

Redox-regulated expression of glycolytic enzymes in resting and proliferating rat thymocytes

Brigitte Hamm-Künzelmann¹, Doris Schäfer, Cora Weigert, Karl Brand*

Institut für Biochemie, Medizinische Fakultät, Universität Erlangen-Nürnberg, Fahrstr. 17, D-91054 Erlangen, Germany

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Abstract Resting rat thymocytes partially degrade glucose aerobically to CO₂ and H₂O and produce reactive peroxide anions. In contrast proliferating cells, due to enhanced induction of glycolytic enzymes, degrade glucose almost completely to lactate thus minimizing the production of reactive oxygen species. In this paper we show that under conditions of oxidative stress the induction of the glycolytic enzymes in cultured rat thymocytes is markedly reduced. Furthermore, transfection assays with a rat hepatoma cell line and *Drosophila* Schneider cells revealed that reactive oxygen intermediates dramatically decrease the transcriptional activities of the Sp1-dependent aldolase A and pyruvate kinase M₂ 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.

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Key words: Glycolytic enzyme induction; Rat thymocyte; Reactive oxygen; Redox regulation; Sp1

1. Introduction

Glucose is the major energy fuel for rat thymocytes. Resting rat thymocytes degrade glucose mainly aerobically to CO₂ and H₂O, whereas proliferating cells metabolize glucose almost completely to pyruvate and lactate [1,2]. The concomitant increase in the activity of glycolytic enzymes with the level of mRNA indicates that these enzymatic activities in proliferating cells are stimulated by activation of gene transcription [3]. The promoter regions of the aldolase A and pyruvate kinase M₂ genes contain various Sp1-binding sites, and Sp1 has been shown to play a critical role in the transcriptional regulation of these glycolytic enzymes [4,5]. Recently we demonstrated with gel shift analyses that peroxide anions inhibit the interaction of Sp1 transcription factor with its consensus sequence, a process which can be reversed by addition of dithioerythritol (DTE). Furthermore, chemiluminescence measurements revealed that resting, but not proliferating thymocytes do produce peroxide anions. This could be the reason for the observed decrease in Sp1-binding efficiency with nuclear extracts from resting thymocytes [5].

The aim of this paper was to study the effect of hydrogen peroxide, added to cultures of Con A-stimulated rat thymocytes on the induction of the glycolytic enzymes. Furthermore

the influence of H₂O₂ on Sp1-dependent transcription efficiency was investigated by transfection assays. The results of these experiments revealed that Sp1 acts as a redox switch regulating gene expression.

2. Materials and methods

2.1. Materials

Female outbred Wistar rats (6–9 weeks old) were used for all experiments. Percoll was obtained from Pharmacia (Freiburg, Germany) and [α -³²P]dATP from Amersham Buchler (Braunschweig, Germany). The pGL3Basic vector, the luciferase assay system and DEAE-dextran was purchased from Promega (Heidelberg, Germany) and the β -galactosidase chemiluminescent assay kit from Boehringer Mannheim (Mannheim, Germany). Catalase was from Sigma (Deisenhofen, Germany).

2.2. Cell preparation and cell culture

Thymocytes from the thymus gland of female Wistar rats were prepared as described in [1] and collected in sterile Krebs saline buffer (KSP) containing 136 mM NaCl, 4.7 mM KCl, 5 mM NaH₂PO₄·H₂O, 1.3 mM CaCl₂·2H₂O, 1.2 mM K₂HPO₄·3H₂O, 0.8 mM MgSO₄·7H₂O at pH 7.4. Large thymocytes, with proliferative activity, were separated from small non-dividing thymocytes by Percoll density gradient centrifugation according to Salisbury et al. [6]. Large thymocytes (10–15% of total thymocytes) were stimulated by the addition of Con A (10 μ g·ml⁻¹) and cultured at a cell density of 1–2 \times 10⁸ per 70 ml of RPMI 1640 medium supplemented with 2 mM glutamine and 70 μ M mercaptoethanol, 10% heat-inactivated fetal calf serum and 5–10 units·ml⁻¹ interleukin-2 at 37°C in a 5% CO₂ incubator for 42–48 h. For exerting oxidative stress the cultured thymocytes were exposed to 50 μ M hydrogen peroxide for periods of 6–12 h during the G₁ phase of the cell cycle.

