BIOCHEMICAL PHARMACOLOGY 77 (2009) 46–53

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Acetylsalicylic acid and salicylic acid decrease tumor cell viability and glucose metabolism modulating 6-phosphofructo-1-kinase structure and activity

Guilherme A. Spitz, Cristiane M. Furtado, Mauro Sola-Penna, Patricia Zancan *

Laboratório de Oncobiologia Molecular (LabOMol) and Laboratório de Enzimologia e Controle do Metabolismo (LabECoM), Departamento de Fármacos, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941-590, RJ, Brazil

ARTICLE INFO

Article history:
Received 25 August 2008
Accepted 15 September 2008

Keywords:
Cancer
NSAID
Metabolism
Glycolysis
Phosphofructokinase
MCF-7
Viability

ABSTRACT

The common observation that cancer cells present higher glycolytic rates when compared to control cells leads to the proposal of glycolysis as a potential target for the development of anti-tumoral agents. Anti-inflammatory drugs, such as acetylsalicylic acid (ASA) and salicylic acid (SA), present anti-tumoral properties, inducing apoptosis and altering tumor glucose utilization. The present work aims at evaluating whether ASA could directly decrease cell glycolysis through inhibition of the major regulatory enzyme within this pathway, 6-phosphofructo-1-kinase (PFK). We show that ASA and SA inhibit purified PFK in a dose-dependent manner, and that this inhibition occurs due to the modulation of the enzyme quaternary structure. ASA and SA promote the dissociation of the enzyme active tetramers into quite inactive dimers, a common regulatory mechanism of this enzyme. The inhibitory effects of ASA and SA on PFK are fully reversible and can be prevented or reverted by the binding of the enzyme to the actin filaments. Both drugs are also able to decrease glucose consumption by human breast cancer cell line MCF-7, as well as its viability, which decrease parallelly to the inhibition of PFK on these cells. In the end, we demonstrate the ability of ASA and SA to directly modulate an important regulatory intracellular enzyme, and propose that this is one of their mechanisms promoting anti-tumoral effects.

1. Introduction

Cancer cells are highly dependent on glycolysis even under normoxia, in a phenomenon called ''Warburg effect'' [1]. Glycolytic inhibitors, depriving cancer cells for their energy supply, are under discussion for a new class of anticancer drugs [2–6]. It has been shown that classical anticancer drugs, such as vinblastine [7], vincristine [8] and paclitaxel [9], also modulate glycolytic enzymes activity and cellular distribution, contributing to their effects on tumor cells viability. Other drugs with potential anticancer activity, such as the antifungal clotrimazole, have been demonstrated to decrease cancer cells viability through inhibition of glycolytic enzymes [3,10,11,4].

Acetylsalicylic acid (ASA), the acetylated derivative of salicylic acid (SA), is one of the leading non-steroidal anti-inflammatory drugs, which can behave as an apoptosis-inducing agent in animal cancer cells [12,13]. The anticancer action of ASA has been attributed to it effects on cyclooxygenases (COx) [13]. However, COx negative colon cancer cell line SW480 undergoes apoptosis upon ASA action, revealing that other mechanisms different from COx inhibition may also be triggered by ASA, resulting in cell apoptosis [14].

* *Corresponding author*. Tel.: +55 21 2260 9192x215.
E-mail address: pzancan@ufrj.br (P. Zancan).

Abbreviations: ASA, acetylsalicylic acid; SA, salicylic acid; PFK, 6-phosphofructo-1-kinase, phosphofructokinase.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.09.020

Recently, it has been demonstrated that apoptosis is closely related to glycolysis, since the pro-apoptotic protein BAD associates to hexokinase [15] and to 6-phosphofructo-1-kinase (PFK, phosphofructokinase) [16], playing a crucial role on these enzymes modulation by external signals. Conversely, anti-apoptotic signals that modulate BAD increase the glycolytic rate through BAD-mediated activation of PFK [16]. Moreover, inhibition of PFK activity in human breast cancer cell line MCF-7 decreases the cells viability inducing apoptosis [3]. The present study aims at evaluating the effects of ASA and SA on surviving, glucose consumption and PFK activity of tumor cells, as well as on the purified enzyme.

2. Materials and methods

2.1. Materials

ATP, ADP and F6P were purchased from Sigma–Aldrich Brasil Ltda (São Paulo, SP, Brazil). The human breast cancer cell line MCF-7 was obtained from Cell Bank of Hospital Universitário Clementino Fraga Filho, UFRJ, Brazil, and maintained in Dubelcco's modified Eagle's medium (DMEM, Gibco, Invitrogen, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Invitrogen, USA). Other chemicals were of the highest purity available. [^{32}P]H_2PO_4 were purchased from Instituto de Pesquisa em Energia Nuclear (IPEN, Brazil) and were used to prepare [γ-^{32}P]ATP as described elsewhere [17]. PFK was purified from rabbit skeletal muscle according to Ref. [18], with the modification introduced by Ref. [19]. Muscle homogenates were prepared according to Ref. [20] and erythrocytes membrane according to Ref. [21]. All protein concentration determinations were performed according to Ref. [22].

2.2. Cell viability assay, glucose consumption and lactate production

MCF-7 cells were grown in DMEN medium supplemented with 10% FBS until confluence was achieved. The medium was removed and fresh medium containing the desired concentrations of ASA or SA was added and cells were returned to the incubator for 24 h. After this incubation, the medium was removed and used to evaluate the glucose consumption and lactate production, while the remaining cells were used for cell viability evaluation through MTT assay. Glucose consumption was performed as described previously [23,3], assessing the glucose content that remained in the culture media using a coupled enzyme system containing glucose oxidase/catalase (Glucox 500, Doles Ltda, GO, Brazil). Lactate production was evaluated as described previously [3] assessing the lactate content in the culture media incubating the media in the presence of lactate dehydrogenase and NAD^+, and measuring the formation of NADH spectrophotometrically at 340 nm. As for MTT assay, cells were washed twice with PBS and 20 μl of 5 mg/ml MTT reagent (3,4,5-dimethiazol-2,5-diphenyltetrazolium bromide, Sigma–Aldrich Co., MA, USA) was added. After 3 h at the incubator, the reagent was removed and the formazan crystals formed were dissolved in 200 μl DMSO. Afterwards, the formazan content was evaluated in a microplate reader set at 560 nm and subtracting the background at 670 nm.

