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Research paper

Resveratrol decreases breast cancer cell viability and glucose metabolism by inhibiting 6-phosphofructo-1-kinase

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ABSTRACT

Cancer cells are highly dependent on glycolysis to supply the energy and intermediates required for cell growth and proliferation. The enzyme 6-phosphofructo-1-kinase (PFK) is critical for glycolysis, and its activity is directly correlated with cellular glucose consumption. Resveratrol is a potential anti-tumoral drug that decreases glucose metabolism and viability in cancer cells. However, the mechanism involved in resveratrol-mediated anti-tumor activity is not entirely clear. In this work, it is demonstrated that resveratrol decreases viability, glucose consumption and ATP content in the human breast cancer cell line MCF-7. These effects are directly correlated with PFK inhibition by resveratrol in these cells. Moreover, resveratrol directly inhibits purified PFK, promoting the dissociation of the enzyme from fully active tetramers into less active dimers. This effect is exacerbated by known negative regulators of the enzyme, such as ATP and citrate. On the other hand, positive modulators that stabilize the tetrameric form of the enzyme, such as fructose-2,6-bisphosphate and ADP, prevent the inhibition of PFK activity by resveratrol, an effect not observed with increased pH. In summary, our results provide evidence that resveratrol directly inhibits PFK activity, therefore disrupting glucose metabolism and reducing viability in cancer cells.

1. Introduction

Energy metabolism is often altered in neoplastic cells [1]. Many studies have shown that cancer cells are highly dependent on glycolytic metabolism [2–4] because this bioenergetic pathway is able to generate metabolic products to support the energy demands of tumor growth and proliferation [5]. Thus, inhibitors of glycolytic metabolism that could reduce the tumoral energy supply are emerging as potential drugs to combat this disease [4,6,7].

The enzyme 6-phosphofructo-1-kinase (EC 2.7.1.11, or PFK) is critical for glycolytic metabolism, catalyzing the transfer of a phosphate from ATP to fructose-6-phosphate (F6P) to produce fructose-1,6-bisphosphate and adenosine diphosphate (ADP). Expression levels of the three different PFK isoforms are tissue-specific: skeletal muscle is unique in that it expresses only PFK-M, whereas all other tissues express distinct levels of the three isoforms [8,9]. This enzyme is characterized by complex regulatory mechanisms, among them allosteric regulation, oligomerization, subcellular localization, interaction with other proteins and covalent modifications [9,10]. The metabolic alterations found in cancer cells have been correlated with changes in the expression pattern and activity of PFK [1], and PFK inhibition can lead to cell death in human breast carcinoma cell lines [4,7] and human breast cancer tissues [11]. Thus, PFK appears to be a promising target for the development of new drugs with anti-tumoral activity.

Resveratrol, a polyphenolic compound found mainly in grapes, can decrease the rate of tumor cell growth [12,13]. This effect may be related to the capacity of resveratrol to reduce glucose uptake and lactate production [14]. However, the mechanism of action of this promising compound is not well understood. Because neoplastic cells depend preferentially on anaerobic metabolism instead of aerobic metabolism, and because PFK is a pacemaker enzyme capable of regulating the glycolytic pathway, we aimed to investigate whether resveratrol modulates survival, glucose metabolism and

Abbreviations: AMP, adenosine monophosphate; ADP, adenosine diphosphate; F6P, fructose-6-phosphate; F2,6BP, fructose-2,6-bisphosphate; PFK, 6-phosphofructo-1-kinase.

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PFK activity in the breast cancer cell line MCF-7. Our results provide further insight into the mechanism of PFK inhibition by resveratrol.

2. Materials and methods

2.1. MCF-7 breast cancer cell culture and assays

The breast cancer cell line MCF-7 was cultured in DMEM supplemented with 4.5 mM glucose, 10% (v/v) fetal bovine serum and 2 mM glutamine (except when indicated that cells were grown in the absence of glutamine) at 37 °C until confluence was achieved. Then, the medium was removed, and cells were seeded in 96-well plates with fresh medium in the absence or presence of different concentrations of resveratrol (1, 5, 15, 50 or 100 μM; Sigma Chemical, St. Louis, MO, USA; ≥99% purity) and incubated for 24 h. Controls were also performed in the absence or presence of 2.5% DMSO to ensure the lack of cytotoxicity of DMSO. After this incubation, the medium was removed and used to evaluate the metabolic effects of resveratrol treatment. Cell viability was assessed by the MTT assay [7]. Glucose consumption was measured using a coupled enzyme system (Glucox 500, Doles Ltda, Brazil) [1]. LDH leakage was determined by measuring the total LDH activity in the culture media based on conversion of NADH to NAD^+ [1]. Lactate production was determined by assessing the lactate content in the culture media through monitoring the formation of NADH in the presence of exogenous LDH and NAD^+ [15]. The intracellular ATP content was measured using a system based on a luciferin-luciferase assay (PerkinElmer ATPLite) with a VICTOR3 multilabel reader (PerkinElmer) [4]. PFK activity was measured spectrophotometrically using a coupled enzyme system that relies on the oxidation of NADH. The basic medium contained 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM F6P, 1 mM ATP, 0.2 mM NADH, 2 U/ml aldolase, 4 U/ml triosephosphate isomerase and 2 U/ml α-glycerophosphate dehydrogenase [4]. Determination of F1,6BP/F6P ratio was performed as described previously [16]. Measurements from cell-based assays were corrected by the number of viable cells.

