ELSEVIER

European Journal of Pharmacology 342 (1998) 113–117

ejp

Short communication

Clotrimazole and bifonazole detach hexokinase from mitochondria of melanoma cells

Julia Penso, Rivka Beitner *

Department of Life Sciences, Health Sciences Research Center, Bar-Ilan University, Ramat-Gan 52900, Israel

Received 21 August 1997; revised 4 November 1997; accepted 7 November 1997

Abstract

Cancer cells are characterized by a high rate of glycolysis. Hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1), the only glycolytic enzyme which binds to mitochondria, is exceptionally high in cancer cells, and believed to play a key role in regulating cell energy metabolism and cancer cell growth rate. We have previously found that clotrimazole (1-(α-2-chlorotrityl)imidazole) and bifonazole (1-(α-biphenyl-4-ylbenzyl)imidazole), the antifungal azole derivatives, which were recently recognized as calmodulin antagonists, are calmodulin antagonists which most effectively reduce glycolysis and ATP level in B16 melanoma cells. They act through allosteric regulation and detachment of glycolytic enzymes from cytoskeleton. Here we report of a novel, additional, mechanism of action of these drugs. We show that they induce a dose-dependent detachment of hexokinase from mitochondria of B16 melanoma cells. This effect preceded the decrease in cell viability. These results suggest that clotrimazole and bifonazole may be promising drugs in treatment of melanoma. © 1998 Elsevier Science B.V.

Keywords: Clotrimazole; Bifonazole; Hexokinase; Melanoma; Glycolysis; Calmodulin antagonist

1. Introduction

Cancer cells are characterized by a high rate of glycolysis, even under aerobic conditions, which is their primary energy source (Eigenbrodt et al., 1985; Fiechter and Gmünder, 1989; Beckner et al., 1990; Greiner et al., 1994). Glycolysis is known to be controlled by allosteric regulators (for reviews, see Beitner, 1979, 1984, 1985, 1990), as well as by reversible binding of the glycolytic enzymes to cyto-skeleton (Arnold and Pette, 1968; for reviews see Clarke et al., 1985; Beitner, 1993; Pagliaro, 1993). All glycolytic enzymes bind to cytoskeleton except hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1), which binds reversibly to mitochondria, where it is linked to oxidative phosphorylation (Gots et al., 1972; Gots and Bessman, 1974; Viitanen et al., 1984; Wilson, 1985; Kottke et al., 1988; Adams et al., 1991). Hexokinase was shown to bind to porin at the contact sites between the mitochondrial inner and outer membranes (Kottke et al., 1988; Adams et al., 1991; Brdiczka, 1991). The mitochondrially-bound hexokinase preferentially utilizes mitochondrially-generated ATP (Gots et al., 1972; Gots and Bessman, 1974; Viitanen et al., 1984). In addition, the contacts were shown to have a higher Ca^{2+}-binding capacity compared to the outer and inner mitochondrial membrane. The mitochondrial-bound hexokinase enhances the uptake of Ca^{2+} by the mitochondria (Kottke et al., 1988), and a rise in mitochondrial Ca^{2+} stimulates intramitochondrial oxidative metabolism and ATP production (Denton and McCormack, 1990; McCormack and Denton, 1990).

Various factors and conditions control the binding of hexokinase to mitochondria. Bessman and coworkers (Bessman, 1966; Viitanen et al., 1984; Bessman and Mohan, 1988; Mohan et al., 1989) have shown that insulin stimulates the binding of hexokinase to mitochondria. Binding of hexokinase to mitochondria is also increased by contractile activity (Weber and Pette, 1990; Parra and Pette, 1995; Parra et al., 1997). Experiments from our laboratory have revealed that Ca^{2+} and Ca^{2+}-mobilizing hormones increase mitochondrially bound hexokinase (Bassukevitz et al., 1992). An increase in mitochondrially bound hexokinase was also found in tumor cells (Arora and Pederson, 1988) and it has been suggested that this binding plays an important role in tumor cell metabolism.

* Corresponding author. Tel.: +972-3-5318224; fax: +972-3-5351824.

We have previously found that the increase in mitochondrially bound hexokinase, induced by insulin, Ca^{2+} and Ca^{2+}-mobilizing hormones, could be prevented by treatment with calmodulin antagonists, which strongly suggest that Ca^{2+}/calmodulin is involved in their action (Bassukevitz et al., 1992; Chen-Zion et al., 1992; Beitner, 1993).