2.3. Radioassay for PFK activity

PFK activity was assessed through the radiometric method developed by Sola-Penna et al. [24] with the modifications introduced by Zancan and Sola-Penna [25,26]. Briefly, PFK was assayed in a 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, otherwise specified, 5 μg/ml purified PFK. Reaction was initiated by the addition of the enzyme and aliquots were withdrawn at increasing reaction times and transferred to tubes containing activated charcoal in 0.1 M HCl to interrupt the reaction and adsorb the unreacted [γ-^{32}P]ATP. After centrifugation, [γ-^{32}P]ATP-adsorbed activated charcoal was pelleted, and aliquots of the supernatant containing [1-^{32}P]fructose-1,6-bisphostate (F1,6BP) were withdrawn and the radioactivity evaluated in a liquid scintillation counter. Blanks in the absence of F6P were run in parallel and subtracted from the data to correct the ATP hydrolysis. The amount of F1,6BP (nmol) measured in each condition was plotted against reaction time, and the linear coefficient of the curve was used to express PFK activity (mU).

2.4. Spectrophotometric assay for PFK activity

PFK activity was assayed as described previously [20] in a medium containing: 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 1 mM fructose 6-P, 1 mM ATP, 0.5 mM NADH, 2 mU/ml aldolase, 2 mU/ml triosephosphate isomerase, 2 mU/ml α-glycerophosphate dehydrogenase and 50 μg/ml of protein in a final volume of 200 μl. Other reagents used are indicated for each experiment. Reaction was started by the addition of protein and NADH oxidation was followed by measuring the decrease in absorbance at 340 nm in a microplate reader. Blanks in the absence of the coupled enzymes were performed to control non-specific NADH oxidation.

2.5. Intrinsic fluorescence measurements

PFK intrinsic fluorescence measurements were performed as described previously [27], in the same conditions described for the radioassay. Excitation wavelength was fixed at 280 nm and fluorescence emission was scanned from 300 to 400 nm. The center of mass of the intrinsic fluorescence spectra (c.m.) was calculated using:

$$\text{c.m.} = \frac{\sum \lambda I_\lambda}{\sum I_\lambda}$$

where λ is the wavelength and I_λ is the fluorescence intensity at a given λ. Center of mass is used to evaluate PFK oligomeric state, since the dissociated enzyme presents its tryptophans more exposed to the aqueous milieu and thus the fluorescence emitted by these tryptophans is of lower energy. Consequently, the center of mass of a population of tetramers is smaller than the parameter measured for a population of dimers, which has been confirmed in many recent publications [4,23,27–30].

2.6. Statistical analyses

Statistical analyses and non-linear regression were performed using the software SigmaPlot 10.0 integrated with SigmaStat

3.1 packages (Systat, CA, USA). Otherwise indicated, Student's *t*-test was used. *P* values ≤ 0.05 were used to consider statistically different mean values.

3. Results

Due to the reported apoptotic effects of ASA on some cancer cells [12,14], we decided to evaluate the effects of ASA, as well as its precursor SA, on the viability of human breast cancer cell line MCF-7. Both drugs decrease MCF-7 cells viability in a dose-dependent manner, in spite of presenting distinct effectiveness (Fig. 1A). It can be observed that SA is more potent than ASA decreasing cells viability, since 1 mM SA diminishes MCF-7 cells viability and similar effects are observed only with 4 mM ASA (Fig. 1A). Moreover, this difference in effectiveness remains up to 10 mM, when both compounds present similar effects on MCF-7 cells viability (Fig. 1A). These results are comparable to the effects of ASA and SA on MCF-7 glucose consumption, where, although, in a lesser extent, SA is also more effective than ASA inhibiting MCF-7 glucose consumption (Fig. 1B). Here, differences between the two drugs are observed only up to 2 mM, since at higher concentrations both compounds present similar inhibitory effects on glucose consumption by the cells (Fig. 1B). Similar results are observed assessing lactate production (Fig. 1C), suggesting that the glycolytic flux is inhibited by the drugs. Furthermore, the major glycolytic regulatory enzyme, PFK, is also inhibited by ASA and SA when MCF-7 cells are incubated in the presence of the compounds and PFK activity is measured 24 h after incubation (Fig. 1D). Altogether, these results confirm that ASA and SA affect cancer cell viability and support evidences that they inhibit cell PFK as well as the whole glucose utilization.

Recently we have observed similar effects of those presented in Fig. 1 promoted by another compound, the antifungal clotrimazole, which also decreased MCF-7 cell viability, glucose utilization and PFK activity [3]. After that, we demonstrated that the effects of clotrimazole occurred through a direct action of the compound over PFK, altering its quaternary structure, intracellular distribution and catalytic activity [4]. This encouraged us to investigate the effects of ASA and SA directly on the purified PFK activity and quaternary structure. Initially, we evaluated the effects of ASA directly on PFK activity, assessed through the direct radiometric method [24], which does not use auxiliary enzymes and allows the observation of the effects on PFK without interference of other proteins (Fig. 2). Similarly to the whole cell system, ASA inhibited purified PFK in a dose–response manner, presenting an $I_{0.5}$ of 2.3 ± 0.2 mM (Fig. 2A). Although

