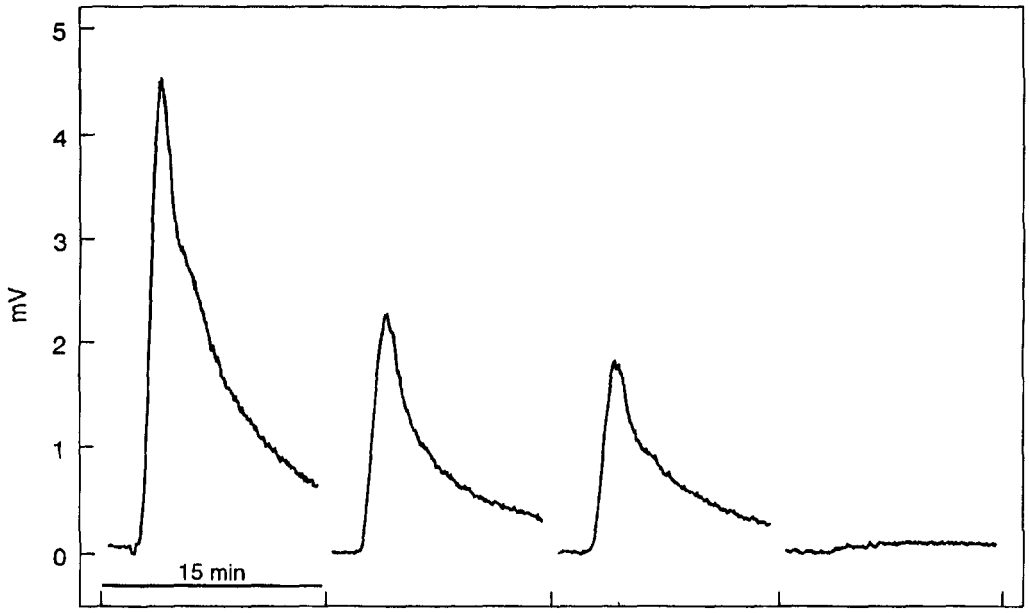


Fig. 3. Superoxide radical formation in rat thymocytes and in HL-60 cells: comparison between proliferating and nonproliferating cells. Nonstimulated and ConA-stimulated rat thymocytes were incubated for 30 min at 37°C in 4 mM glucose containing phosphate-buffered saline, pH 7.4, at a cell density of 5×10^6 cells per 1 ml. The incubation medium with proliferating and DMSO-differentiated HL-60 cells contained 1×10^6 cells per 1 ml. Initiation of superoxide anion production was achieved by the addition of 2 μg PMA per 1 ml to each vial. Superoxide dismutase-sensitive reduction of ferricytochrome *c* was measured spectrophotometrically at 550 nm and calculated from the difference in the absorbance (O.D.) determined in the absence (sample) or presence (control) of superoxide dismutase. For calculation of the amount of nmol $\cdot O_2^-$ produced, the extinction coefficient of 21 liter $\times$ mmol^{-1} $\times$ cm^{-1} was used.

Activation of genes for glycolytic enzymes has been shown to require the transcription factor Sp1 in its reduced form. By preventing increased ROS formation and effective scavenging of peroxide anions, aerobic glycolysis provides sufficient amounts of the reduced activating transcription factor Sp1. Thus, transition to aerobic glycolysis by proliferating thymocytes appears to be a simple, ingenious yet effective strategy to minimize production of ROS in the critical S-phase of the cell cycle. This conclusion may apply not only to thymocytes but also to a number of other normally proliferating as well as malignant tumor cells.

However, several reports in the literature suggest transcriptional activation by ROS. Los *et al.* (1995) report that activation of the transcription factor NFκB and as a consequence interleukin 2 gene expression in primary T lymphocytes through CD28 requires reactive oxygen production. Using cysteamine as an antioxidant, Goldstone *et al.* (1995) demonstrated that the transcription factors such as AP-1 and NFκB are molecular targets for oxidative signaling during lymphocyte activation. From these data it appears that activation of transcription factors by moderate ROS



Pyruvate	none	10μM	100μM	1000μM
rel. AUC_{15}	100	48.1	39.8	4.8

Fig. 4. Effect of pyruvate on PMA-primed luminol chemiluminescence of nonproliferating rat thymocytes. The incubation conditions, initiation, and measurement of chemiluminescence are described in the legend to Fig. 2. In each experiment, 2×10^7 cells were preincubated with Na-pyruvate at the indicated concentration for 10 min. Chemiluminescence was continuously recorded for 15 min and the total light emission was quantified by integration. For each analysis, the area under the curve (AUC15) was related to the control without pyruvate (set to 100). Curves of one representative experiment out of three are shown.

production is an early event during entry of lymphocytes into the cell cycle and during the G1-phase. This interpretation is in agreement with our observation (Schäfer *et al.*, 1996) that the redox-regulated DNA binding efficiency of Sp1 to GC-boxes of the aldolase and pyruvate kinase promoter correlates with the altered redox states in resting and proliferating rat thymocytes. Recently the team of Pascal J. Goldschmidt-Clermont at Ohio State University in Columbus (Irani *et al.*, 1997) reported that NIH 3T3 fibroblasts stably transformed with a constitutively active isoform of $p21^{Ras}$ ($H\text{-}Ras^{V12}$) produce large amounts of superoxide anions and production of these reactive oxygen species correlates with the ability of these cell lines to progress through the cell cycle in the absence of growth factors. The mitogenic activity of the cells expressing $H\text{-}Ras^{V12}$ was inhibited by treatment with the chemical antioxidant N-acetyl-L-cysteine. It will be interesting to see whether superoxide anions also participate in Ras signaling in cells other than transformed fibroblasts, and whether it is possible to extrapolate these results to synchronized proliferating cells as well.

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