Recent experiments from our laboratory (Glass-Marmor et al., 1996) have revealed that different calmodulin antagonists decrease the levels of glucose 1,6-bisphosphate and fructose 1,6-bisphosphate, the two allosteric stimulatory signal molecules of glycolysis, and reduce ATP content and cell viability in B16 melanoma cells. The most effective compounds were clotrimazole (1-(α-2-chlorotrityl)imidazole) and bifonazole (1-(α-biphenyl-4-ylbenzyl)imidazole), the antifungal azole derivatives, that were recently recognized as calmodulin antagonists (Hegemann et al., 1993; Mac Neil et al., 1993). We have also recently found (Glass-Marmor and Beitner, 1997) that different calmodulin antagonists cause a detachment of glycolytic enzymes from cytoskeleton of melanoma cells. Here again, clotrimazole and bifonazole were the most effective compounds. In the present study, we investigated whether these azole derivatives also exert an effect on the mitochondrial-bound hexokinase in B16 melanoma cells.

2. Materials and methods

2.1. Materials

Clotrimazole and bifonazole were purchased from Sigma Chemical. Other chemicals and enzymes were either from Sigma Chemical or from Boerhinger Mannheim. Tissue culture reagents were purchased from Biological Industries, Beit Haemek.

2.2. Cell culture

B16 F10 mouse melanoma cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics, at 37°C in humidified atmosphere at 5% CO_2 and 95% air. Cells were passaged two to three times weekly.

2.3. Treatment of culture

Melanoma cells (8×10^5 cells ml^{-1}) were seeded in tissue culture plates (10 cm). After 48 h, cells were washed twice with phosphate-buffered saline (PBS). The cells were incubated at 37°C in PBS containing 5 mM glucose in the absence and presence of azole derivatives for 1 h at different concentrations. Clotrimazole and bifonazole were dissolved in water containing acidified 6% polyethylene glycol 400. The solvent was added to the controls.

2.4. Cell viability determination

After incubation in absence and presence of azole derivatives, the cells were harvested with trypsin (0.25%)-EDTA (0.05%) and centrifuged for 10 min at $270 \times g$. The precipitated cells were suspended in PBS and counted in a hemozitometer (Neubauer). Cell viability was determined by trypan blue dye exclusion.

2.5. Protein measurement

Protein was measured by the method of Bradford (1976) with crystalline bovine serum albumin as a standard.

2.6. Separation and assay of mitochondrial bound and soluble hexokinase

After the incubation, the medium was collected in a centrifuge tube and the cells, harvested with trypsin (0.25%)-EDTA (0.05%), were added. Then the cells were pelleted by centrifugation for 10 min at $270 \times g$.

Mitochondrial and soluble fractions were separated by the following method: the precipitated cells were homogenized in Potter homogenizer for 90 s, in 0.5 ml of ice cold 0.25 M sucrose. Samples (2–3 μl) were removed for protein determination. The homogenate was centrifuged at 4°C for 10 min at $1000 \times g$ to remove the cell debris, and the supernatant was centrifuged for 15 min at $27\,000 \times g$. The $27\,000 \times g$ supernatant is referred to as the 'soluble fraction' and the $27\,000 \times g$ pellet is referred to as the 'mitochondrial fraction'. The pellet was redissolved in 0.1 ml of 0.25 M sucrose.

Hexokinase activity was assayed by a fluorometric method as described previously (Beitner et al., 1983), with slight modifications. The reaction mixture contained in a final volume of 1 ml: 0.1 M Tris–HCl buffer (pH 6.9), 0.25 mM $MgCl_2$, 0.05 mM $NADP^+$, 0.25 u/ml of glucose-6-P dehydrogenase, 0.1 mM glucose, 0.75 mM ATP and 20–40 μl of the enzyme fraction. The reaction was initiated by the addition of ATP and fluorescence of NADPH was read on a Perkin-Elmer (MPF-44) fluorescence spectrophotometer. Controls were recorded for each experiment in which ATP was omitted. One unit of hexokinase represents the amount of enzyme activity which forms 1 μmol of NADPH in 1 min at 25°C.

3. Results

Fig. 1 shows that clotrimazole (A) or bifonazole (B) induced a dose-dependent decrease in mitochondrial-bound hexokinase in B16 melanoma cells, with a corresponding increase in soluble activity.

