# Cells overexpressing fructose-2,6-bisphosphatase showed enhanced pentose phosphate pathway flux and resistance to oxidative stress

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**Abstract Changes in the content of fructose-2,6-bisphosphate, a modulator of glycolytic flux, also affect other metabolic fluxes such as the non-oxidative pentose phosphate pathway. Since this is the main source of precursors for biosynthesis in proliferating cells, PFK-2/FBPase-2 has been proposed as a potential target for neoplastic treatments. Here we provide evidence that cells with a low content of fructose-2,6-bisphosphate have a lower energy status than controls, but they are also less sensitive to oxidative stress. This feature is related to the activation of the oxidative branch of the pentose phosphate pathway and the increased production of NADPH.** 

*Key words:* Glycolysis; 6-Phosphofructo-2-kinase; Fructose-2,6-bisphosphatase; Fructose-2,6-bisphosphate; Pentose phosphate pathway; Microcalorimetry

## 1. Introduction

Fru-2,6-$P_2$ is a potent allosteric stimulator of 6-phosphofructokinase-1 and an inhibitor of fructose-1,6-bisphosphatase, key enzymes of the F6P/fructose-1,6-bisphosphate cycle [1–3]. The synthesis and degradation of Fru-2,6-$P_2$ are catalysed by the bifunctional enzyme PFK-2/FBPase-2. The intracellular concentration of Fru-2,6-$P_2$ depends on the balance between kinase and phosphatase activities, which, in turn, are regulated by allosteric effectors, phosphorylation/dephosphorylation processes and by regulation of its gene expression. Consequently, PFK-2/FBPase-2 is a switch between glycolysis and gluconeogenesis in mammalian liver and contributes to the regulation of glycolytic flux in extrahepatic tissues [1–3].

An increase in Fru-2,6-$P_2$ concentration in growing cells activates glycolysis and correlates with an enhancement of PFK-2/FBPase-2 expression and activity [4–8]. Transformation of chick embryo fibroblasts by retrovirus carrying either the v-src or v-fps oncogene induced Fru-2,6-$P_2$ synthesis and increased glycolytic flux and cell proliferation [5]. Additionally, the modulation of PFK-2/FBPase-2 expression by growth factors is concomitant with their mitogenic response [4]. Recent results [9,10] indicate that changes in Fru-2,6-$P_2$ content may affect a wide diversity of metabolic processes. Durante et al. reported an enhancement of the apoptosis induced by serum withdrawal in Rat-1 fibroblasts overexpressing the kinase domain of PFK-2/FBPase-2 [10]. Chesney et al. identified an inducible regulatory PFK-2/FBPase-2 isoenzyme constitutively expressed in several human cancer cell lines and required for tumour cell growth [9]. Inhibition of inducible PFK-2 protein expression decreased the intracellular levels of PRPP, a product of the PPP and a precursor for nucleic acid biosynthesis. This observation suggested that the Fru-2,6-$P_2$ regulation of flux through PFK-1 may affect the non-oxidative PPP [11,12], which is the main source of ribose 5-P in proliferating cells [13–15]. Moreover, the pentoses cycle contributes to the control of glucose metabolism and provides cells with NADPH for proliferation [16]. These functions may also be altered by changes in Fru-2,6-$P_2$ content. To evaluate the role of PPP in cells with a low Fru-2,6-$P_2$ content, we measured the activities of the main controlling enzymes and the flux through this metabolic pathway in transfected cells overexpressing FBPase-2 (pFBPase-2 cells). Since changes in the oxidative PPP may affect the reduction power of cells, we also measured their sensitivity to the oxidative stress induced by hydrogen peroxide.

## 2. Materials and methods

### 2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum (FCS) were obtained from Biological Industries (Kibbutz Reit, Haewek, Israel). Annexin V/FITC was purchased by Bender MedSystems (Vienna, Austria). Enzymes and biochemical reagents were obtained from Boehringer Mannheim (Mannheim, Germany) or Sigma Chemical (St. Louis, MO, USA).

### 2.2. Cell transfections and measurement of culture metabolites

Fru-2,6-$P_2$ defective cells were obtained by stable transfection of a plasmid containing a truncated construction of the rat liver PFK-2/FBPase-2 enzyme with only the FBPase-2 domain, into a mink lung epithelial cell line Mv1Lu (CCL-64, American Type Culture Collection), as previously described [17]. Control cells were transfected with the empty pcDNA3 vector.

