Mitochondrial Oxidative Phosphorylation Is Regulated by Fructose 1,6-Bisphosphate

A POSSIBLE ROLE IN CRABTREE EFFECT INDUCTION?*

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In numerous cell types, tumoral cells, proliferating cells, bacteria, and yeast, respiration is inhibited when high concentrations of glucose are added to the culture medium. This phenomenon has been named the “Crabtree effect.” We used yeast to investigate (i) the short term event(s) associated with the Crabtree effect and (ii) a putative role of hexose phosphates in the inhibition of respiration. Indeed, yeast divide into “Crabtree-positive,” where the Crabtree effect occurs, and “Crabtree-negative,” where it does not. In mitochondria isolated from these two categories of yeast, we found that low, physiological concentrations of glucose 6-phosphate and fructose 6-phosphate slightly (20%) stimulated the respiratory flux and that this effect was strongly antagonized by fructose 1,6-bisphosphate (F16bP). On the other hand, F16bP by itself was able to inhibit mitochondrial respiration only in mitochondria isolated from a Crabtree-positive strain. Using permeabilized spheroplasts from Crabtree-positive yeast, we have shown that the sole effect observed at physiological concentrations of hexose phosphates is an inhibition of oxidative phosphorylation by F16bP. This F16bP-mediated inhibition was also observed in isolated rat liver mitochondria, extending this process to mammalian cells. From these results and taking into account that F16bP is able to accumulate in the cell cytoplasm, we propose that F16bP regulates oxidative phosphorylation and thus participates in the establishment of the Crabtree effect.

In aerobic organisms, glycolysis and oxidative phosphorylation are coordinated to fulfill the cell energy demand. In some conditions, such as glucose addition to the cells, one can observe an increase in glycolytic flux, whereas respiration is inhibited. This has been observed in tumoral cells (1), nontumoral proliferating cells (2), some bacteria (3), and some yeast species (4). In all of these cases, glucose induces a transition to a mostly fermentative metabolism. This phenomenon has been named the “Crabtree effect,” after its discoverer (1).

The physiological events that could clearly explain the occurrence of the Crabtree effect are currently unknown, although many hypotheses have been laid (4–7). It has been proposed, for instance, that it could originate from a competition between mitochondria and glycolytic enzymes for free ADP and inorganic phosphate (5, 8). Indeed, the respiration of isolated mitochondria is decreased in the presence of ADP-consuming systems, such as reconstituted glycolysis or the phosphoglucone/creatine kinase system (5). Nevertheless, after glucose addition, ADP levels remain constant or even increase in yeast (6, 9) and hepatoma cells (7). Furthermore, in both models, there is a transient decrease in cytoplasmic Pi levels (7, 10), pointing to a possible role of Pi, or phosphate potential (ΔGp), in this process.

It has been proposed that one of the short term events leading to the Crabtree effect is an overflow through pyruvate decarboxylase, since it has been observed that in Crabtree-positive yeast strains, its activity increases after a glucose pulse (4). Nonetheless, pyruvate decarboxylase seems to be an important bypass of pyruvate dehydrogenase during oxidative metabolism (11).

It was also proposed that changes in mitochondrial outer membrane permeability could be critical for the regulation of the Crabtree effect (12, 13). From results obtained with reconstituted systems (12) and with mitochondria isolated from potato tubers (13), it was suggested that cytosolic NADH produced by glycolysis could close the voltage-dependent anionic channel and consequently limit the passage of molecules such as ADP toward the intermembrane space. In permeabilized yeast cells, it has been shown that NADH is not involved in the voltage-dependent anionic channel closure and that in situ produced NADH is channeled through voltage-dependent anionic channel to the intermembrane space, where the external NADH dehydrogenases are located (14).