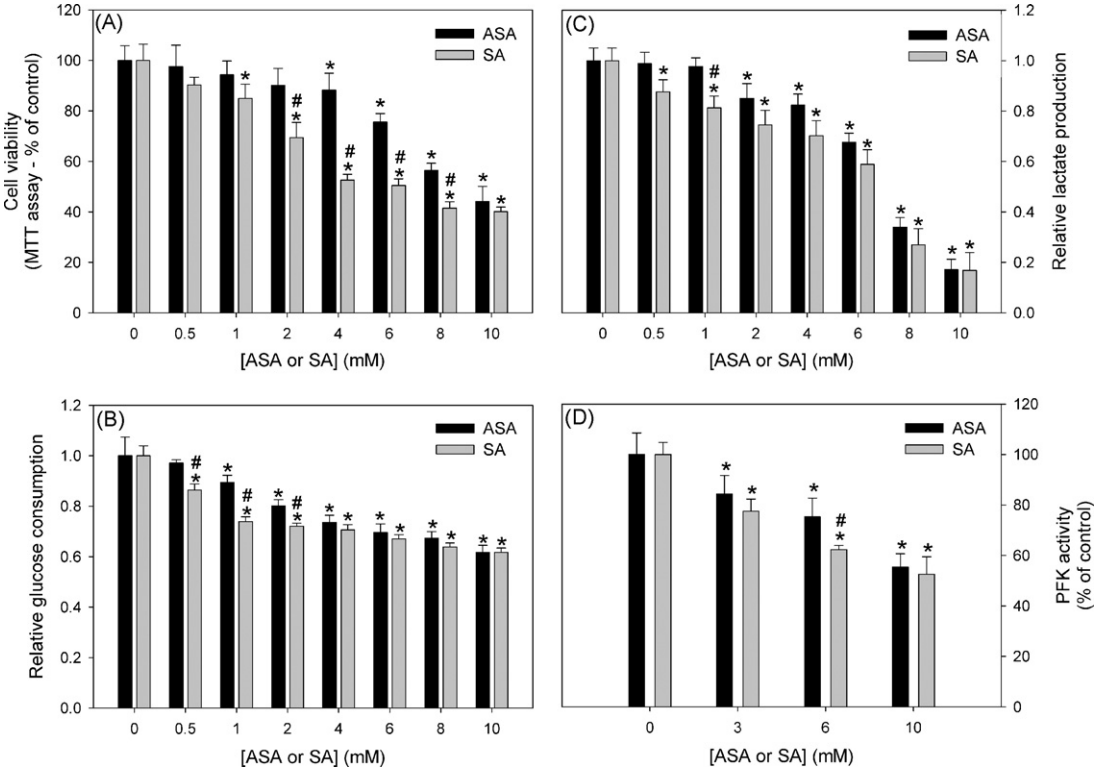

Fig. 1 – Effects of ASA and SA on MCF-7 cells viability, glucose consumption, lactate production and PFK activity. MCF-7 cells were grown to confluence in DMEM as indicated under Section 2. After that, the grown medium was removed and fresh medium containing the desired concentrations of ASA or SA was added and cells were incubated for 24 h under these conditions. Treated cells were used to evaluate viability (panel A), glucose consumption (panel B), lactate production (panel C) and PFK activity (panel D) as indicated under Section 2. For PFK activity, the spectrophotometric method was used. Plotted values are mean ± standard errors of at least five independent experiments ($n \geq 5$). *$P < 0.05$ comparing to control, in the absence of drugs. #$P < 0.05$ comparing to the same concentrations of ASA. Student's *t*-test was used to evaluate statistically significant differences between the values.

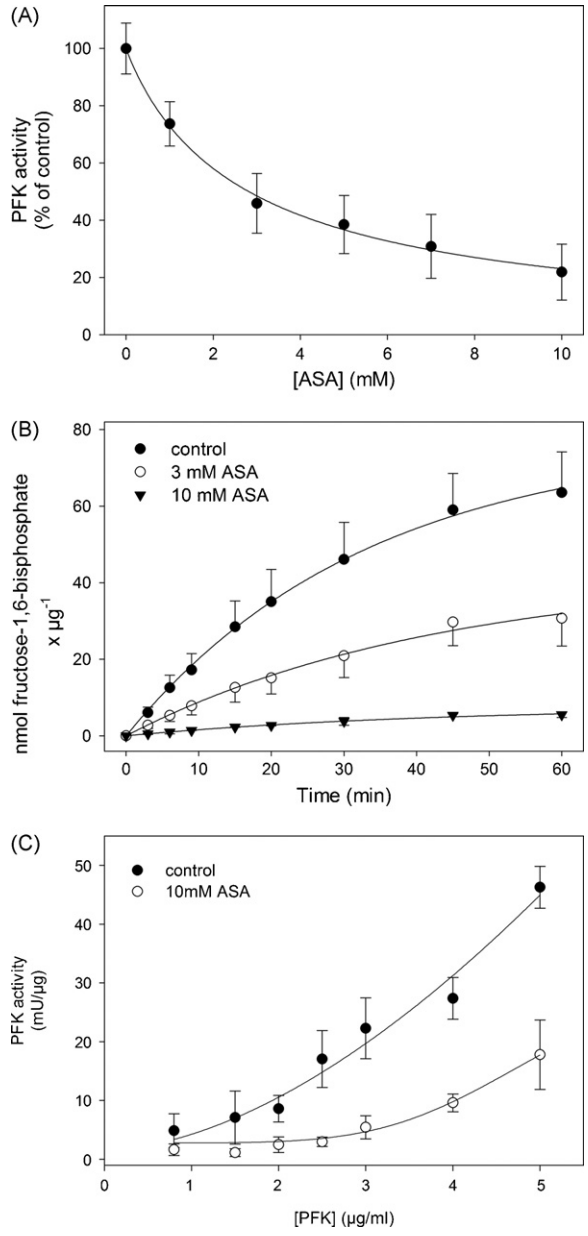

Fig. 2 – Effects of ASA on catalytic activity of purified PFK. Enzyme activity was evaluated as described under Section 2 through the radiometric assay, in the presence of the desired concentrations of ASA. Panel A: dose–response curve for the effects of ASA on PFK activity. Panel B: time course of PFK activity in the absence and in the presence of 3 mM and 10 mM ASA. Panel C: PFK titration curve of enzyme-specific activity in the absence and in the presence of 10 mM ASA. Plotted values are mean ± standard errors of at least five independent experiments ($n \geq 5$).