The results presented in Fig. 2 show the effects of clotrimazole (A) and bifonazole (B) on cell viability and its relation to their reducing action on mitochondrial-bound hexokinase. At high concentrations (50 μM) both compounds caused a significant reduction in viable cells, that resulted in marked cell detachment from culture plates.

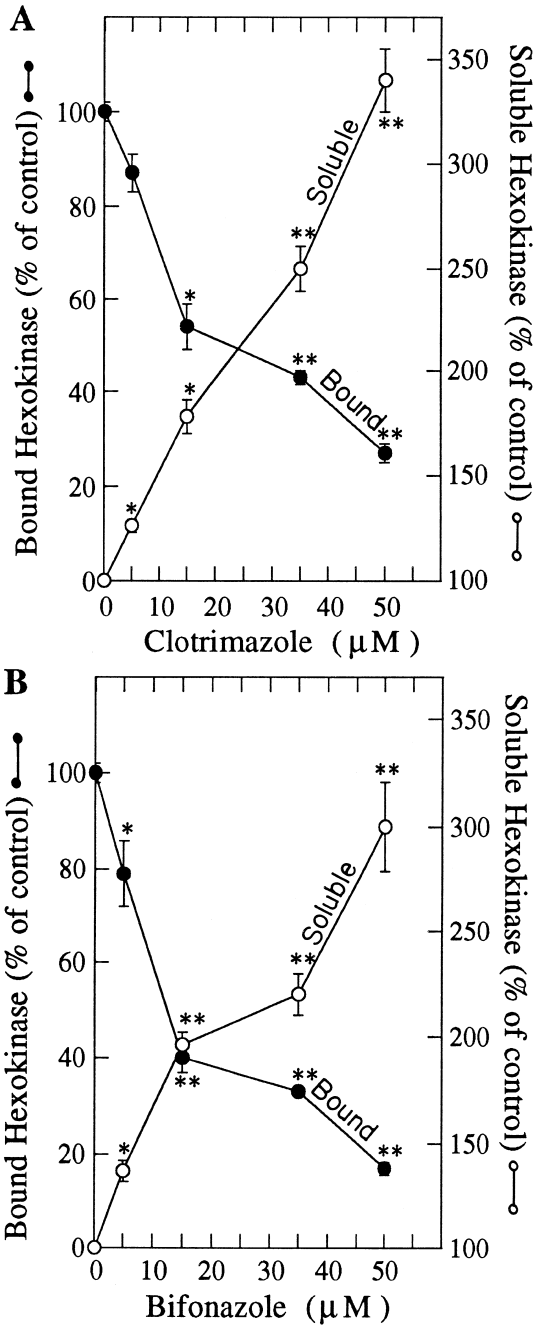

Fig. 1. Dose–response curves of the effects of clotrimazole (A) and bifonazole (B) on mitochondrial-bound and soluble hexokinase in B16 melanoma cells. Cells were incubated for 1 h in absence and presence of different concentrations of clotrimazole or bifonazole. 100% activity of bound and soluble hexokinase was 6.25 ± 0.05 and 12.7 ± 0.6 (u/mg protein), respectively. Each point is the mean $\pm$ S.E.M. of 2–3 separate experiments which were performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.005$.