2.2. Radioassay for PFK activity

Purified PFK activity was assessed through the radiometric method developed by Sola-Penna et al. [17], with the modifications introduced by Zancan and Sola-Penna [18]. Briefly, PFK was assayed in a basic medium containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 1 mM F6P, 1 mM [γ-^{32}P]ATP and 1 μg/ml purified PFK. The pH and the concentrations of substrates, enzyme, and resveratrol may vary depending on the experiment, as indicated in the legends or figures. All reactions were performed at 37 °C with the exception of those shown in Fig. 6 (50 °C). All experiments were performed in the absence or presence of 2.5% DMSO. Controls throughout the work were performed only in the presence of DMSO because the enzyme activity is not affected by this concentration of DMSO. PFK activity was reported in m-units, where 1 m-unit is equal to the formation of 1 nmol of F1,6BP per minute of reaction. PFK was purified from rabbit skeletal muscle, and the purity of the enzyme was 99.9%, such as evaluated by SDS-PAGE (Fig. 1) performed according to [19]. Purified PFK for all experiments was obtained according to the method developed by Kemp [20] with the modifications introduced by Hesterberg et al. [21].

2.3. Intrinsic fluorescence measurements

PFK intrinsic fluorescence measurements were performed as described previously by Marinho-Carvalho [22] and Leite et al. [16]. Measurements were taken in the presence of different enzyme concentrations (0.2, 1 and 5 μg/ml) and in the absence or presence of 15 μM resveratrol. The excitation wavelength was fixed at 280 nm, and the fluorescence emission was scanned from 300 to 400 nm. The centers of mass of the intrinsic fluorescence spectra (c.m.) were calculated using equation (1):

$$c.m. = \frac{\sum \lambda \cdot I_\lambda}{\sum I_\lambda} \quad (1)$$

where λ is the wavelength, and I_λ is the fluorescence intensity at a given λ.

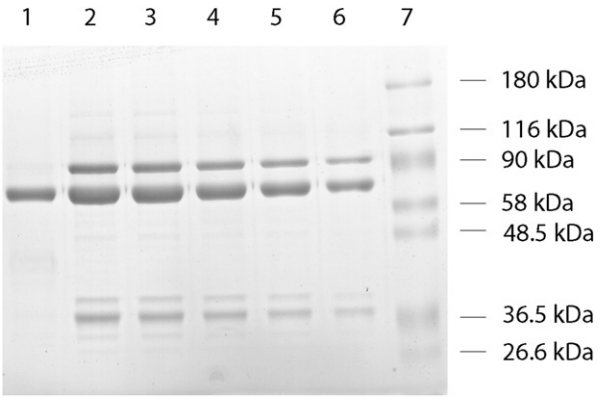

Fig. 1. SDS-PAGE of the purified PFK from rabbit skeletal muscle. SDS-PAGE (8% acrylamide) was performed as described in materials and methods. Lane 1: Final purified PFK. Lanes 2–6: Previous *salting-out* steps. Lane 1: Molecular weight markers used - α_2-macroglobulin from equine serum (180 kDa), β-galactosidase from *E. coli* (116 kDa), Lactoferrin from human milk (90 kDa), pyruvate kinase from rabbit muscle (58 kDa), fumarase from porcine heart (48.5 kDa), lactic dehydrogenase from rabbit muscle (36.5 kDa) and triosephosphate isomerase from rabbit muscle (26.6 kDa). This is a representative gel of a series of purifications presenting the same pattern.

2.4. Statistical analysis and calculations

The results are expressed as means ± standard error. Analyzes of the data and linear and non-linear regression were performed using Sigma Plot software (v. 10.0, Systat Inc., CA, USA) integrated with the software SigmaStat (v. 3.2, Systat Inc. CA, USA). Values for each group were compared by Student's *t*-test, and the differences were considered significant if $P < 0.05$.

Kinetic parameters for ATP effects on PFK were calculated through non-linear regression using the experimental data to fit the parameters of the equation:

$$v = \frac{\dfrac{V_{max}[ATP]^{n1}}{K_{0.5}^{n1} + [ATP]^{n1}} \cdot [ATP]^{n2}}{I_{0.5}^{n2} + [ATP]^{n2}} \quad (2)$$

where v is the PFK activity calculated for a given concentration of ATP ([ATP]), V_{max} is the maximal velocity calculated, $K_{0.5}$ is the affinity constant for the catalytic component of the ATP curve, which is equal to the concentration of F6P responsible for half activation of the enzyme by ATP, $n1$ is the cooperativity index for this catalytic component, $I_{0.5}$ is the affinity constant for the inhibitory component of the ATP curve, which is equal to the concentration of ATP responsible for 50% of the maximal inhibition of the enzyme by ATP, and $n2$ is the cooperativity index for this inhibitory component.

Kinetic parameters for F6P effects on PFK were calculated through non-linear regression using the experimental data to fit the parameters of the equation:

$$v = \frac{V_{max}[F6P]^{n}}{K_{0.5}^{n} + [ATP]^{n}} \quad (3)$$

where v is the PFK activity calculated for a given concentration of F6P ([F6P]), V_{max} is the maximal velocity calculated at saturating concentrations of F6P, $K_{0.5}$ is the affinity constant for F6P, which is equal to the concentration of F6P responsible for half-activation of the enzyme by F6P, and n is the cooperativity index for this phenomenon.