these experiments were performed without pre-incubation of PFK with the drug, inhibition occurs since the first moment of reaction, decreasing the rate of the products formation during all the recorded time (Fig. 2B). Moreover, ASA is able to inhibit PFK in a broad range of the enzyme concentrations (Fig. 2C). Particularly, enzyme concentration curves have been used as indicative of the oligomerization state of PFK in many recent publications [27,29,30]. This is possible due to the distinct catalytic rates of the major oligomeric conformations of PFK: the quite inactive dimers and the fully active tetramers [29,30]. Since the equilibrium between the oligomers is shifted to the more complex structures at higher concentrations of enzyme, it is observed that the specific activity of the enzyme increases when the tetramers are stabilized at high PFK concentrations. This have allowed us to predict the more stable structure calculating the transition between a conformation with lower specific activity (dimers) and high specific activity (tetramers) in a PFK concentration curve of the enzyme-specific activity [29,30], as presented in Fig. 2C. It can be seen that, in the presence of 10 mM ASA, the specific activity is not altered up to 3 µg/ml PFK, differing from the control experiments, where specific activity augments gradually as PFK concentration increases (Fig. 2C). This is a strong indication that ASA is stabilizing the dimeric conformation of PFK, and so inhibiting the enzyme.

Aiming at evaluating the effects of ASA and SA on PFK oligomeric structure, we assessed the effects of the compounds on the center of mass of the intrinsic fluorescence spectra of PFK. We have validated that tetramers present this parameter more shifted to the blue region (higher energy, lower wavelength) of the spectrum, when compared to dimers, due to the lower exposition of the tryptophans presented in the interface of interaction between two dimers to form a tetramer [4,23,27,29,30]. Moreover, when dimers are formed, it can be observed a small (2–3 nm in average) shift of the center of mass to the red region (lower energy, higher wavelength). Here we show that ASA and SA promote a red-shift of the center of mass of intrinsic fluorescence spectra of PFK (Fig. 3A), which occurs parallelly to the inhibition of the enzyme activity (Fig. 3B), strongly suggesting that ASA and SA stabilize the dimeric conformation of PFK and thus inhibit the enzyme. These results corroborate the findings of Fig. 2C, reported above. Furthermore, in these experiments SA was also more efficient than ASA, corroborating the results presented in Fig. 1, and indicating that the inhibitory effects of the ASA are not dependent on the acetylation of PFK, since SA, that is not able to acetylate proteins, promotes similar and even more potent effects. This encouraged us to test the reversibility of the effects of ASA and SA on purified PFK. Fig. 4 shows the PFK activity of the enzyme pre-incubated for 1 h in the presence of 1 mM or 10 mM ASA and SA, and then assayed for catalytic activity immediately or after 24 h dialysis to remove the compounds. It is clear that after removal of ASA or SA, PFK recover the same activity as control, revealing that the inhibitory effects of ASA and SA are fully reversible.

The ability of PFK to interact with actin filaments (f-actin) within cell cytoskeleton has been recognized as an important issue of the enzyme regulatory mechanism, participating on the regulation of glycolysis by metabolites [23,25,27,29–32], drugs [3,4], hormones [33,20,34,26] and in diseases [2–4,32,35]. The interaction of PFK and f-actin promotes an increase in the enzyme-specific activity and thus in the whole glycolytic rate, due to the stabilization of the tetrameric (fully active) conformation of PFK [33,20,23]. Hence, the presence of f-actin is able to shift the equilibrium between PFK dimers and tetramers towards the formation of the later [23]. Therefore,

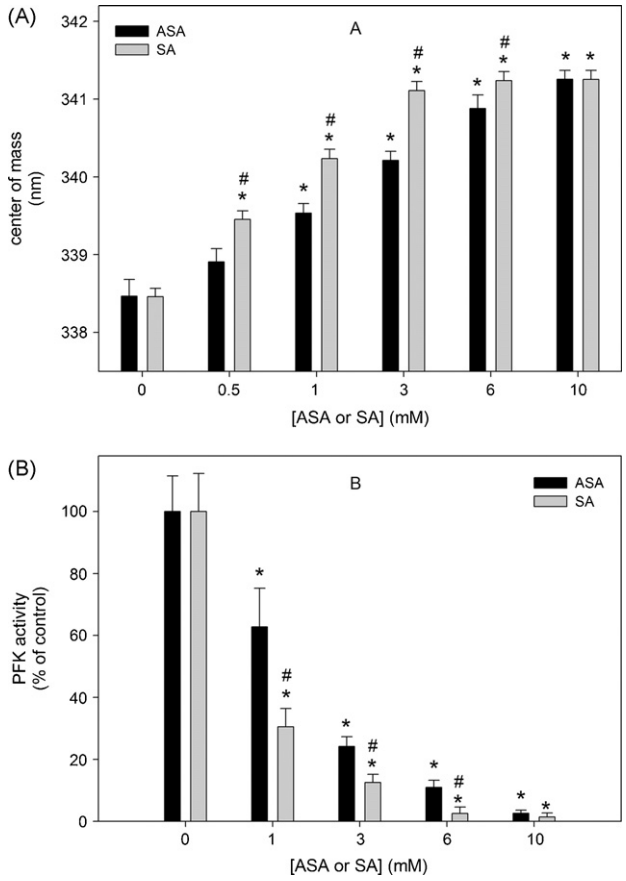

Fig. 3 – Effects of ASA and SA on PFK function-structure relationship. Purified PFK was incubated for 1 h in the presence of the concentrations of ASA or SA indicated in each panel. The treated enzyme was used to evaluate its quaternary structure, assessing the center of mass of the intrinsic fluorescence spectra (panel A), and the catalytic activity, through the spectrophotometric assay (panel B), as indicated under Section 2. Plotted values are mean ± standard errors of at least four independent experiments ($n \geq 4$). *$P < 0.05$ comparing to control, in the absence of drugs. #$P < 0.05$ comparing to the same concentrations of ASA. Student's t-test was used to evaluate statistically significant differences between values.