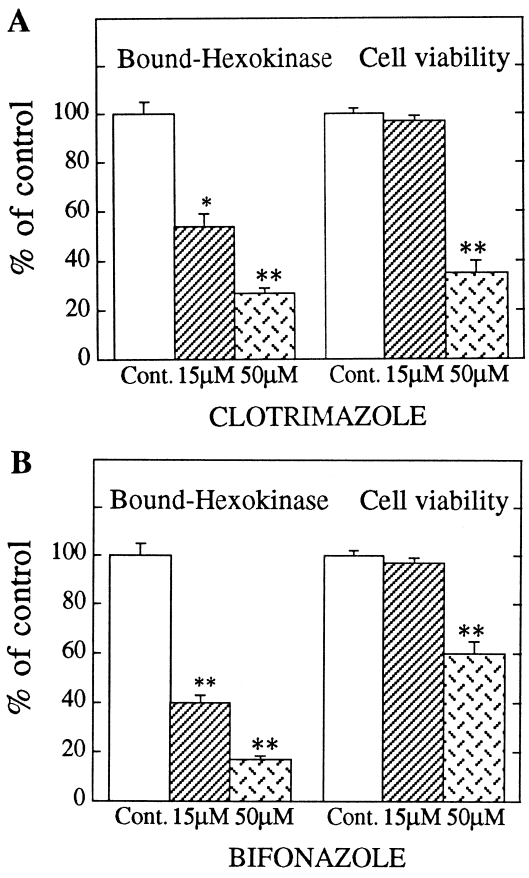

Fig. 2. Effects of clotrimazole (A) and bifonazole (B) on cell viability and its relation to the activity of mitochondrial-bound hexokinase in B16 melanoma cells. Cells were incubated with and without 15 μM and 50 μM clotrimazole or bifonazole for 1 h. 100% cell viability refers to 5×10^6 cells ml^{-1}; 100% activity of bound hexokinase refers to 7.53 ± 0.93 (u/mg protein). Values are the means $\pm$ S.E.M. of 2–3 separate experiments which were performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.005$.

The results in Fig. 2 also clearly show that the decrease in mitochondrial-bound hexokinase induced by both compounds, preceded the decrease in cell viability.

4. Discussion

The results presented here reveal that the antifungal imidazole derivatives, clotrimazole and bifonazole, induced a marked dose-dependent detachment of hexokinase from mitochondria in B16 melanoma cells (Fig. 1). Their IC_{50} in detaching mitochondrial hexokinase was approximately 10–15 μM which is close to their reported IC_{50} for calmodulin inhibition (Hegemann et al., 1993). However, our experiments have revealed that clotrimazole and bifonazole were more effective than other different calmodulin antagonists with similar IC_{50} for calmodulin inhibition (results not shown). These findings suggest that in addition to calmodulin inhibition, other actions of the azole deriva-

tives (e.g., inhibition of cytochrome P-450) may potentiate their effect on mitochondrial hexokinase. We found no correlation between the degree of hydrophobicity of the different calmodulin antagonists and their ability to detach hexokinase from mitochondria. Similarly, we previously found that clotrimazole and bifonazole were more potent than other calmodulin antagonists in detaching glycolytic enzymes from cytoskeleton in B16 melanoma cells (Glass-Marmor and Beitner, 1997), as well as in inducing a reduction of glycolysis through changes in allosteric effectors (Glass-Marmor et al., 1996). They also more effectively reduced melanoma cells viability, which occurred in close correlation with the fall in ATP level induced by these drugs (Glass-Marmor et al., 1996).

The levels of mitochondrial bound hexokinase in highly glycolytic tumor cells greatly exceeds that of normal tissues, and the bound enzyme was shown to have a preferred access to mitochondrially generated ATP (Arora and Pederson, 1988). As compared to soluble hexokinase, mitochondrial binding increases the catalytic activity of hexokinase, leading to stimulation of glucose 6-phosphate production (Parra et al., 1997).

Glucose 6-phosphate is a critical precursor not only for glycolysis for supplying cellular ATP, but also for pentose phosphate pathway, leading to synthesis of excessive amounts of nucleic acids and lipids, which would be available for maintenance of cancer cell proliferation. It was found that NIH/3T3 cells transfected with tumor hexokinase cDNA, showed increased hexokinase, mainly located in the particulate fraction, increased glycolytic rate and enhanced growth rate (Fanciulli et al., 1994). These findings strengthen the belief that the exceptionally high mitochondrial bound hexokinase in cancer cells plays a key role in regulating cell energy metabolism and cell growth rate. Therefore, as shown here, the detachment of hexokinase from mitochondria of melanoma cells induced by clotrimazole or bifonazole, would lead to a reduction in cell metabolism and cell growth. The decrease in the mitochondrial bound hexokinase induced by these drugs preceded the decrease in cell viability (Fig. 2), which indicates that this is an early effect and not a result of cell death. This novel mechanism of action of clotrimazole and bifonazole on mitochondrial hexokinase occurs concomitantly with the detachment of other glycolytic enzymes from cytoskeleton as well as a reduction in glycolysis through allosteric effectors induced by these drugs, which we found previously (Glass-Marmor et al., 1996; Glass-Marmor and Beitner, 1997). Thus these compounds affect all levels of control of glucose metabolism (for review see Beitner, 1993).

Taken together, the present and our previous findings, as well as the reports showing that antifungal azole derivatives, like other calmodulin antagonists, have an antiproliferative action (Mac Neil et al., 1993), suggest that clotrimazole and bifonazole are most promising drugs for treatment of melanoma.

Acknowledgements

The skillful technical assistance of Mrs. H. Morgenstern and Mrs. H. Ben-Porat is acknowledged with thanks. This work was supported in part by the ALSAM Foundation, Los Angeles, CA, USA; the Health Sciences Research Center and by the Research Committee, Bar-Ilan University.