Another possible effector involved in the Crabtree effect is Ca2+ (15). In Ehrlich ascites tumors and in Zajdela hepatoma cells, it has been observed that there is a glucose-induced increase in cytoplasmic calcium levels along with an enhanced mitochondrial uptake of this cation. Inside the mitochondria, Ca2+ would inhibit ATP synthase by enhancing the interaction with IF1, its inhibitory subunit (16). However, it is not clear whether this Ca2+ accumulation is a common event in all Crabtree-positive cells, since in AS-D30 hepatoma cells, cal-

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Results and taking into account that F16bP accumulates in the cell cytoplasm under certain conditions, we propose that F16bP has an effector role in the repression of oxidative metabolism observed in the course of the Crabtree effect.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions—** *S. cerevisiae* (Yeast Foam, an industrial strain) and *C. utilis* (laboratory strain) were used for mitochondria preparation. Cultures were obtained by growing cells in YPL medium (1% yeast extract, 0.1% potassium phosphate, 0.12% ammonium sulfate, supplemented with 2% lactate as carbon source, pH 5.5). Yeast cells were harvested in midlog growth phase for spheroplasts and mitochondria preparation. The laboratory strains W303 1-A (wild type: Mat a, ade 2-1, trp1-1, leu 2-3/112, his 3-11-15, ura 3-1, can 1-100, GAL, SUC2) and YSH 648 (Mat a, ade 2-1, trp1-1, leu 2-3/112, his 3-11-15, ura 3-1, can 1-100, GAL, SUC, tps1::TRP1) (27) were grown in YPGal medium (1% yeast extract, 0.1% potassium phosphate, 0.12% ammonium sulfate, pH 5.5, supplemented with 2% galactose as carbon source) and collected at 1 unit of optical density.

**Spheroplasts and Mitochondria Preparation—** Spheroplasts were obtained according to Avéret et al. (28) and were suspended in buffer A (1 M sorbitol, 1.7 mM NaCl, 0.5 mM EGTA, 10 mM KCl, 1 mM potassium phosphate, 10 mM Tris-HCl, 4 mM iodoacetate, and 1% bovine serum albumin, pH 6.8). Yeast mitochondria were isolated from spheroplasts as described elsewhere (29), and they were suspended in buffer B (0.6 M mannitol, 5 mM MES, 10 mM KCl, 1 mM potassium phosphate, pH 6.8). Rat liver mitochondria were obtained according to Saavedra-Molina et al. (30) from male Wistar rats weighing 180–200 g and suspended in buffer C (250 mM sucrose, 10 mM Tris-HCl, 1 mM EGTA, pH 7.2). Protein determination was done using the biuret method with BSA as a standard.

**Respiration Assay—** The rate of oxygen consumption was measured in a thermostatically controlled chamber at 28 °C equipped with a Clark electrode connected to a recorder. Spheroplasts (1 mg of protein/ml) or mitochondria (0.3 mg of protein/ml) were suspended in buffer A or B, respectively. NADH (1 mM for mitochondria or 10 mM for spheroplasts) or 100 mM ethanol was used as respiratory substrate. For rat liver mitochondria, 5 mM glutamate/malate was used. In order to achieve proper permeabilization, spheroplasts were incubated for 10 min with nystatin (20 μg/ml) at 28 °C before each experiment. For cytochrome c oxidase-mediated respiration, mitochondria (0.3 mg of protein/ml) were incubated in the presence of antimycin (2.5 μg/mg of protein), 2.5 mM ascorbate, and 100 μM N,N,N′,N′′-tetramethyl-p-phenylenediamine. To induce state 3 respiration, ADP was added at the concentration indicated in the figure legends. To determine cellular respiration, galactose-grown cells (wild type W303 1-A and Δtps1) or lactate-grown cells (Yeast Foam and *C. utilis*) collected in midlog phase were placed in an oxygraph.

**Determination of Mitochondrial Complex III (Ubiquinol:Cytochrome c Oxidoreductase) Activity—** Mitochondria (0.3 mg of protein/ml) were incubated in buffer B in the presence of 1 mM KCN, 2 mM ferricyanide, and 100 mM ethanol as respiratory substrate. Since isolated *S. cerevisiae* mitochondria do not have
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...a complex I (31) but rather internal and external NADH dehydrogenases that donate their electrons to the quinone pool, the ferricyanide reduction rate in these conditions is representative of the ubiquinol:cytochrome c oxidoreductase activity. Absorbance changes were followed at 436 nm in a Safas spectrophotometer (Monaco). The rate of ferricyanide reduction was calculated from the slope of absorbance change as a function of time. A molar extinction coefficient (ε) of 0.21 mm⁻¹ cm⁻¹ was used.