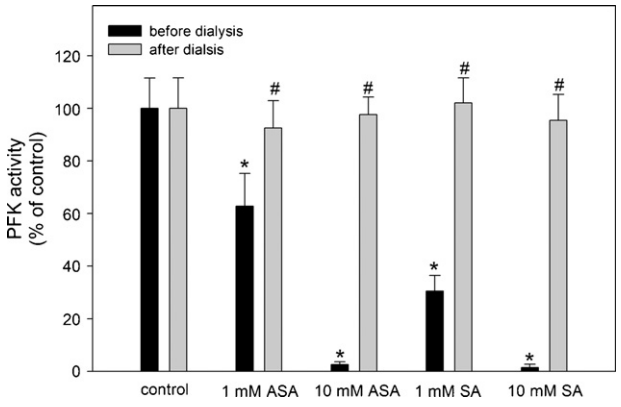

Fig. 4 – Reversibility of the effects of ASA and SA on purified PFK activity. Purified PFK was incubated for 1 h in the presence of the concentrations of ASA or SA indicated in each panel. After incubation, an aliquot of the samples were subjected to dialysis for 24 h to remove the dugs. PFK catalytic activity was assayed immediately after the treatment with the drugs (before dialysis) and after removal of the drugs (after dialysis). Plotted values are mean ± standard errors of at least five independent experiments ($n \geq 5$). *$P < 0.05$ comparing to control, in the absence of drugs. #$P < 0.05$ comparing to the same condition, before the dialysis. Student's t-test was used to evaluate statistically significant differences between the values.

we decided to test whether the presence of f-actin would prevent the inhibitory effects of ASA and SA on PFK or not. This hypothesis proved right, as shown in Fig. 5, where it can be clearly seen that the presence of 0.1 mg/ml f-actin totally prevents PFK from inhibition promoted by ASA (Fig. 5A) and SA (Fig. 5B). This result definitely establishes that ASA and SA inhibit PFK through inducing the dissociation of the active tetrameric conformation of the enzyme into less active dimers, as well as confirm the reversibility of the effects of the drugs. However, a question rose from this last result: would the f-actin present in cells prevent the inhibitory effects of the drugs? At first sight, we could answer that the drugs inhibit PFK in f-actin containing cells, since we have already observed this inhibition in MCF-7 cells, where we have previously proved that PFK interacts with f-actin being activated [3]. Aiming to discard any doubts about the inhibitory properties of ASA and SA in a cell presenting high f-actin content, we tested the effects of the drugs on PFK activity of mouse skeletal muscle homogenates. These experiments reveal that both ASA and SA inhibit PFK activity in a dose-dependent manner, even in cellular systems containing high amounts of f-actin, such as skeletal muscle (Fig. 5C). Furthermore, we observed very similar effects of these compounds in intact human erythrocytes, which present low f-actin content (data not shown).

4. Discussion

The relationship between cancer and glucose metabolism has recovered its importance due to some relevant recent publications [36–41,6]. It is clear from the literature the necessity of tumors to use glucose as the major source of energy, as well as the close correlation between glucose usage and tumor aggressiveness [36,37,2]. Despite the necessity of finding some therapeutic selectivity without affecting normal cell, when searching for new drugs and strategies for cancer control, glycolysis has been proposed as a promising target for the development of new anti-tumoral drugs [38–41].

Within glycolytic pathway, there is a consensus that the key regulatory role is played by PFK, probably the most complex regulatory enzyme in this pathway [18,42–44]. PFK is regulated by a series of physiological and non-physiological ligands including metabolites [44,23,29,30], proteins [33,20,27,34,16] and