3. Results

Many studies have shown that resveratrol is able to control the growth of neoplastic cells [12,14]. First, we investigated whether resveratrol modulates survival, glucose metabolism and PFK activity in MCF-7 breast cancer cells after 24 h of preincubation with this compound. Fig. 2A shows that resveratrol decreases the reduction of MTT in a concentration-dependent manner. The higher concentration of resveratrol (100 μM) decreases cell viability by approximately 40%. In addition, resveratrol increases lactate dehydrogenase (LDH) leakage in the cell culture media (Fig. 2A), indicating the loss of cell integrity and viability. Thus, these results confirm data from previous literature showing that resveratrol decreases cell viability in the MCF-7 cell line [23]. Seeking to elucidate the mechanisms of these effects, the glucose consumption and lactate production were analyzed in MCF-7 cells cultured in the presence of different concentrations of resveratrol for 24 h Fig. 2B shows that resveratrol decreases glucose consumption in a concentration-dependent manner, resulting in significant effects even at lower resveratrol concentrations such as 1 μM. Interestingly, resveratrol increases lactate production at concentrations higher than 15 μM, which is probably related to increased glutamine oxidation. This hypothesis is confirmed evaluating glucose consumption and lactate production in a glutamine-free culture medium (Fig. 2B). Under this condition, resveratrol also decreases lactate production by MCF-7 cells. It is important to notice that glucose consumption is affected in a similar manner in the presence or the absence of glutamine in the culture medium (Fig. 2B), as well as cell viability (data not shown). Similar to the effects of resveratrol upon the MTT reduction and glucose consumption, this compound also decreases cellular ATP content (Fig. 2C) by approximately 16% at 1 μM resveratrol and 50% at 100 μM resveratrol. To understand the mechanism by which resveratrol affects energy metabolism, we analyzed the activity of PFK, the major glycolytic regulatory enzyme, in the presence of different concentrations of resveratrol. Fig. 2D shows that PFK activity is inhibited by resveratrol in a concentration-dependent manner. Moreover, 100 μM resveratrol decreases the F1,6BP/F6P ratio in ~25%, corroborating that resveratrol intracellularly inhibits PFK. Altogether, the degree of