FTO2B rat hepatoma cells were grown in DMEM/Ham's F-12 medium supplemented with 5% heat-inactivated fetal calf serum, 200 U·ml⁻¹ penicillin G and 100 U·ml⁻¹ streptomycin at 37°C. Oxidative stress was achieved by treating the cells with 200 μ M hydrogen peroxide for 48 h.

Drosophila SL2 cells were maintained in Schneider medium supplemented with 10% fetal calf serum at 25°C.

2.3. Enzyme activity measurements

Thymocytes were harvested after 42–48 h of culture, which corresponds to the maximum of the S phase of the cell cycle [1,7]. Cell extracts were obtained by ultrasonication of the cells in KSP for five separate periods of 6 s at 70 W with a Branson sonifier. The extracts were maintained between 0 and 4°C during sonication. The activities of glycolytic enzymes were assayed at 37°C immediately after sonication by the following methods: aldolase as described by Bergmeyer et al. [8], pyruvate kinase according to Fujii and Miwa [9], and lactate dehydrogenase as described by Vassault [10].

2.4. Luciferase reporter constructs

Aldolase A and pyruvate kinase M₂ promoters were cut with the appropriate restriction enzymes and inserted 5' upstream of the luciferase reporter gene in the commercially available luciferase basic vector (pGL3Basic). The aldolase promoter fragment –727 to +141 and the pyruvate kinase M₂ fragment –454 to +78 were cloned into the *Sst*I-*Bgl*II sites of pGL3Basic. The expression plasmid pPacSp1

*Corresponding author. Fax: (49) (9131) 852484.

E-mail: K.Brand@bigapple.biochem.uni-erlangen.de

¹The first two authors contributed equally to this work.

Abbreviations: Con A, concanavalin A; DTE, dithioerythritol; PBS, phosphate buffered saline; ROS, reactive oxygen species

was used for cotransfections. In SL2 cells the plasmid p97b was used for normalization of transfection efficiencies. In this plasmid the β -galactosidase gene is driven by an Sp1 independent *Drosophila* promoter. pPacSp1 and p97b were kindly provided by Dr. G. Suske.

2.5. Cell transfections and treatments

FTO2B cells were transfected by DEAE/dextran with 5 μ g of the indicated plasmid construct according to the standard protocol of the technical manual of the Promega mammalian transfection system. After treatment with the DNA/DEAE-dextran mixture the cells were incubated with fresh medium and varying amounts of hydrogen peroxide as indicated. Cells were harvested 48 h after transfection.

Drosophila SL2 cells were transfected by the calcium phosphate coprecipitation method described by Di Nocera and Dawid [11]. One day prior to transfection 4.5×10^6 cells in 5 ml medium were seeded in 6 cm culture dishes. To the cultures 500 μ l of a transfection mix containing 9 μ g reporter plasmid, 9 μ g p97b, variable amounts of pPacSp1 and 250 mM CaCl_2 in $1 \times \text{HeBS}$ ($2 \times \text{HeBS}$ contains: 16 g l^{-1} NaCl, 0.7 g l^{-1} KCl, 0.4 g l^{-1} Na_2HPO_4 , 2 g l^{-1} glucose, 10 g l^{-1} HEPES at pH 7.1) were added. After 4 h the medium was removed and the cells were incubated with 10% glycerol in Schneider medium for 3 min. The glycerol medium was then replaced by fresh Schneider medium, with different amounts of hydrogen peroxide as indicated. After 48 h the cells were harvested.