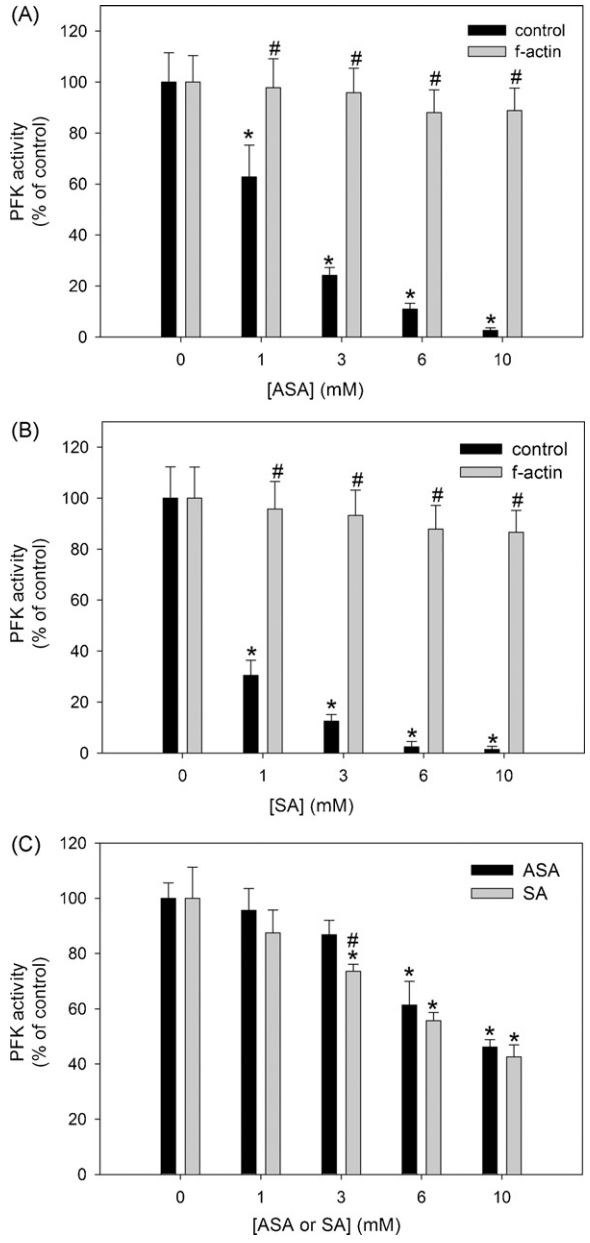

Fig. 5 – Modulation of PFK activity by ASA and SA in the presence of f-actin and of muscle homogenates. Purified PFK was incubated for 1 h in the presence of the concentrations of ASA (panel A) or SA (panel B) indicated, in the absence or in the presence of 0.1 mg/ml f-actin. PFK activity was evaluated through the radiometric method, as indicated under Section 2. Plotted values are mean ± standard errors of at least three independent experiments ($n \geq 3$). $^{*}P < 0.05$ comparing to each control, in the absence of drugs. $^{\#}P < 0.05$ comparing to the same concentrations of drugs in the absence of f-actin. Panel C: muscle homogenates were treated for 1 h in the presence of the drugs and PFK activity was evaluated through the spectrophotometric assay, as indicated under Section 2. Plotted values are mean ± standard errors of at least four independent experiments ($n \geq 4$). $^{*}P < 0.05$ comparing to control, in the absence of drugs. $^{\#}P < 0.05$ comparing to the same concentrations of ASA. Student's t-test was used to evaluate statistically significant differences between the values.

drugs [3,4,9,11,45–47]. Apparently, most of these signals modulate PFK affecting the equilibrium between the two major oligomeric conformations of the enzyme: the quite inactive dimers and the fully active tetramers [4,20,23,27–30]. PFK activity is increased in tumor tissues [2] and cells [3], and some drugs able to decrease tumor cells growth also decrease PFK activity, two phenomena that are probably correlated [3,9–11,47]. More recently, it has been demonstrated that PFK is also directly correlated to the control of apoptosis, binding to the pro-apoptotic protein BAD, and causing its activity to decrease when apoptosis initiates [16]. The role of PFK on apoptosis is still unknown, but it is proposed that the decrease in its activity would diminish the glycolytic pathway and ATP supply to the cell, phenomena that occur simultaneously to apoptosis [16].

The recent finding that ASA induce apoptosis in tumor cell lines [14,12] reinforces the use to non-steroidal anti-inflammatory drugs (NSAID), such as ASA, as a treatment for cancer [48–50]. In the present work, we demonstrate that ASA, as well as its precursor SA, decrease the human breast tumor cell line MCF-7 viability, diminishing its glucose consumption and PFK activity. Although PFK is very sensitive to pH changes, it is not the inhibitory mechanism observed here, since the drugs were diluted in buffered solutions and the final pH was monitored before the experiments. Moreover, we also show that the drugs are able to directly inhibit PFK, altering its quaternary structure through a mechanism common to other modulators of the enzyme. These findings might be directly correlated to the pro-apoptotic effects of ASA, since modulation of PFK quaternary structure (such as the effects of ASA and SA on PFK) recognizably alters its association to other proteins [20,23,27,4,29,30], and can also modulate its association to BAD modulating apoptosis. It would not be the first time that it is observed that inhibition of PFK is followed by cell death through apoptosis, as it was observed upon treatment of MCF-7 with clotrimazole [3]. Despite being speculative, it sounds reasonable to correlate the inhibitory effects of ASA on PFK with its anti-tumoral and pro-apoptotic activities, and certainly a lot of studies and efforts must be done before proving this hypothesis. On the other hand, the anti-tumoral properties of ASA could not be ignored, and it is strongly supported by a recent clinical study where it was demonstrated that breast cancer risk dramatically decreases with chronic use of ASA [51]. In this trial, it was established that volunteers receiving low doses of ASA (~100 mg/day) did not experience a reduction in breast cancer risk, supporting that high doses of the drug are required for this property [51]. The concentrations used in the present study are very high, when compared to plasma concentrations achieved upon oral administration of ASA or SA (<0.1 mM) [52]. However, it is very difficult to determine the cellular concentration of the drugs, since they can accumulate in the cytosol at concentrations higher than those found in plasma [52]. Therefore, we cannot infer whether the concentrations used here are achieved or not under therapeutic conditions, nor if these drugs could be used as preventive drugs. Nevertheless, several studies evaluating the effects of ASA on cellular viability, apoptosis and other properties have used the same concentrations found in the present study, reinforcing our results [1,14,12,53,54].

Furthermore, our results show that SA is more effective than ASA inhibiting PFK in purified system (Figs. 2 and 3), muscle homogenate (Fig. 5), in MCF-7 cells (Fig. 1), as well as in

intact human erythrocytes (data not shown). This difference in effectiveness is also observed on the ability to modulate PFK quaternary structure (Fig. 4), as well as in decreasing tumor cells glucose consumption and viability (Fig. 1). This might be due to the higher solubility of SA over ASA. This corroborates our hypothesis that the effects of these compounds occur (non-exclusively, but partially) over PFK that, being an intracellular enzyme, is more susceptible to be affected by small and soluble compounds that easily diffuse within the cytosol. Finally, we can propose that not just ASA could be used as an anti-tumoral agent, but also SA.

Acknowledgements

Grateful thanks are due to Rafael Machado for critical reading of the manuscript. This work was supported by grants from Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), Fundação Ary Frauzino/Fundação Educacional Charles Darwin (FAF/FECD Programa de Oncobiologia), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Programa de Núcleos de Excelência (PRONEX).