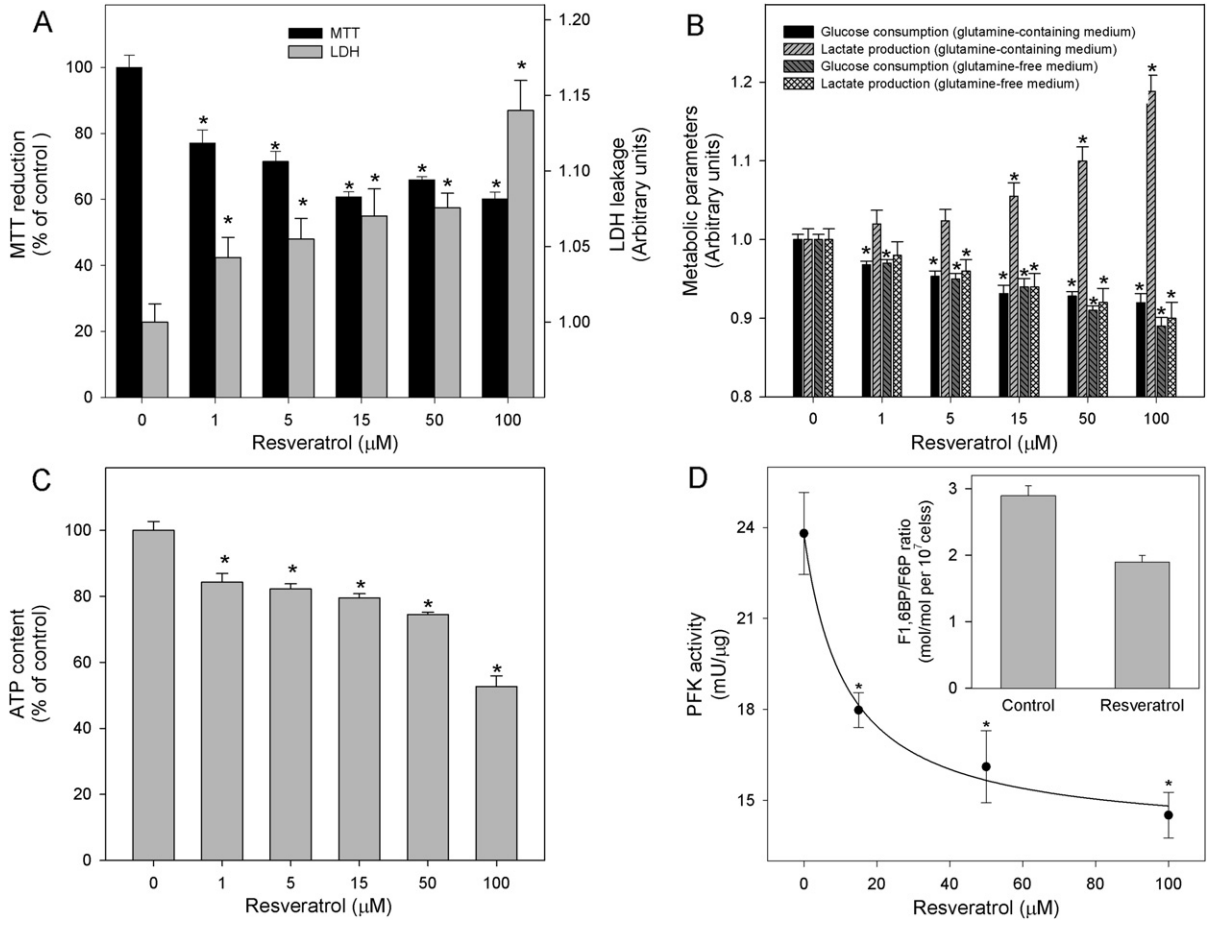

Fig. 2. Effects of different concentrations of resveratrol on MCF-7 cells viability, glucose consumption, lactate production, ATP content and PFK activity. MCF-7 cells were grown in DMEM until confluence. After that, the medium was removed and fresh medium containing the desired concentrations of resveratrol was added and cells were incubated for 24 h under these conditions. Controls were done in the presence of DMSO. Treated cells were used to evaluate: Panel A, viability by MTT reduction (black bars) and LDH leakage (gray bars); panel B, glucose consumption (black bars) and lactate production (gray bars); panel C, intracellular ATP content; panel D, PFK activity; panel D *inset*, F1,6BP/F6P ratio. The absolute glucose consumption rate in the absence of resveratrol was 31.3 ± 0.8 nmol/h × 10^7 cells. The absolute lactate production rate in the absence of resveratrol was 10.6 ± 0.3 nmol/h × 10^7 cells. The absolute value for ATP content in the absence of resveratrol was 17.3 ± 1.1 nmol/10^7 cells. The absolute amount of F1,6BP was 2.1 ± 1.5 nmol/10^7 cells and of F6P was 0.7 ± 0.1 nmol/10^7 cells. The experimental procedures are described in material and methods. Data represent the mean ± standard error of 4–8 independent experiments (n = 4–8). *$P < 0.05$ compared with respective control in the absence of resveratrol (Student's t-test). The origin of the ordinates is suppressed in panels B and D.

PFK inhibition by resveratrol is highly correlated with decreased cell viability, glucose consumption and intracellular ATP content, suggesting PFK as a target of resveratrol in MCF-7 cells.

Our group has previously shown that other drugs that cause these effects, such as clotrimazole [24] and acetylsalicylic acid [7], can directly inhibit PFK, glucose consumption and cell viability in MCF-7 cells. Thus, to investigate whether resveratrol could be acting directly on PFK, the activity of the purified enzyme was analyzed in the presence of different concentrations of this compound. Fig. 3 shows that resveratrol inhibits the activity of PFK in a concentration-dependent manner, with approximately 50% inhibition of total enzyme activity achieved in the presence of 15 μM of the polyphenol. This concentration was used for subsequent experiments because it resulted in the greater degree of inhibition. These experiments were performed without pre-incubation of the enzyme with resveratrol, demonstrating the rapid effect of this inhibitor.

To investigate whether the inhibitory effect of resveratrol could be due to alterations in the kinetic parameters of PFK, we measured the enzyme activity in the presence of different concentrations of its substrates, ATP and F6P. Resveratrol inhibits PFK activity at all concentrations of F6P tested (Fig. 4A). PFK is also inhibited by resveratrol at all concentrations of ATP (Fig. 4B). Moreover, for the F6P substrate, resveratrol increases the Km (from 0.14 ± 0.01 to 0.22 ± 0.02 mM; $P < 0.05$), thus decreasing the affinity, and also decreases the V_{max} (from 65 ± 3 to 33 ± 3 mU/μg; $P < 0.05$) without altering the cooperativity index (from 1.5 ± 0.4 to 1.1 ± 0.3) (Fig. 4A). In relation to ATP, resveratrol also decreases the affinity of the PFK catalytic site for this substrate, as supported by the increase in $K_{0.5}$ (from 0.34 ± 0.01 to 0.40 ± 0.01; $P < 0.05$); without modifying the cooperativity index for the stimulatory component of the enzyme (1.3 ± 0.1 and 1.5 ± 0.1 for control and resveratrol, respectively). However, despite the significantly reduced apparent maximal velocity of PFK (from 65 ± 3 to 36 ± 2 mU/μg; $P < 0.05$), resveratrol does not alter either the affinity for the inhibitory component, $I_{0.5}$, (1.9 ± 0.1 and 2.2 ± 0.1 mM for control and resveratrol, respectively) or the cooperativity index (3.0 ± 0.4 and 4.0 ± 0.3 for control and resveratrol, respectively) of PFK for ATP (Fig. 4B).

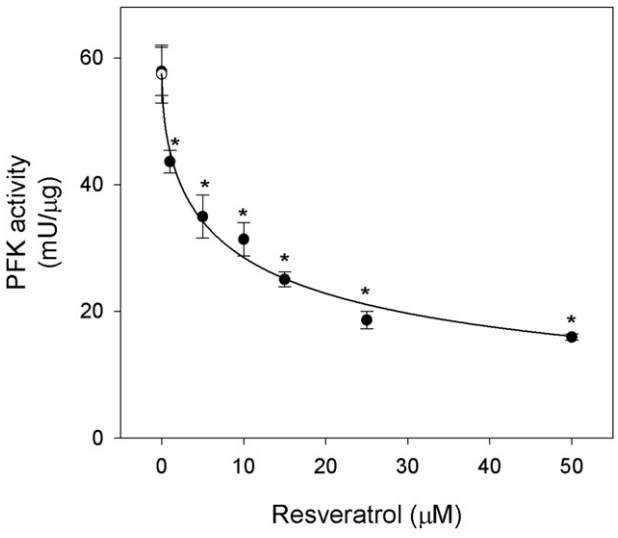

Fig. 3. Effects of different concentrations of resveratrol on purified PFK activity. The PFK activity (1 μg/ml) was assayed in the absence (filled circle, control) or presence of 1, 5, 10, 15, 25 or 50 μM of resveratrol (filled circles) at pH 7.4. The PFK activity was measured as described in material and methods. *$P < 0.05$ compared to control (Student's *t*-test). Values are means ± standard error of six independent experiments ($n = 6$).