Transfected cells were harvested with a rubber policeman, washed with TEN buffer (40 mM Tris-HCl, 1 mM EDTA, 15 mM NaCl, pH 7.5), and resuspended in 150 μ l of 0.25 M Tris-HCl pH 7.5. After three freeze-thaw cycles the extracts were centrifuged for 10 min and the supernatant was used for luciferase and β -galactosidase assay or determination of protein concentration.

2.6. Nuclear extracts and electrophoretic mobility shift assays (EMSA)

Nuclear extracts from FTO2B cells were prepared according to Dignam et al. [12]. Protein concentration was quantified with the Bradford reagent (BioRad Laboratories, Richmond, CA, USA). For mobility shift assays the following probes were used: (A) a fragment of the aldolase A promoter containing two GC boxes and spanning from position +14 to –84; (B) a fragment of the pyruvate kinase M₂ promoter containing two GC boxes and spanning from position +15 to –86.

10 fmol ^{32}P end-labeled fragments, 2 μ g double-stranded poly-(dIdC) and 5 μ g nuclear protein were incubated for 30 min at room temperature in binding buffer (20 mM Tris-HCl, 50 mM KCl, 5 mM MgCl_2 , 0.5 mM DTE, 5% (v/v) glycerol, pH 8.0). The binding complex was separated by 5% polyacrylamide gel electrophoresis at 4°C in $1 \times \text{TGE}$ (25 mM Tris-HCl, 190 mM glycine, 1 mM EDTA, pH 8.3) at 25 mA.

3. Results and discussion

3.1. Effect of hydrogen peroxide on the induction of glycolytic enzymes in Con A-stimulated rat thymocytes

Primary cultures of rat thymocytes (10^8 cells/50 ml medium) were exposed to 50 μM hydrogen peroxide, which was added to the culture medium 14 h after Con A stimulation (G₁ phase of the cell cycle [1]). H_2O_2 exposure was in the range between 6 and 12 h. Termination was achieved by the addition of 300

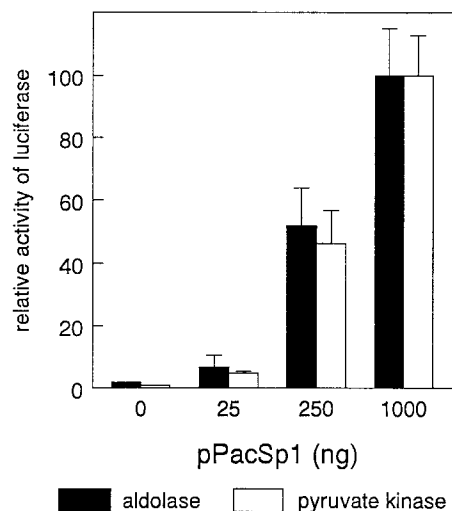


Fig. 1. Transcriptional activation of Sp1 in SL2 cells. 9 μ g of the reporter plasmids (pGL3Basic) containing the aldolase A or pyruvate kinase M₂ promoter fragments were cotransfected in SL2 cells along with the β -galactosidase plasmid p97b and variable amounts of pPacSp1 as indicated. Cells were subsequently lysed, luciferase activities determined and normalized to the β -galactosidase activities. Data are means \pm S.E.M. of three independent experiments.

U catalase to the cultures. After 42 h of culture (during S phase [1]) thymocytes were harvested, the number of cells counted, and the glycolytic enzyme activities measured in the cell extracts. Exposure to 50 μM H_2O_2 between 16 and 28 h of culture resulted in a more than 60% time-dependent decrease in the enzyme activities (Table 1). This finding points to a reduced gene expression of these enzymes during the S phase when the cells were transiently exposed to oxidative stress during the G₁ phase of the cell cycle.