REFERENCES

[1] Cuesta E, Boada J, Perales JC, Roig T, Bermudez J. Aspirin inhibits NF-[kappa]B activation in a glycolysis-depleted lung epithelial cell line. Eur J Pharmacol 2005;517:158–64.
[2] El-Bacha T, de Freitas MS, Sola-Penna M. Cellular distribution of phosphofructokinase activity and implications to metabolic regulation in human breast cancer. Mol Genet Metab 2003;79:294–9.
[3] Meira DD, Marinho-Carvalho MM, Teixeira CA, Veiga VF, Da Poian AT, Holandino C, et al. Clotrimazole decreases human breast cancer cells viability through alterations in cytoskeleton-associated glycolytic enzymes. Mol Genet Metab 2005;84:354–62.
[4] Zancan P, Rosas AO, Marcondes MC, Marinho-Carvalho MM, Sola-Penna M. Clotrimazole inhibits and modulates heterologous association of the key glycolytic enzyme 6-phosphofructo-1-kinase.. Biochem Pharmacol 2007;73:1520–7.
[5] Meier-Ruge WA, Bruder E. Current concepts of enzyme histochemistry in modern pathology. Pathobiology 2008;75:233–43.
[6] Shaw RJ. Glucose metabolism and cancer. Curr Opin Cell Biol 2006;18:598–608.
[7] Vertessy BG, Kovacs J, Low P, Lehotzky A, Molnar A, Orosz F, et al. Characterization of microtubule-phosphofructokinase complex: specific effects of MgATP and vinblastine. Biochemistry 1997;36:2051–62.
[8] Orosz F, Vertessy BG, Salerno C, Crifo C, Capuozzo E, Ovadi J. The interaction of a new anti-tumour drug, KAR-2 with calmodulin. Br J Pharmacol 1997;121:955–62.
[9] Glass-Marmor L, Beitner R. Taxol (paclitaxel) induces a detachment of phosphofructokinase from cytoskeleton of melanoma cells and decreases the levels of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate and ATP. Eur J Pharmacol 1999;370:195–9.
[10] Penso J, Beitner R. Detachment of glycolytic enzymes from cytoskeleton of Lewis lung carcinoma and colon adenocarcinoma cells induced by clotrimazole and its correlation to cell viability and morphology. Mol Genet Metab 2002;76:181–8.
[11] Penso J, Beitner R. Clotrimazole decreases glycolysis and the viability of lung carcinoma and colon adenocarcinoma cells. Eur J Pharmacol 2002;451:227–35.
[12] Garcia-Heredia J, Hervas M, De la Rosa M, Navarro J. Acetylsalicylic acid induces programmed cell death in Arabidopsis cell cultures. Planta 2008;228:89–97.
[13] Loll PJ, Picot D, Garavito RM. The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H_2 synthase. Nat Struct Biol 1995;2:637–43.
[14] Lai MY, Huang JA, Liang ZH, Jiang HX, Tang GD. Mechanisms underlying aspirin-mediated growth inhibition and apoptosis induction of cyclooxygenase-2 negative colon cancer cell line SW480. World J Gastroenterol 2008;14:4227–33.
[15] Danial NN, Gramm CF, Scorrano L, Zhang CY, Krauss S, Ranger AM, et al. BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis. Nature 2003;424:952–6.
[16] Deng H, Yu F, Chen J, Zhao Y, Xiang J, Lin A. Phosphorylation of Bad at Thr-201 by JNK1 promotes glycolysis through activation of phosphofructokinase-1. J Biol Chem 2008;283:20754–60.
[17] Maia, JCC, Gomes, SL, Juliani, MH, Preparation of [á-32P] and [ã-32P]-nucleoside triphosphate, with high specific activity; first edition. 1983: 146–157.
[18] Kemp RG. Phosphofructokinase from rabbit skeletal muscle. Methods Enzymol 1975;42:71–7.
[19] Kuo HJ, Malencik DA, Liou RS, Anderson SR. Factors affecting the activation of rabbit muscle phosphofructokinase by actin. Biochemistry 1986;25:1278–86.
[20] Coelho WS, Costa KC, Sola-Penna M. Serotonin stimulates mouse skeletal muscle 6-phosphofructo-1-kinase through tyrosine-phosphorylation of the enzyme altering its intracellular localization. Mol Genet Metab 2007;92:364–70.
[21] Alves GG, Lima LM, Favero-Retto MP, Lemos AP, Peres-Sampaio CE, Meyer-Fernandes JR, et al. p-Nitrophenylphosphatase activity catalyzed by plasma membrane ($Ca^{(2+)}$ + $Mg^{(2+)}$ATPase: correlation with structural changes modulated by glycerol and Ca^{2+}). Biosci Rep 2001;21:25–32.
[22] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–75.
[23] Leite TC, Da Silva D, Coelho RG, Zancan P, Sola-Penna M. Lactate favours the dissociation of skeletal muscle 6-phosphofructo-1-kinase tetramers down-regulating the enzyme and muscle glycolysis. Biochem J 2007;408:123–30.
[24] Sola-Penna M, dos Santos AC, Alves GG, El-Bacha T, Faber-Barata J, Pereira MF, et al. A radioassay for phosphofructokinase-1 activity in cell extracts and purified enzyme. J Biochem Biophys Methods 2002;50:129–40.
[25] Zancan P, Sola-Penna M. Calcium influx: a possible role for insulin modulation of intracellular distribution and activity of 6-phosphofructo-1-kinase in human erythrocytes. Mol Genet Metab 2005;86:392–400.
[26] Zancan P, Sola-Penna M. Regulation of human erythrocyte metabolism by insulin: cellular distribution of 6-phosphofructo-1-kinase and its implication for red blood cell function. Mol Genet Metab 2005;86:401–11.
[27] Marinho-Carvalho MM, Zancan P, Sola-Penna M. Modulation of 6-phosphofructo-1-kinase oligomeric equilibrium by calmodulin: formation of active dimers. Mol Genet Metab 2006;87:253–61.
[28] Faber-Barata J, Sola-Penna M. Opposing effects of two osmolytes—trehalose and glycerol—on thermal inactivation of rabbit muscle 6-phosphofructo-1-kinase. Mol Cell Biochem 2005;269:203–7.