Cells were maintained in DMEM, supplemented with 10% (v/v) FCS, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 400 μg/

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*Abbreviations:* PRPP, 5-phosphoribosyl-1-pyrophosphate; PFK-2/FBPase-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; F6P, fructose-6-phosphate; Fru-2,6-$P_2$, fructose-2,6-bisphosphate; Gluc-6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; G3P, glyceraldehyde-3-phosphate; PPP, pentose phosphate pathway; pentoses-5-P, pentoses-5-phosphate; TK, transketolase

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ml of G418 in 100 mm culture dishes (TPP) [17]. To measure heat production and metabolite production, cell suspensions attached to microcarriers were used. For a 100 ml culture, approximately $3\times10^7$ cells were added to 0.3 g (dry weight) of pre-swollen Cytodex 1 microcarriers (Pharmacia Biotech). Cell concentrations were determined by a cell counter (Coulter Electronics, UK). To achieve a maximum yield of cells attached to microcarriers, the cultures were stirred for 5 min every 30 min. After 4 h, the medium was changed and the cultures were stirred continuously at 60 rpm. Microcarrier cultures were incubated for up to 48 h. Before each determination, cells attached to microcarriers were rinsed and suspended in Krebs bicarbonate buffer containing 10 mM glucose, 10 mM HEPES, 2.5 mM $CaCl_2$, and 2% BSA. Cell suspensions were incubated in glass vials for 30 min at 37°C using an orbital shaking bath, and afterwards, samples for metabolite determination collected in duplicate at 0, 1.5 and 3 h. ATP and lactate were measured spectrophotometrically in neutralised perchloric extracts using standard enzymatic methods [4,18,19]. Protein concentration was determined by the Bio-Rad assay. Fru-2,6-$P_2$ was determined in supernatants by its ability to activate pyrophosphate-dependent 6-phosphofructo-1-kinase from potato tubers as described elsewhere [20].

### 2.3. Heat production

To measure heat production, 2.7 ml of the microcarrier suspension ($10^5$ cells/ml) was introduced into each measurement vessel of a Thermal Activity Monitor (LKB-Thermometric AB, Järfalla, Sweden) thermostatted at 37°C, as previously described [21]. The air remaining in the vessels (0.8 ml) and the stirring at 120 rpm provided aerobic conditions during the measurements. The resulting power–time curves indicate the heat evolved from the metabolic activity of cell suspensions.

### 2.4. Measurement of PPP flux, enzyme activities, and NADPH/NADP$^+$ ratio

Mv1Lu cells were scraped into a medium composed of 20 mM Tris–HCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 0.2 g/l Triton X-100 and 0.2 g/l sodium deoxycholate, at pH 7.5. The cell extracts were further homogenised in an ultrasound bath (Branson) for 6 min at 0°C. The enzymes were partially purified by ultracentrifugation at $140\,000\times g$ and 4°C. G6PDH activity was assayed by measuring the rate of NADPH production from 0.5 mM NADP$^+$ and 2 mM Gluc-6P at pH 7.6 [22]. TK was assayed by measuring the rate of NADH consumption from 0.2 mM NADH, 5 mM $MgCl_2$, 0.1 mM TPP, 2 mM ribose-5-phosphate and 1 mM xylulose-5-phosphate, at pH 7.6 [23]. Metabolic flux through the PPP was determined by measuring the rate of NADPH production from 1 mM NADP$^+$, 5 mM $MgCl_2$, 0.2 mM TPP, 4 mM ribose-5-phosphate and 2 mM xylulose-5-phosphate, at pH 7.6.

Alkaline extraction of cells was performed with 0.5% Nonidet P-40 buffer containing KOH at pH 9, as previously described [16]. The cell lysates were centrifuged at 3000 rpm for 5 min at 4°C. The supernatants were immediately filtered through 10 000 NMWL Ultrafree-MC centrifugal filter units (Millipore), collected and stored at −20°C. Reverse-phase HPLC was performed with a C-18 ($25\times0.46$ cm) analytical column (Tracer) on a Perkin-Elmer HPLC. The mobile phase and the chromatographic conditions were the same as reported in [16]. Retention times and peak areas were monitored at 254 nm with an UV detector, and analysed with Turbochrom Navigator software (Perkin-Elmer). NADPH and NADP$^+$ standards were dissolved in extraction buffer and immediately injected in the HPLC instrument.