3.2. Influence of oxidative stress on Sp1-dependent transactivation and DNA binding

The promoter regions of the aldolase A and pyruvate kinase M₂ genes are rich in G and C and contain various Sp1-binding sites [13–15]. To study the interaction of Sp1 with these promoter sequences, transcription analyses using *Drosophila* cells (SL2), which are completely devoid of endogenous Sp1, were performed. Luciferase reporter plasmids carrying (i) a fragment of the aldolase A promoter spanning from position –727 to +141 or (ii) a fragment of the pyruvate kinase M₂ promoter spanning from position –454 to +78 were cotransfected with varying amounts of an Sp1 expression vector (pPacSp1). Both promoters showed Sp1-dependent stimulation of the transcription activity (Fig. 1).

Previous experiments [5] with nuclear extracts from proliferating

Table 1

Effect of transient H_2O_2 exposure on the induction of aldolase A, pyruvate kinase M₂ and lactate dehydrogenase activity in cultured rat thymocytes

H_2O_2 exposure (h of culture)	Percentage of enzyme activities		
	aldolase	pyruvate kinase	lactate dehydrogenase
–	100	100	100
14–20	53.7	56.3	51.2
14–24	48.1	49.7	45.5
16–28	38.8	35	39.5

Preparation of cell extracts and measurements of enzyme activity were performed as described in Section 2. Data of 3 experiments with various times of H_2O_2 exposure are shown.

erating rat thymocytes revealed that hydrogen peroxide decreases the binding activity of Sp1 to the aldolase A and pyruvate kinase M₂ promoters. Addition of DTE fully restores DNA binding efficiency. In the present study we examined the effect of peroxide anions on the transcription of the luciferase gene fused to the aldolase A and pyruvate kinase M₂ promoter. H₂O₂ can permeate the plasma membrane, whereby the level of reactive oxygen species in the cell can be experimentally modulated. Since thymocytes are not suitable for transfection assays, two different cell lines were used: SL2 cells and rat hepatoma cells (FTO2B), the latter ones containing the same isoenzymes with respect to aldolase A and pyruvate kinase M₂ as thymocytes [4]. Both cell lines were transfected with the indicated luciferase reporter constructs. SL2 cells were additionally transfected with the Sp1 expression vector pPacSp1. As shown in Fig. 2A,B, increasing amounts of hydrogen peroxide added to the cultures immediately after transfection resulted in a marked, concentration-dependent decrease of the transcription activity with both promoters. SL2 cells were slightly less responsive to H₂O₂ than FTO2B cells (Fig. 2B). The low transcription efficiency of the aldolase A and pyruvate kinase M₂ genes under condition of oxidative stress may reflect the sensitivity of Sp1 to reactive oxygen intermediates.

To find out if the observed decrease in transcription activity by hydrogen peroxide is the consequence of reduced Sp1 binding to its cognate DNA sequence, mobility shift assays were performed (Fig. 3). Nuclear extracts were prepared from FTO2B cells cultured for 48 h in the presence of 200 μ M