[29] Zancan P, Almeida FV, Faber-Barata J, Dellias JM, Sola-Penna M. Fructose-2,6-bisphosphate counteracts guanidinium chloride-, thermal-, and ATP-induced dissociation of skeletal muscle key glycolytic enzyme 6-phosphofructo-1-kinase: a structural mechanism for PFK allosteric regulation. Arch Biochem Biophys 2007;467: 275–82.
[30] Zancan P, Marinho-Carvalho MM, Faber-Barata J, Dellias JM, Sola-Penna M. ATP and fructose-2,6-bisphosphate regulate skeletal muscle 6-phosphofructo-1-kinase by altering its quaternary structure. IUBMB Life 2008;60:526–33.
[31] Alves GG, Marinho-Carvalho MM, Atella GC, Silva-Neto MA, Sola-Penna M. Allosteric regulation of 6-phosphofructo-1-kinase activity of fat body and flight muscle from the bloodsucking bug *Rhodnius prolixus*. An Acad Bras Cienc 2007;79:53–62.
[32] Sola-Penna M. Metabolic regulation by lactate. IUBMB Life 2008;60:605–8.
[33] Alves GG, Sola-Penna M. Epinephrine modulates cellular distribution of muscle phosphofructokinase. Mol Genet Metab 2003;78:302–6.
[34] Silva AP, Alves GG, Araujo AH, Sola-Penna M. Effects of insulin and actin on phosphofructokinase activity and cellular distribution in skeletal muscle. An Acad Bras Cienc 2004;76:541–8.
[35] El-Bacha T, Menezes MM, Azevedo e Silva MC, Sola-Penna M, Da Poian AT. Mayaro virus infection alters glucose metabolism in cultured cells through activation of the enzyme 6-phosphofructo 1-kinase. Mol Cell Biochem 2004;266:191–8.
[36] Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? Nat Rev Cancer 2004;4:891–9.
[37] Ashrafian H. Cancer's sweet tooth: the Janus effect of glucose metabolism in tumorigenesis. Lancet 2006;367: 618–21.
[38] Chesney J. 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and tumor cell glycolysis. Curr Opin Clin Nutr Metab Care 2006;9:535–9.
[39] Clem B, Telang S, Clem A, Yalcin A, Meier J, Simmons A, et al. Small-molecule inhibition of 6-phosphofructo-2-kinase activity suppresses glycolytic flux and tumor growth. Mol Cancer Ther 2008;7:110–20.
[40] Pelicano H, Martin DS, Xu RH, Huang P. Glycolysis inhibition for anticancer treatment. Oncogene 2006;25:4633–46.
[41] Xu RH, Pelicano H, Zhou Y, Carew JS, Feng L, Bhalla KN, et al. Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. Cancer Res 2005;65:613–21.
[42] Uyeda K. Phosphofructokinase. Adv Enzymol Relat Areas Mol Biol 1979;48:193–244.
[43] Hers HG, Van Schaftingen E. Fructose 2,6-bisphosphate 2 years after its discovery. Biochem J 1982;206:1–12.
[44] Kemp RG, Gunasekera D. Evolution of the allosteric ligand sites of mammalian phosphofructo-1-kinase. Biochemistry 2002;41:9426–30.
[45] Glass-Marmor L, Beitner R. Detachment of glycolytic enzymes from cytoskeleton of melanoma cells induced by calmodulin antagonists. Eur J Pharmacol 1997;328:241–8.
[46] Karniel M, Beitner R. Local anesthetics induce a decrease in the levels of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate, and ATP, and in the viability of melanoma cells. Mol Genet Metab 2000;69:40–5.
[47] Schwartz D, Beitner R. Detachment of the glycolytic enzymes, phosphofructokinase and aldolase, from cytoskeleton of melanoma cells, induced by local anesthetics. Mol Genet Metab 2000;69:159–64.
[48] Reddy BS, Rao CV, Rivenson A, Kelloff G. Inhibitory effect of aspirin on azoxymethane-induced colon carcinogenesis in F344 rats. Carcinogenesis 1993;14:1493–7.
[49] Garcia Rodriguez LA, Huerta-Alvarez C. Reduced incidence of colorectal adenoma among long-term users of nonsteroidal antiinflammatory drugs: a pooled analysis of published studies and a new population-based study. Epidemiology 2000;11:376–81.
[50] Garcia-Rodriguez LA, Huerta-Alvarez C. Reduced risk of colorectal cancer among long-term users of aspirin and nonaspirin nonsteroidal antiinflammatory drugs. Epidemiology 2001;12:88–93.
[51] Kirsh VA, Kreiger N, Cotterchio M, Sloan M, Theis B. Nonsteroidal antiinflammatory drug use and breast cancer risk: subgroup findings. Am J Epidemiol 2007;166:709–16.
[52] Yoovathaworn KC, Sriwatanakul K, Thithapandha A. Influence of caffeine on aspirin pharmacokinetics. Eur J Drug Metab Pharmacokinet 1986;11:71–6.
[53] Abbadessa G, Spaccamiglio A, Sartori ML, Nebbia C, Dacasto M, Di CF, et al. The aspirin metabolite, salicylate, inhibits 7,12-dimethylbenz[a]anthracene–DNA adduct formation in breast cancer cells. Int J Oncol 2006;28: 1131–40.
[54] Ho CC, Yang XW, Lee TL, Liao PH, Yang SH, Tsai CH, et al. Activation of p53 signalling in acetylsalicylic acid-induced apoptosis in OC2 human oral cancer cells. Eur J Clin Invest 2003;33:875–82.