### 2.5. Flow cytometric analysis

For synchronisation, Mv1Lu cells were cultured in DMEM supplemented with 10% FCS for 4 days, up to confluence. After this period, cells were harvested, countered and plated (250 000 cells) into 60 mm dishes to start the experiment. After 48 h, different amounts of hydrogen peroxide were added. 24 h later cells were washed twice in PBS and once in binding buffer composed of 140 mM NaCl, 10 mM HEPES and 2.5 mM $CaCl_2$, at pH 7.4. Then, cells were stained with 1 μl/ml annexin V/FITC and 0.5 μg/ml propidium iodide for 30 min at room temperature in the dark. Stained cells were analysed using a FACSCalibur cytometer and the CellQuest software (Becton Dickinson, CA, USA). Apoptosis was analysed using the MODFIT program (Becton Dickinson, CA, USA).

### 2.6. Data analysis

Results are shown as mean ± S.E.M. of the values obtained from the indicated number of experiments. Differences between groups were tested by ANOVA and the appropriate a priori contrast methods and were considered to be statistically significant at $P<0.05$.

## 3. Results

Metabolic activity, as assessed by microcalorimetry, and energy status of cells were analysed in cultures of transfected cells adhered to microcarriers. Our measurements were in good agreement with those previously reported by Perez et al. [17] for the same clones. Briefly, the contents of Fru-2,6-$P_2$ of control and FBPase-2 cells were 23.6 ± 4 pmol/mg protein and 5.5 ± 3 pmol/mg protein, respectively. Cells overexpressing FBPase-2 showed a 22% reduction in metabolic activity and a 26% reduction in lactate production. The ATP content of FBPase-2 cells was also reduced by 20%.

As a consequence of these metabolic alterations FBPase-2 cells accumulate hexoses-P and reduce trioses-P [17]. These changes in the glycolytic metabolite concentrations could drive the activation of the PPP as an alternative metabolic pathway to provide substrate for the lower part of glycolysis. To assess this assumption, we evaluated the activity of G6PDH (Table 1) as the main rate-limiting enzyme of the PPP [16]. Results shown in Table 1 indicate that G6PDH activity, and consequently the oxidative flux of PPP, was higher in cells with a low content of Fru-2,6-$P_2$ than in control cells. The PPP may produce precursors for nucleic acid biosynthesis or it may generate glycolytic intermediates through TK activation and contribute to energy production [16]. To ascertain this possibility we evaluated TK activity, as well as the maximal capacity of pentoses cycle to convert xylulose-5-phosphate and ribose-5-phosphate into G3P and F6P. F6P can be isomerised to Gluc-6P and re-enter the cycle through its oxidative branch. Results shown in Table 1 indicate that both TK activity and PPP flux were higher in pFBPase-2 cells than in control cells.

G6PDH is the main source of NADPH utilised in redox regulation [16] and the activation of this enzyme supplies pFBPase-2 cells with extra NADPH and reduction power [24]. The ratio NADPH/NADP$^+$ measured by HPLC was 20% higher in cells overexpressing FBPase-2 than in control cells (Table 1).

To ascertain the effect of these changes on the sensitivity to oxidative stress, exponentially growing cells were incubated

Table 1
Effect of overexpression of the bisphosphatase domain of PFK-2/FBPase-2 on G6PDH and TK activities, and flux through the PPP

| | Control | pFBPase-2 |
|---|---|---|
| G6PDH | 297 ± 29 | 412 ± 9** |
| *mU/mg protein* | (4) | (4) |
| NADPH/NADP$^+$ | 0.55 ± 0.02 | 0.64 ± 0.03** |
| | (4) | (4) |
| TK | 22.7 ± 0.9 | 27.7 ± 0.5** |
| *mU/mg protein* | (4) | (4) |
| Flux | 2.54 ± 0.24 | 3.58 ± 0.24* |
| *mU/mg protein* | (4) | (4) |

Cells were incubated with DMEM supplemented with 10% FCS. G6PDH and TK activities were assayed as described in Section 2. Results are the mean ± S.E.M. from four different experiments, each in duplicate. Statistically significant differences $P<0.05$ and $P<0.01$ vs. control cells are indicated by * and **, respectively.