Measurement of Mitochondrial Transmembranal Electrical Potential Difference (ΔΨ) by Fluorescent Probe Distribution—ΔΨ was estimated from fluorescent quenching of the lipophilic cationic dye rhodamine 123 (32). Isolated mitochondria (0.3 mg/ml) were incubated in the mitochondrial buffer supplemented with rhodamine 123 (Sigma) (0.5 μg/ml) in the presence of 100 mM EtOH as respiratory substrate. The rhodamine fluorescence signal at each steady state was recorded with a Kontron SFM 25 fluorimeter at 28 °C. The excitation wavelength was 485 nm, and fluorescence emission was continuously collected at 525 nm.

Metabolite Separation and Quantification by HPIC (High Performance Ionic Chromatography)—High Performance Ionic Chromatography (HPIC) was carried out on a DX 500 chromatography work station (Dionex, Sunnyvale, CA) equipped with GP50 gradient pump and ED₁₀₀ electrolytico and UV detectors. System management and data acquisition were controlled through Peaknet 4.3 software (Dionex). Separation and quantification of sugar phosphates was carried out on a CarboPac PA10 column (250 × 4 mm) equipped with a Dionex PA1 guard column according to Ref. 33.

RESULTS

In yeast cells, the addition of large amounts of glucose results in a metabolic shift toward fermentation and in the accumulation of glycolysis hexose phosphates (25, 26). Since accumulation of hexose phosphates occurs concomitantly with an inhibition of respiration, we tested the possibility of a direct role of these intermediates in the regulation of mitochondrial oxidative phosphorylation. Each one of the three glycolysis hexose phosphates (G6P, F6P, and F16bP) was tested. Fig. 1 shows the effect of G6P, F6P, and F16bP on nonphosphorylating respiration. The respiration was stimulated in a concentration-dependent manner in the presence of G6P and F6P. Although the stimulation mediated by G6P was considerable, it should be stressed that cytosolic concentrations from 1 to 6 mM have been reported; thus, higher concentrations of G6P are not physiological, and only the stimulation induced by up to 5 mM G6P is physiologically meaningful. In the presence of F6P, this increase was less important, and for physiological concentrations (less than 1 mM), there was no effect. On the other hand, F16bP, at physiological concentrations (2–10 mM) (25, 26, 34) induced an inhibition of the respiratory rate that reached 25%. This effect is not dependent on the respiratory substrate, since the same effect was also observed when using ethanol as substrate instead of NADH (data not shown). Under phosphorylation conditions, G6P and F6P had no significant effect on the respiratory rate (Fig. 2), whereas the inhibition in the presence of F16bP was still observed (20%). This indicates that the F16bP-mediated inhibition is present regardless of the respiratory state.

In order to determine whether the stimulation mediated by G6P and F6P was due to uncoupling, the mitochondrial transmembrane electrical potential (ΔΨₘ) was assessed in the presence of each glycolysis hexose phosphate. Up to 20 mM, the addition of either G6P or F6P did not change the transmembrane potential value (Table 1). Furthermore, ΔΨₘ did not vary up to 8 mM F16bP and slightly (10 mV) decreased upon the addition of 20 mM F16bP (Table 1). Based on these results, the hypothesis of a possible uncoupling effect induced by G6P and F6P was ruled out. The F16bP-mediated decrease of the respiratory rate in either state, plus the null to slight decrease in ΔΨₘ, strongly suggested that at least one of the mitochondrial respiratory complexes is inhibited.

During the fermentative shift, every hexose phosphate increases in the cytosol. Thus, the mitochondria are in the presence of all of these hexoses at the same time. Therefore, we tested whether the G6P-induced stimulation of the respiratory rate could be inhibited by F16bP. G6P (3 mM)-induced stimulation was reverted by 1.5 mM F16bP (Fig. 3). Moreover, the F16bP (7 mM)-induced inhibition was present (i.e. 25%; 154 nanoatoms of oxygen/min/mg of protein versus 204 nanoatoms of oxygen/min/mg of protein). This indicates that under physiological conditions, the effect of glycolysis hexose phosphates on oxidative phosphorylation is that of F16bP (i.e. an inhibition of the respiratory rate).