The PFK concentration influences the oligomerization state of the enzyme. Lower concentrations of PFK favor the formation of dimers, which are quite inactive, while higher concentrations stabilize the tetrameric form, which is fully active [9,16,25]. In previous work, it was shown that the strong inhibitory effects of lactate and acetylsalicylic acid on PFK are attenuated when the enzyme concentration is increased [3,7]. Therefore, the inhibitory effect of these substances may be due to dissociation of the PFK from the tetrameric (higher specific activity) to the dimeric (lower specific activity) form. After observing that resveratrol directly inhibits PFK activity, we sought to investigate the mechanism by which this effect was occurring. First, we analyzed the effect of 15 μM resveratrol on the activity of PFK in the presence of increasing concentrations of the enzyme (0.2, 1, 5, 10 and 15 μg/ml). Fig. 5 shows that resveratrol markedly inhibits PFK activity at low enzyme concentrations (0.2 μg/ml). However, increasing the enzyme concentration decreases the inhibition caused by resveratrol, and this inhibitory effect is completely abolished at 10 μg/ml enzyme. This effect correlates with the tetramerization and increased enzyme stability at high enzyme concentrations, as previously shown in other studies [3,7,26]. In addition, we evaluated the effects of resveratrol on the intrinsic fluorescence spectrum of the PFK in order to verify whether resveratrol modulates the PFK oligomeric structure. The results show that resveratrol increases the center of mass of the PFK intrinsic fluorescence spectrum, especially at lower enzyme concentrations (0.2 μg/ml) (Fig. 6). However, increasing the concentration of PFK to 5 μg/ml prevents this resveratrol-induced effect. The results shown in Fig. 6 are highly correlated with the data shown in Fig. 5, suggesting that a high enzyme concentration increases the formation and stabilization of more complex oligomeric forms of the enzyme (Fig. 6), thus preventing the inhibitory effects of resveratrol (Fig. 5).

Because high temperature can be used to dissociate the enzyme, and hence destabilize it and reduce its activity [24], we evaluated the effect of resveratrol (15 μM) on the activity of PFK (1 μg/ml) pre-incubated at 50 °C for different periods of time (Fig. 7). Resveratrol is able to inhibit PFK activity by approximately 50% at 37 °C (time 0). In either the absence or presence of resveratrol, PFK activity is lost over time during incubation at 50 °C due to the denaturation of the enzyme. However, in the presence of resveratrol, there is marked inhibition of PFK activity, as can be better observed when the absolute values of activity (Fig. 7A) are expressed as relatives values, with the activity of the enzyme at room temperature set as 100% (Fig. 7B). These results, together with Figs. 5 and 6, suggest that resveratrol may interact directly with PFK, resulting in a synergistic effect with temperature-mediated enzyme destabilization. Furthermore, these results indicate that the formation of inactive dimers may be the mechanism by which resveratrol mediates PFK inhibition.

PFK loses most of its regulatory properties at pH 8.2, when the tetrameric conformation of the enzyme is favored and the enzyme functions at its maximal velocity. In contrast, a lower pH renders the enzyme more susceptible to inhibition by allosteric regulators [3,9,10,27]. Thus, the effect of resveratrol on the downregulation of PFK activity was analyzed at different pH values. Surprisingly, and in contrast to previous studies demonstrating that PFK is not susceptible to inhibitors at higher pH [3,9], our results show that resveratrol inhibits PFK activity at all pH values above 6 (Fig. 8). In addition, even at pH values at which the enzyme reaches its maximum velocity (pH 8.0 and 8.5), resveratrol maintains its inhibitory effect on enzyme activity (Fig. 9). This result suggests that resveratrol could be modulating PFK activity and, consequently, the energy supply to cancer cells even over a large range of intracellular pH values.

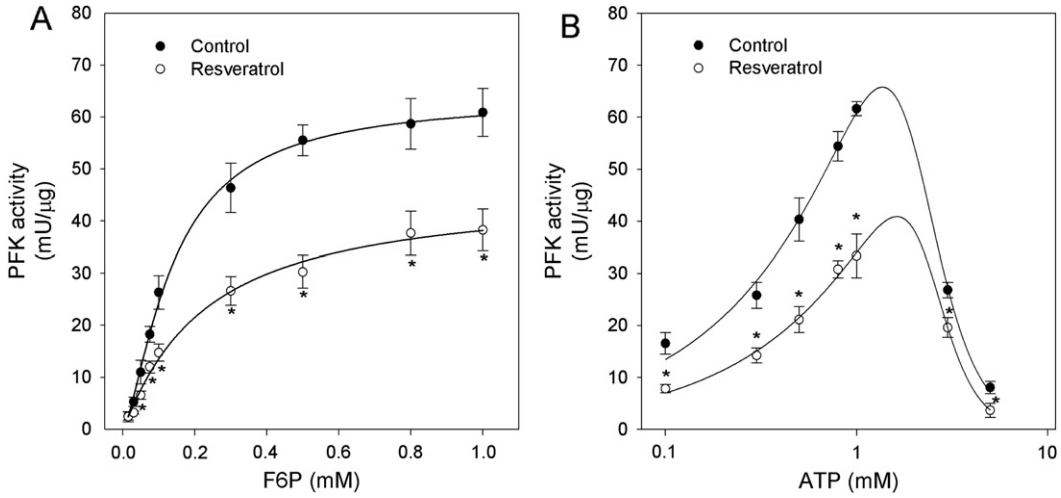

Fig. 4. Effects of resveratrol on purified PFK activity assessed at different concentrations of F6P or ATP. The PFK activity (1 μg/ml) was assayed in the absence (filled circles, control) or presence of 15 μM of resveratrol (white circles) in the presence of different concentrations of the substrates of enzyme at pH 7.4. Panel A: PFK activity in the presence of varying concentrations of F6P (0.025, 0.05, 0.075, 0.1, 0.3, 0.5, 0.8, 1.0 and 2.0 mM). Panel B: PFK activity in the presence of varying concentrations of ATP (0.1, 0.3, 0.5, 0.8, 1.0, 3.0 and 5.0 mM). The PFK activity was measured as described in material and methods. $^*P < 0.05$ compared to control (Student's *t*-test). Values are means ± standard error of five to seven independent experiments ($n = 5–7$).