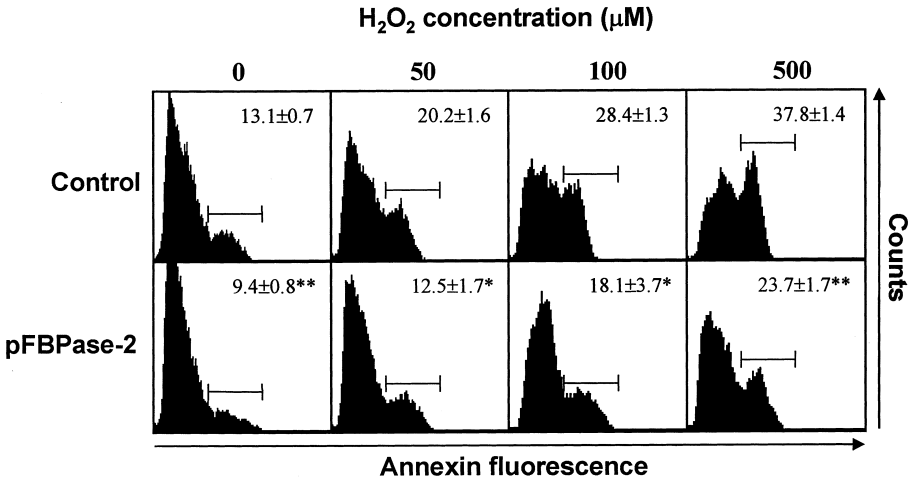


Fig. 1. FACS analysis of apoptosis induced in pFBPase-2 and control cells incubated for 24 h in the presence of the indicated concentrations of $H_2O_2$. Each plot represents the result of one of four independent experiments. The inserted figures indicate the mean ± S.E.M. of the percentage of apoptotic cells from the four experiments. Statistically significant differences $P < 0.05$ and $P < 0.01$ vs. control cells are indicated by * and **, respectively.

for 24 h in the presence of increasing amounts of hydrogen peroxide and analysed by flow cytometry. Even in the absence of $H_2O_2$, apoptosis was lower for pFBPase-2 cells than for control cells. These differences were amplified in the presence of increasing amounts of hydrogen peroxide (Fig. 1). Consistent with these findings, cells containing the lowest Fru-2,6-$P_2$ concentration showed a lower extent of membrane blebbing and chromatin condensation in the presence of hydrogen peroxide (data not shown).

## 4. Discussion

### 4.1. Higher activity of PPP in FBPase-2 cells

These results indicate a significant decrease (20%) in the energy status of Fru-2,6-$P_2$ defective cells, probably as a consequence of a reduction of the glycolytic rate [17]. Energy equilibrium implies that metabolic fluxes are regulated to balance the ATP production and consumption, either by reducing active processes, for instance biosynthesis and transport, or by increasing other metabolic pathways [17].

Results shown in Table 1 indicate an enhancement of the oxidative PPP, probably associated with the accumulation of hexoses-6-P observed in pFBPase-2 cells [17]. Activation of G6PDH increases intracellular redox level and provides pentoses-5-P. These may be transformed into glycolytic metabolites by TK, or into precursors for biosynthesis by PRPP synthase [11,12,16]. The higher capacity of pFBPase-2 cells to convert ribose-5-P into glycolytic metabolites (Table 1) suggests that PPP may be activated in response to a demand for energy, supplying substrate for glycolysis rather than providing precursors for biosynthesis. However, conversion of Gluc-6P into trioses-phosphate through the PPP means to lose one carbon atom as $CO_2$ for each Gluc-6P entering the pathway, which is a less efficient way than glycolysis [11,12].

### 4.2. Lower sensitivity of pFBPase-2 transfectants to oxidative stress

We attempted to assess whether pFBPase-2 cells derived advantage from the enhanced PPP flux since the extra NADPH produced by the activation of G6PDH could increase resistance of such cells to oxidative stress [16,24,25]. Thus, using male mouse embryonic stem cells, in which G6PDH gene was disrupted, it was demonstrated that this enzyme is essential to protect cells against oxidative stress [26]. Moreover, the clones in which the activity of this enzyme was undetectable were extremely sensitive to $H_2O_2$. Similar results were recently obtained using a G6PDH-deficient cell line [27].

The results shown in Table 1 and Fig. 1 indicate that cells with a low content of Fru-2,6-$P_2$ increased their reduction power and resistance to oxidative insult, which is consistent with the increased G6PDH activity and NADPH production. Reciprocally, these results suggest that overproduction of Fru-2,6-$P_2$ may have the opposite effect: an enhancement of the glycolytic rate and a reduction of the G6P concentration, which would limit the flux through G6PDH and NADPH production. The concomitant loss of reduction power would render these cells more susceptible to induced apoptosis [10]. Moreover, biosynthesis depends on the rate of NADPH production [16,27] and its restriction could be a limiting factor for growth of cells with a high content of Fru-2,6-$P_2$ [10].

In conclusion, these results show that a low concentration of Fru-2,6-$P_2$ reduced energy production, which was partly compensated by enhancing the flux through PPP. The activation of G6PDH provided pFBPase-2 cells with extra reduction power, which increased their resistance against oxidative stress.