As mentioned above and in view of the results observed for flux and ΔΨₘ in the presence of each hexose phosphate (see above), we identified the respiratory chain complex(es) whose activity is affected by these hexose phosphates. Complex IV (cytochrome c oxidase) catalyzes an irreversible step of the respiratory chain and has been identified as an important step for controlling the respiratory fluxes both in phosphorylating and

FIGURE 1. Effect of glycolysis-derived hexose phosphates on the JO₂ of isolated yeast mitochondria under nonphosphorylating conditions. Yeast Foam mitochondria (0.3 mg of protein/ml) were suspended in buffer B (see “Experimental Procedures”) using 1 mM NADH as respiratory substrate, and oxygen consumption was monitored as described under “Experimental Procedures.” Various concentrations of glucose 6-phosphate (■), fructose 6-phosphate (○), and fructose 1,6-bisphosphate (▲) were added as indicated. Results are expressed as mean values ± S.D. (n = 3). natO, nanoatoms of oxygen.
nonphosphorylating conditions (35, 36). Complex IV activity is strongly inhibited by F16bP (Fig. 4). This inhibition, which is similar regardless of the respiratory state, is important at low concentrations and reaches ~30% of inhibition for 5 mM F16bP. Furthermore, G6P and F6P had no effects on cytochrome c oxidase activity (data not shown).

We then assessed complex III (ubiquinol:cytochrome c oxidoreductase) activity in the presence of these glycolysis intermediates. Both hexose monophosphates stimulated the basal activity of complex III (Fig. 5). This activation was similar for both hexose monophosphates, reaching a maximal stimulation at ~150% of the basal value. However, this maximal stimulation was obtained for nonphysiological concentrations of these intermediates, and only a slight stimulation was observed in the presence of physiological (3 mM G6P and 1 mM F6P) concentrations of hexose monophosphates. An F16bP-induced inhibition was also observed on this respiratory complex. Furthermore, hexose phosphate-mediated effects shown in Fig. 5 were sensitive to antimycin A (data not shown), indicating that the ferricyanide reduction rate assessed here was indeed mediated by complex III.

Since hexose monophosphates and F16bP have, respectively, stimulatory and inhibitory effects over complex III activity (Fig. 5), we decided to determine whether these antagonistic effects could be observed on the functionally isolated complex III. Indeed, G6P-mediated stimulation of complex III could be reverted by F16bP, and basal activity was almost restored (Fig. 6).

To this point, we have used mitochondria isolated from S. cerevisiae as an experimental model. However, to extrapolate our results to the in vivo situation, it was necessary to study mitochondria in a more physiological context. One approach that has been used to undertake bioenergetic studies in situ is the use of permeabilized cells (28, 37). Nystatin-permeabilized spheroplasts have been successfully employed to study yeast energetic metabolism (28, 38, 39). To determine the effect of F16bP on the mitochondrial respiratory rate in situ, we evaluated nystatin-permeabilized spheroplast respiration in the presence of this hexose. To avoid F16bP metabolism, we omitted the cofactors required for adequate function of the glycolytic enzymes and added iodoacetate in respiration buffer. Fig. 7 shows that in nystatin-permeabilized spheroplasts, F16bP inhibited the nonphosphorylating respiratory rates by 50%. The range of concentrations used to obtain the maximum inhibition was similar to that employed on isolated mitochondria: 6–10 mM for state 4 and 2–4 mM for state 3 (data not shown). Of note, these concentrations are within the physiological range (25, 26) (see Table 3). The additions of G6P and F6P were also tested. However, an exceedingly high concentration was required to observe a stimulation of respiratory flux (~120 mV) (data not shown), indicating that there might be a constraint for metabolite diffusion toward mitochondria or the intermembrane space. A similar situation was observed in permeabilized cells for NADH in yeast (28) and in hepatocytes (37). In each of these cases, it was found that closure of the voltage-dependent anion channel was responsible for restraining molecule flux to the intermembrane space (14, 40). Since the voltage-dependent anionic channel is closed in permeabilized spheroplasts, it is possible that the hexose monophosphate diffusion is constrained. According to our results, this would also imply that