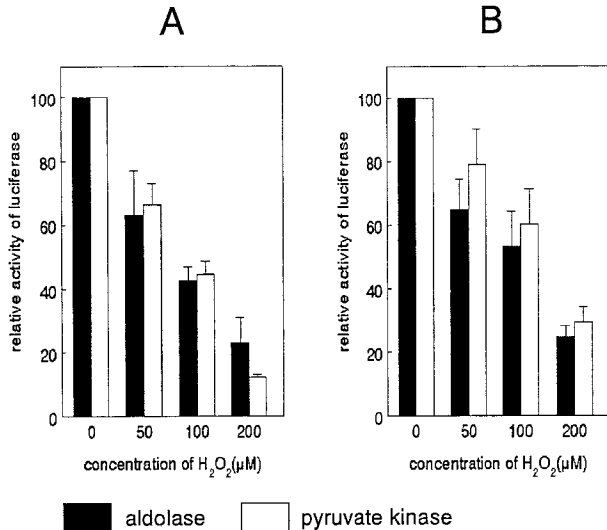


Fig. 2. The effect of H₂O₂ on the aldolase A and pyruvate kinase M₂ promoter-controlled expression of a luciferase reporter gene. A: The effect of H₂O₂ treatment in FTO2B cells. Cells were transfected with 5 μ g of the luciferase reporter gene constructs containing the aldolase A or pyruvate kinase M₂ promoters. For redox modification cells were exposed to varying amounts of hydrogen peroxide for 48 h. Luciferase activities were determined and normalized to equal amounts of protein. B: The effect of H₂O₂ treatment in SL2 cells. 9 μ g of the indicated reporter plasmids were cotransfected in SL2 cells along with 250 ng of the expression plasmid pPacSp1 and the β -galactosidase plasmid p97b. For exerting oxidative stress cells were treated with varying amounts of hydrogen peroxide for 48 h. The luciferase activities were determined and normalized to the β -galactosidase activities. Data are means \pm S.E.M. of three independent experiments.

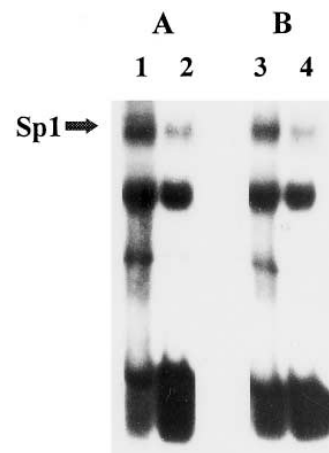


Fig. 3. Effect of hydrogen peroxide on Sp1 binding in vivo. Electrophoretic mobility shift assays were performed using (A) a fragment of the aldolase A promoter spanning from position +14 to –84 containing two GC boxes, and (B) a fragment of the pyruvate kinase M₂ promoter spanning from position +15 to –86 containing two GC boxes. The following nuclear extracts were used: lanes 1 and 3: nuclear extracts from FTO2B cells; lanes 2 and 4: nuclear extracts from FTO2B cells cultured in the presence of 200 μ M H₂O₂ for 48 h.

H₂O₂. The arrow indicates the retarded Sp1-DNA complex. The faster migrating band is an unknown nuclear factor from FTO2B cells, which can associate with the DNA fragments. Consistent with the results from the transactivation assays, Sp1-binding activity was dramatically decreased upon treatment of cells with 200 μ M H₂O₂.

Taken together we conclude that the reduced glycolytic enzyme induction in proliferating rat thymocytes exposed to H₂O₂ is caused by oxidation of Sp1. Obviously the intracellular redox balance is intimately linked to the cellular proliferative capacity. Resting thymocytes, which produce reactive oxygen species, cannot induce glycolytic enzyme expression due to oxidative inactivation of Sp1. In contrast the presence of an effective scavenging system and/or a suppression of ROS formation in proliferating cells [5] enables them to induce glycolytic enzymes and progress orderly through the somatic cell division cycle [1,2].

Several recent studies have reported about oxidative stress affecting gene expression. The transcription factors NF- κ B and AP-1 have been implicated in the inducible expression of a variety of genes involved in responses to oxidative stress and cellular defense mechanisms [16,17]. The results of Miyamoto et al. [18] suggest that catalase is a growth promoting molecule and that its H₂O₂-decomposing activity is important for cell proliferation. Ammendola et al. [19] previously demonstrated that the DNA binding efficiency of Sp1 is greatly decreased in nuclear extracts from old rat tissues compared to those from young ones. As reactive oxygen intermediates are known to accumulate in aged animals, Sp1 appears to become progressively oxidized during aging, resulting in a reversible loss of binding activity.

In this context, our suggestion that reversible oxidative inactivation of Sp1-binding is a means to regulate glycolytic enzyme induction is another example how oxidative stress regulates metabolic activity.

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