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## References

[1] Van Schaftingen, E. (1987) Adv. Enzymol. Relat. Areas Mol. Biol. 59, 315–395.
[2] Hue, L. and Rider, M.H. (1987) Biochem. J. 245, 313–324.

[3] Pilkis, S.J., Claus, T.H., Kurland, I.J. and Lange, A.J. (1995) Annu. Rev. Biochem. 64, 799–835.
[4] Joaquin, M., Salvado, C., Bellosillo, B., Lange, A.J., Gil, J. and Tauler, A. (1997) J. Biol. Chem. 272, 2846–2851.
[5] Hue, L. and Rousseau, G.G. (1993) Adv. Enzyme Regul. 33, 97–110.
[6] Boscá, L., Rousseau, G.G. and Hue, L. (1985) Proc. Natl. Acad. Sci. USA 82, 6440–6444.
[7] Colomer, D., Vives, J.L. and Bartrons, R. (1991) Biochim. Biophys. Acta 1097, 270–274.
[8] Dalmau, M., Bartrons, R. and Gil, J. (1994) Exp. Cell Res. 212, 93–96.
[9] Chesney, J., Mitchell, R., Benigni, F., Bacher, M., Spiegel, L., Al Abed, Y., Han, J.H., Metz, C. and Bucala, R. (1999) Proc. Natl. Acad. Sci. USA 96, 3047–3052.
[10] Durante, P., Gueuning, M.A., Darville, M.I., Hue, L. and Rousseau, G.G. (1999) FEBS Lett. 448, 239–243.
[11] Belyaeva, N.F., Golubev, M.A., Grigorovich, J.A., Dubinsky, Z.V., Semenova, N.A., Pitkanen, E. and Korovkin, B.F. (1994) Experientia 50, 780–784.
[12] Blackmore, P.F. and Shuman, E.A. (1982) FEBS Lett. 142, 255–259.
[13] Boros, L.G., Puigjaner, J., Cascante, M., Lee, W.N., Brandes, J.L., Bassilian, S., Yusuf, F.I., Williams, R.D., Muscarella, P., Melvin, W.S. and Schirmer, W.J. (1997) Cancer Res. 57, 4242–4248.
[14] Eigenbrodt, E. and Glossmann, H. (1980) Trends Pharmacol. Sci. 1, 240–245.
[15] Gumaa, K.A., McLean, P., Boros, L.G., Puigjaner, J., Cascante, M., Lee, W.N., Brandes, J.L., Bassilian, S., Yusuf, F.I., Williams, R.D., Muscarella, P., Melvin, W.S. and Schirmer, W.J. (1969) Biochem. J. 115, 1009–1029.
[16] Tian, W.N., Braunstein, L.D., Pang, J., Stuhlmeier, K.M., Xi, Q.C., Tian, X. and Stanton, R.C. (1998) J. Biol. Chem. 273, 10609–10617.
[17] Perez, J.X., Roig, T., Manzano, A., Dalmau, M., Boada, J., Ventura, F., Rosa, J.L., Bermudez, J. and Bartrons, R. (2000) Am. J. Physiol., in press.
[18] Gutman, I. and Wahlefeld, A.W. (1974) in: Methods of Enzymatic Analysis, Academic Press, New York.
[19] Trauchold, I., Lamprecht, W. and Schweitzer, G. (1974) in: Methods of Enzymatic Analysis, Academic Press, New York.
[20] Van Schaftingen, E., Lederer, B., Bartrons, R. and Hers, H.G. (1982) Eur. J. Biochem. 129, 191–195.
[21] Roig, T., Bartrons, R. and Bermudez, J. (1997) Am. J. Physiol. 273, C473–C478.
[22] Kornberg, A. and Horecker, B.L. (1955) Methods in Enzymology, Academic Press, New York.
[23] Pekovich, S.R., Martin, P.R. and Singleton, C.K. (1996) J. Nutr. 126, 1791–1798.
[24] Mazurek, S., Boschek, C.B. and Eigenbrodt, E. (1997) J. Bioenerg. Biomembr. 29, 315–330.
[25] Ursini, M.V., Parrella, A., Rosa, G., Salzano, S. and Martini, G. (1997) Biochem. J. 323, 801–806.
[26] Pandolfi, P.P., Sonati, F., Rivi, R., Mason, P., Grosveld, F. and Luzzato, L. (1995) EMBO J. 14, 5209–5215.
[27] Cheng, M.L., Ho, H.Y., Liang, C.M., Chou, Y.H., Stern, A., Lu, F.J. and Chiu, D.T.Y. (2000) FEBS Lett. 475, 257–262.