The effect of pH prompted us to test if the enzyme could respond to other well-characterized negative and positive regulators of PFK. This enzyme is positively modulated by ATP (at concentrations below 1 mM), fructose-2,6-bisphosphate (F2,6BP), phosphorylation, adenosine monophosphate (AMP), ADP and F-actin. In addition, ATP (at concentrations greater than 1 mM), citrate and lactate function as negative modulators of the enzyme [9,10,16]. Therefore, we evaluated the effects of resveratrol on PFK activity in the presence of different positive and negative modulators of the enzyme. Resveratrol inhibits PFK activity by approximately 50% (Fig. 9), in accordance with the effect observed previously for 15 μM resveratrol (Fig. 2). However, the inhibitory effect of resveratrol is attenuated by the presence of 100 nM F2,6BP. Furthermore, F-actin (50 μg/ml) and ADP (10 mM) are able to partially reverse the resveratrol-mediated inhibition of PFK. ATP (5 mM), citrate (10 mM) and lactate (10 mM) inhibit PFK activity (Fig. 9). In addition to these effects, resveratrol is able to increase the degree of inhibition by citrate and ATP (Fig. 9). Together, these results show that resveratrol can modulate the activity of PFK independently of some positive allosteric factors, such as F-actin and ADP and that the presence of some negative regulatory factors may have additional effects on resveratrol-mediated PFK inhibition.

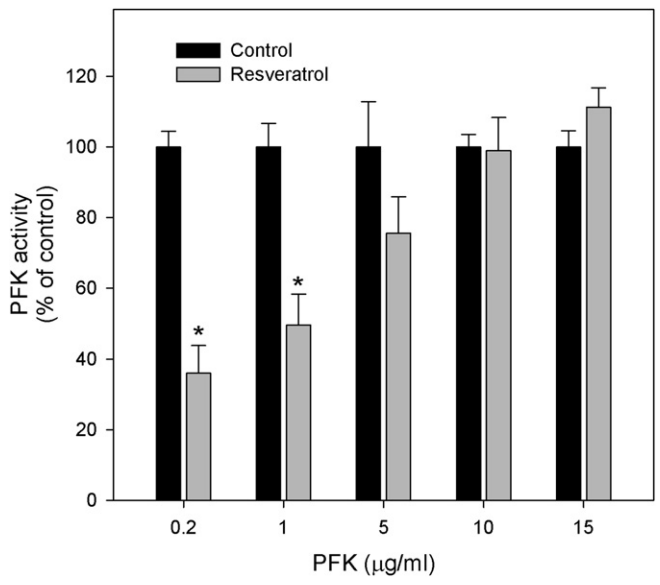

Fig. 5. Effects of resveratrol on purified PFK activity assessed at different concentrations of the enzyme. The PFK activity was assayed in the presence of different enzyme concentrations (0.2, 1, 5, 10 or 15 μg/ml) in the absence (black bars, control) or presence of 15 μM of resveratrol (gray bars) at pH 7.4. The PFK activity was measured as described in material and methods. Values are means ± standard error of seven to nine independent experiments ($n = 7–9$) and PFK activity for each enzyme concentration was considered as 100%. $^*P < 0.05$ compared to respective control (Student's *t*-test).

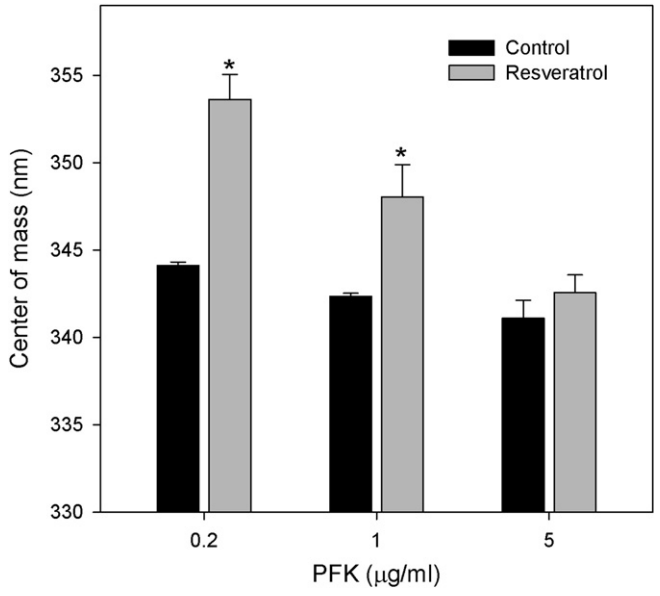

Fig. 6. Effects of resveratrol on the center of mass of PFK intrinsic fluorescence spectra measured at different enzyme concentrations. The center of mass was determined in the presence of different enzyme concentrations (0.2, 1 or 5 μg/ml) in the absence (black bars, control) or presence (gray bars) of 15 μM of resveratrol at pH 7.4. The center of mass was measured as described in material and methods. Values are means ± standard error of four independent experiments ($n = 4$). $^*P < 0.05$ compared to respective control (Student's *t*-test).