References

Adams, V., Griffin, L., Towbin, J., Gelb, B., Worley, K., McCabe, E.R.B., 1991. Porin interaction with hexokinase and glycerol kinase: Metabolic microcompartmentation at the outer mitochondrial membrane. Biochem. Med. Metab. Biol. 45, 271–291.

Arnold, H., Pette, D., 1968. Binding of glycolytic enzymes to structure proteins of the muscle. Eur. J. Biochem. 6, 163–171.

Arora, K.K., Pederson, P.L., 1988. Functional significance of mitochondrial-bound hexokinase in tumor cell metabolism. J. Biol. Chem. 263, 17422–17428.

Bassukevitz, Y., Chen-Zion, M., Beitner, R., 1992. Ca^{2+}-ionophore A23187 and the Ca^{2+}-mobilizing hormones serotonin, vasopressin, and bradykinin increase mitochondrially bound hexokinase in muscle. Biochem. Med. Metab. Biol. 47, 181–188.

Beckner, M.E., Stracke, M.L., Liotta, L.A., Schiffmann, E., 1990. Glycolysis as primary energy source in tumor cell chemotaxis. J. Natl. Cancer Inst. 82, 1836–1840.

Beitner, R., 1979. The role of glucose-1,6-diphosphate in the regulation of carbohydrate metabolism in muscle. Trends Biochem. Sci. 4, 228–230.

Beitner, R., 1984. Control of levels of glucose 1,6-bisphosphate. Int. J. Biochem. 16, 579–585.

Beitner, R., 1985. Glucose 1,6-bisphosphate – the regulator of carbohydrate metabolism. In: Beitner, R. (Ed.), Regulation of Carbohydrate Metabolism, Vol. I. CRC Press, Boca Raton, Florida, pp. 1–27.

Beitner, R., 1990. Regulation of carbohydrate metabolism by glucose-1,6-bisphosphate in extrahepatic tissues; comparison with fructose-2,6-bisphosphate. Int. J. Biochem. 22, 553–557.

Beitner, R., 1993. Control of glycolytic enzymes through binding to cell structures and by glucose 1,6-bisphosphate under different conditions. The role of Ca^{2+} and calmodulin. Int. J. Biochem. 25, 297–305.

Beitner, R., Lilling, G., Frucht, H., Ben-Porat, H., Sofer, Y., 1983. Age-dependent changes in glucose 1,6-bisphosphate levels and in the activities of glucose 1,6-bisphosphatase, and particulate hexokinase and 6-phosphogluconate dehydrogenase in rat skin. Biochem. Med. 30, 369–380.

Bessman, S.P., 1966. A molecular basis for the mechanism of insulin action. Am. J. Med. 40, 740–749.

Bessman, S.P., Mohan, C., 1988. The intracellular mechanism of insulin action. In: Kleinkauf, H., von Dohren, H., Jaenicke, L. (Eds.), The Roots of Modern Biochemistry. Walter de Gruyter, Berlin, pp. 663–670.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248–254.

Brdiczka, D., 1991. Contact sites between mitochondrial envelope membranes. Structure and function in energy and protein-transfer. Biochim. Biophys. Acta 1071, 291–312.

Chen-Zion, M., Bassukevitz, Y., Beitner, R., 1992. Sequence of insulin effects on cytoskeletal and cytosolic phosphofructokinase, mitochondrial hexokinase, glucose 1,6-bisphosphate and fructose 2,6-bisphosphate levels, and the antagonistic action of calmodulin inhibitors, in diaphragm muscle. Int. J. Biochem. 24, 1661–1667.

Clarke, F., Stephan, P., Morton, D., Weidemann, J., 1985. Glycolytic enzyme organization via the cytoskeleton and its role in metabolic regulation. In: Beitner, R. (Ed.), Regulation of Carbohydrate Metabolism, Vol. II. CRC Press, Boca Raton, FL, pp. 1–31.

Denton, R.M., McCormack, J.G., 1990. Ca^{2+} as a second messenger within mitochondria of the heart and other tissues. Annu. Rev. Physiol. 52, 451–466.

Eigenbrodt, E., Fister, P., Reinacher, M., 1985. New perspectives on carbohydrate metabolism in tumor cells. In: Beitner, R. (Ed.), Regulation of Carbohydrate Metabolism, Vol. II. CRC Press, Boca Raton, FL, pp. 141–179.