### Table 1

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<tr>
<th>Hexose Phosphate</th>
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<td>G6P</td>
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<tr>
<td>F6P</td>
<td>178 ± 4</td>
<td>177 ± 5</td>
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<td>180 ± 3</td>
<td>178 ± 3</td>
</tr>
<tr>
<td>F16bP</td>
<td>178 ± 4</td>
<td>177 ± 5</td>
<td>175 ± 3</td>
<td>175 ± 4</td>
<td>176 ± 7</td>
<td>171 ± 6</td>
<td>168 ± 3</td>
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**FIGURE 2. Effect of glycolysis-derived hexose phosphates on the respiratory flux of isolated yeast mitochondria under phosphorylating conditions.** Reaction mixture and respiratory substrate were the same as in Fig. 1 except that 1 mM ADP was added. Respiratory rates were measured after the addition of various concentrations of glucose 6-phosphate (●), fructose 6-phosphate (○), and fructose 1,6-bisphosphate (▲), as indicated. Results are expressed as mean values ± S.D. (n = 3). nato, nanomoles of oxygen.

**TABLE 1**

Mitochondrial transmembranal electrical potential difference in the presence of hexose phosphates

ΔΨ was estimated from fluorescent quenching of the lipophilic cationic dye rhodamine 123 as described under “Experimental Procedures.” ΔΨ was expressed in mV. Results are means ± S.D. of at least three independent experiments.

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<td>F6P</td>
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<td>F16bP</td>
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<td>171 ± 6</td>
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**Oxidative Phosphorylation Regulation by Hexose Phosphates**
F16bP would be specifically channeled through the outer membrane in permeabilized spheroplasts.

Once F16bP had been shown to regulate mitochondrial oxidative phosphorylation in situ, it was necessary to determine respiration of intact cells in conditions where F16bP accumulates. We thus induced the Crabtree effect in galactose-grown cells and in the mutant $\Delta tps1$, which lacks the enzyme trehalose 6-phosphate synthase. Several reports have demonstrated that this mutant overaccumulates glycolysis hexose phosphates, particularly F16bP, in response to glucose addition (24, 41, 42). Spontaneous and uncoupled respiration values were decreased in the $\Delta tps1$ strain (Table 2). Furthermore, when comparing the percentage of spontaneous respiration inhibition for each strain, glucose addition (30 mM) inhibited by 36 and 62% the respiration of wild type and $\Delta tps1$ strains, respectively (Table 2). We assessed the intracellular concentration of each of the considered hexose phosphates after glucose addition. Table 3 shows that in the wild type strain, the intracellular concentration of both G6P and F6P did not significantly change after glucose addition. On the other hand, the concentration of F16bP largely increased. In the mutant strain ($\Delta tps1$), there is a significant increase in the concentration of G6P and F6P and a drastic (10-fold) increase in F16bP concentration. Taking into account our data indicating that F16bP inhibits the respiratory flux of isolated mitochondria, permeabilized spheroplasts, and whole cells, we propose that the accumulation of F16bP is
rate (Fig. 8). This effect is reverted by F16bP. Moreover, it is isolated from F16bP was 10-fold increased (see Table 3). In mitochondria with nystatin (20 μM) in buffer A (nonphosphorylating conditions). The substrate was 10 mM NADH.

**DISCUSSION**

In aerobic organisms, the phosphate potential is maintained both through the glycolytic and respiratory pathways. Glycolysis provides reducing equivalents to the mitochondrial respiratory chain. In the presence of high concentrations of glucose, facultative aerobic organisms shift their metabolism toward fermentation, hence implicating a modification of the glycolysis/respiration balance. The glucose-induced respiration shift toward fermentation associated with respiration inhibition was named the “Crabtree effect.” As mentioned in the Introduction, different hypotheses have been laid out, and no clear mechanisms apply to all Crabtree-positive cells. It has been stressed that this phenomenon is induced by different factors, such as changes in the phosphate potential or in pH (7). Indeed, since glycolytic and respiratory fluxes both depend on the phosphate potential,
it is possible that changes in ATP or P_i could modify the glycolysis/oxidative phosphorylation coordination in aerobic conditions. In this regard, during the Crabtree effect in hepatoma cells (7) and in yeast (10), a glucose-induced decrease of cytoplasmic phosphate has been measured. Moreover, a cytoplasmic accumulation of fructose 1,6-bisphosphate has been determined as well for hepatoma, Ehrlich ascites, and yeast Crabtree-positive cells (7, 25, 45).