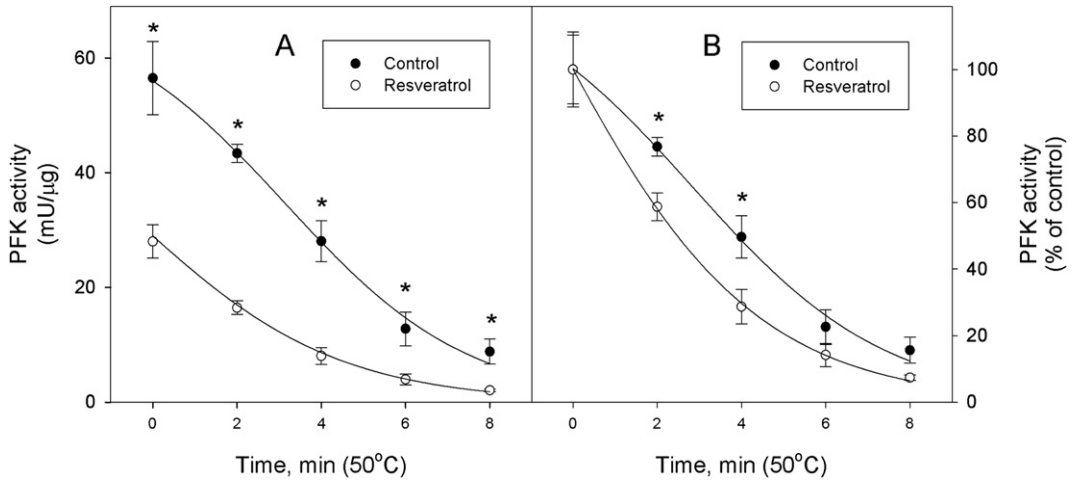

Fig. 7. Effects of resveratrol upon the time course of thermal inactivation of purified PFK. PFK (1 μg/ml) was pre-incubated at 50 °C in the absence (filled circles, control) or presence of 15 μM of resveratrol (empty circles) at pH 7.4. At the times indicated on abscissa, aliquots were withdrawn and the PFK activity was measured as described in material and methods. Panel A and panel B represents the absolute and relative PFK activity, respectively. PFK activity without pre-incubation (time 0) was considered as 100% in panel B. Values are means ± standard error of six independent experiments ($n = 6$). $^{*}P < 0.05$ compared to resveratrol at the same time of pre-incubation (Student's *t*-test).

4. Discussion

Cancer cells are highly dependent on glucose metabolism to maintain high levels of glycolytic intermediates and to meet the additional requirements for growth, division and proliferation [2,4,5]. This dependence of cancer cells on glucose metabolism, even in the presence of oxygen, is known as the Warburg Effect. Recent studies have provided evidence that inhibition of PFK, the key glycolytic enzyme, may control cell viability in cancer cells [7,24,28,29]. Although the mechanisms mediating these effects are still not clear, PFK inactivation is directly correlated to apoptosis induction, reduced glycolysis and a reduction in the cellular ATP supply [30].

Resveratrol is a phytoalexin that has been demonstrated to possess a wide variety of biological activities, including longevity, antidiabetic and anticancer properties [12,31]. Recently, it was demonstrated that resveratrol increases cyclic AMP levels by competitively inhibiting phosphodiesterases (PDE), thus leading to the activation of AMP-activated protein kinase (AMPK) and ameliorating aging-related metabolic phenotypes [32]. In addition, resveratrol has received wide attention for its ability to induce apoptotic cell death in many different cancer cell lines [33–35]. However, its mechanism of action is still unclear.

Here, we have shown that resveratrol decreases MCF-7 breast cancer cell viability, glucose consumption, intracellular ATP content and PFK activity (Fig. 2). Curiously, resveratrol promotes increased lactate production due to the augmented oxidation of glutamine (Fig. 2). This whole picture, i.e. diminished glucose consumption and augmented lactate production due to an increased glutamine

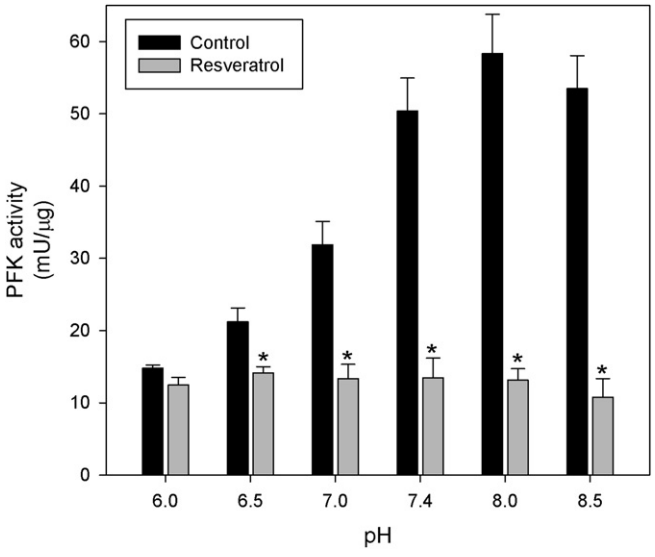

Fig. 8. Effects of resveratrol on purified PFK activity assessed at different pHs. The PFK activity (1 μg/ml) was assayed in the absence (black bars, control) or presence (gray bars) of 15 μM of resveratrol at different pHs (6.0, 6.5, 7.0, 7.4, 8.0 or 8.5). The PFK activity was measured as described in material and methods. Values are means ± standard error of six independent experiments ($n = 6$). $^{*}P < 0.05$ compared to respective control (Student's *t*-test).