Fanciulli, M., Paggi, M.G., Bruno, T., DelCarlo, C., Bonetto, F., Gentile, F.P., Floridi, A., 1994. Glycolysis and growth rate in normal and in hexokinase-transfected NIH/3T3 cells. Oncol. Res. 6, 405–409.

Fiechter, A., Gmünder, F.K., 1989. Metabolic control of glucose degradation in yeast and tumor cells. Adv. Biochem. Eng. Biotechnol. 39, 1–28.

Glass-Marmor, L., Beitner, R., 1997. Detachment of glycolytic enzymes from cytoskeleton of melanoma cells induced by calmodulin antagonists. Eur. J. Pharmacol. 328, 241–248.

Glass-Marmor, L., Morgenstern, H., Beitner, R., 1996. Calmodulin antagonists decrease glucose 1,6-bisphosphate, fructose 1,6-bisphosphate, ATP and viability of melanoma cells. Eur. J. Pharmacol. 313, 265–271.

Gots, R.E., Bessman, S.P., 1974. The functional compartmentation of mitochondrial hexokinase. Arch. Biochem. Biophys. 163, 7–14.

Gots, R.E., Gorin, F.A., Bessman, S.P., 1972. Kinetic enhancement of bound hexokinase activity by mitochondrial respiration. Biochem. Biophys. Res. Commun. 49, 1249–1255.

Greiner, E.F., Guppy, M., Brand, K., 1994. Glucose is essential for proliferation and the glycolytic enzyme induction that provokes a transition to glycolytic energy production. J. Biol. Chem. 269, 31484–31490.

Hegemann, L., Toso, S.M., Lahijani, K.I., Webster, G.F., Uitto, J., 1993. Direct interaction of antifungal azole-derivatives with calmodulin: A possible mechanism for their therapeutic activity. J. Invest. Dermatol. 100, 343–346.

Kottke, M., Adams, V., Riesinger, I., Bremm, G., Bosch, W., Brdiczka, D., Sandri, G., Panfili, E., 1988. Mitochondrial boundary membrane contact sites in brain: Points of hexokinase and creatine kinase location, and control of Ca^{2+} transport. Biochim. Biophys. Acta 935, 87–102.

Mac Neil, S., Dawson, R.A., Crocker, G., Tucker, W.F.G., Bittiner, B., Singleton, J.G., Hunter, T., Tierney, D.F., 1993. Antiproliferative effects on keratinocytes of a range of clinically used drugs with calmodulin antagonist activity. Br. J. Dermatol. 128, 143–150.

McCormack, J.G., Denton, R.M., 1990. The role of mitochondrial Ca^{2+} transport and matrix Ca^{2+} in signal transduction in mammalian tissues. Biochim. Biophys. Acta 1018, 287–291.

Mohan, C., Geiger, P.J., Bessman, S.P., 1989. The intracellular site of action of insulin: The mitochondrial Krebs cycle. In: Horecker, B.L., Stadtman, E.R. (Eds.), Current Topics in Cellular Regulation, Vol. 30. Academic Press, New York, pp. 105–142.

Pagliaro, L., 1993. Glycolysis revisited – a funny thing happened on the way to the Krebs cycle. NIPS 8, 219–223.

Parra, J., Pette, D., 1995. Effects of low-frequency stimulation on soluble and structure-bound activities of hexokinase and phosphofructokinase in rat fast-twitch muscle. Biochim. Biophys. Acta 1251, 154–160.

Parra, J., Brdiczka, D., Cusso, R., Pette, D., 1997. Enhanced catalytic activity of hexokinase by work-induced mitochondrial binding in fast-twitch muscle of rat. FEBS Lett. 403, 279–282.

Viitanen, P.V., Geiger, P.J., Erickson-Viitanen, S., Bessman, S.P., 1984. Evidence for functional hexokinase compartmentation in rat skeletal muscle mitochondria. J. Biol. Chem. 259, 9679–9684.

Weber, F.E., Pette, D., 1990. Changes in free and bound forms and total amount of hexokinase isozyme II of rat muscle in response to contractile activity. Eur. J. Biochem. 191, 85–90.

Wilson, J.E., 1985. Regulation of mammalian hexokinase activity. In: Beitner, R. (Ed.), Regulation of Carbohydrate Metabolism, Vol. 1. CRC Press, Boca Raton, FL, pp. 45–85.