Further, in yeast cells, it has been reported that the addition of 100 mM glucose induced an increase in cytosolic F16bP concentration from 2 mM up to 9 mM in a wild type strain (46), which is in good agreement with concentrations presented in this work (see Table 3). Furthermore in a Δtps1 strain, this concentration increases largely upon glucose addition (see Table 3). In mammalian cells and more precisely hepatocytes, this metabolite exhibits a cytosolic concentration lower than 1 mM. However, in hepatoma cells, this concentration is highly increased and reaches values up to 25 mM (42). This is clearly within the range of concentrations used in this study.

Thus, in this paper, we clearly show that the mitochondrial respiratory rate is inhibited by physiological concentrations of F16bP both in nonphosphorylating and phosphorylating conditions. This is true for both isolated mitochondria and permeabilized spheroplasts from the Crabtree-positive yeast *S. cerevisiae*. Whatever the respiratory state, when hexose mono- and bisphosphates are used together, the resulting effect is an inhibition of the respiratory rate by F16bP. We have shown that electron transfer through complex III was slightly inhibited by F16bP and that this metabolite inhibited cytochrome c oxidase activity. These results point to an *in situ*, F16bP-mediated regulation of the mitochondrial respiratory chain.

This effect seems specific to Crabtree-positive yeast species. Indeed, in mitochondria isolated from the Crabtree-negative species *C. utilis*, hexose monophosphates are able to stimulate the respiratory rate, and this stimulation is inhibited by F16bP. However, in this strain, the respiratory rate itself is never inhibited by F16bP. Altogether, these results indicate that in yeast, F16bP probably participates in the Crabtree effect.

We clearly show that F16bP induces an inhibition of the respiratory rate, acting directly on two complexes of the mitochondrial respiratory chain. We propose that *in vivo*, this hexose acts as an inhibitor of the respiratory flux. Indeed, here, we bring to light several elements that sustain this hypothesis. Several reports in the literature show that the respiration of cells that accumulate F16bP to different degrees changes proportionally. It has been shown that the rate of oxygen consumption of the Δhxk2 mutant, lacking isoform 2 of hexokinase, which has lower levels of F16bP, is not inhibited by high glucose concentrations (25, 47). A strain over-expressing phosphofructokinase has lower JO2 values (48). Furthermore, the Δtps1 strain, well known for accumulating hexose phosphates (24, 26, 41, 42), has an enhanced Crabtree effect (see Tables 2 and 3).
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In mammalian cells, we have demonstrated that physiological concentrations of F16bP inhibited the respiration of isolated rat liver mitochondria as well. One of the early theories on the Crabtree effect in cancer cells was the impairment of oxidative phosphorylation (reviewed in Ref. 49). This was challenged by the finding that hepatoma cells maintained their phosphate potential mainly through respiration (50). Our results point to the fact that an impairment of mitochondrial function is not a prerequisite for Crabtree effect induction since the same arrest of respiration caused by F16bP can be observed in mitochondria obtained from a nontumor source. G6P is another hexose phosphate that seems to have an impact on mitochondrial function (51). The presence of this hexose-monophosphate is important for the release of mitochondria-bound hexokinase and for enhancing the Bax-mediated release of cytochrome c (51). Thus, the participation of hexose phosphates as metabolic messengers and their influence on tumor cell oxidative metabolism needs to be further evaluated.

In conclusion, we propose that F16bP acts as a true metabolic messenger, inducing a decrease in respiratory flux during the transition to fermentative metabolism (i.e. participating in the induction of the Crabtree effect). F16bP accumulation must be one of several events (e.g. changes in pH or phosphate potential) that lead to this inhibition of respiration.

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