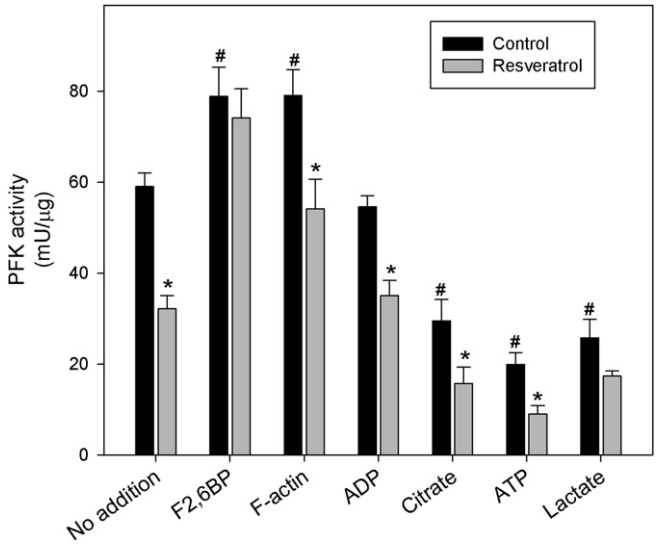

Fig. 9. Effects of resveratrol on purified PFK activity assessed in the presence of different negatives and positives enzyme regulators. The effects of positive modulators (100 nM F2,6BP, 50 μg/ml F-actin or 10 mM ADP) and negative modulators (10 mM citrate, 5 mM ATP or 10 mM lactate) upon PFK activity (1 μg/ml) was assayed in the absence (black bars, control) or presence (gray bars) of 15 μM of resveratrol at pH 7.4. The PFK activity was measured as described in materials and methods. Values are means ± standard error of seven independent experiments ($n = 7$). $^{*}P < 0.05$ compared to respective control in the absence of resveratrol (Student's *t*-test). $^{\#}P < 0.05$ compared to control in the absence of resveratrol (Student's *t*-test).

oxidation, has been reported as a consequence of activation of hedgehog signaling pathway [36], which has been described to be activated by resveratrol [37]. These metabolic alterations are directly correlated with the induction of apoptosis [30,37]. Furthermore, with the exception of increased lactate production, these effects were observed in the presence of low resveratrol concentrations (2.5–10 μM) similar to those used in other studies [38,39].

Previous work from our group has shown that clotrimazole, an antifungal agent, and acetylsalicylic acid, an anti-inflammatory drug, modulate cancer cell viability by directly targeting PFK [4,7,24,28]. In addition, clotrimazole disrupts glycolysis in human breast cancer without affecting non-tumoral tissues [11]. In the present study, we found that resveratrol also specifically inhibits purified PFK in a concentration-dependent manner (Fig. 3). The specificity for resveratrol inhibition of PFK is even stronger, as evidenced by the observation that the inhibitory concentrations are similar in the cell culture (Fig. 2D) and for the purified PFK (Fig. 3). However, the intracellular concentrations of resveratrol were not determined.

Cancer cells express all isoforms of PFK [1], while skeletal muscle cells express only PFK-M [21], and once translated, PFK monomers can rapidly associate into homo- or heteroligomers [21,27]. Thus, the results suggest that PFK-M is a possible target of resveratrol. However, it is difficult to know if resveratrol could specifically modulate the other isoforms (L and P) given the rapid formation of heteroligomers composed of M, L and P isoforms.

PFK can be regulated by the oligomeric transition between tetramers and dimers, which are much less active than tetramers. This enzyme can also be negatively regulated by physiological effectors such as ATP (>1 mM), citrate and lactate. Some drugs also negatively modulate the enzyme by promoting dissociation of tetramers into dimers [7,24,28]. However, these inhibitors are counteracted by positive regulators such as F2,6BP and ADP [3,22,26,29,40]. Our results suggest that resveratrol also downregulates PFK through dissociation of the enzyme tetramers into dimers, as detected by the increased center of mass of the PFK intrinsic fluorescence spectrum (Fig. 6). In agreement with these results, resveratrol has an additional effect on PFK inhibition by ATP (>1 mM) (Fig. 9), citrate (Fig. 9) and high temperature (50 °C) (Fig. 7), all of which accelerate the loss of enzyme activity by promoting dimerization. However, increasing the concentration of the enzyme, which favors the formation of tetramers, prevents the PFK inhibition caused by resveratrol. In addition, it is interesting that resveratrol inhibits PFK even at higher pH values (Fig. 8) because we expected that these conditions (pH 8.0 and 8.5) would stabilize more complex oligomeric forms of the enzyme and thereby prevent inhibition. Thus, resveratrol may prevent oligomerization of the enzyme and reduce its maximal activity. This effect suggests that resveratrol could act over the wide range of intracellular pH values observed in tumors [41].

Altogether, our results indicate that resveratrol decreases cell viability in the human breast cancer cell line MCF-7 by reducing glucose consumption and ATP content, an effect that could be related to PFK inhibition. This relationship is supported by results demonstrating that resveratrol directly inhibits the activity of purified PFK, thus suggesting a new target in the mechanism through which resveratrol induces its antitumorigenic effects.

Author contributions

Lilian Sales Gomez performed most of the experiments with the purified enzyme. Patricia Zancan and Mariah C. Marcondes performed the cell culture experiments. Livia Ramos-Santos performed some of the experiments with the purified enzyme. Mauro Sola-Penna and José Roberto Meyer-Fernandes designed the study and were involved in the analysis and interpretation of the results. Daniel da Silva designed the study performed the experiments with the purified enzyme was involved in the analysis and interpretation of the results and wrote the manuscript.

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