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Clotrimazole disrupts glycolysis in human breast cancer without affecting non-tumoral tissues

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ABSTRACT

Human breast cancer tissues, as well as normal tissues from the same patients, were treated with clotrimazole (CTZ) and have their capacities for glucose consumption and lactate production evaluated. This treatment strongly decreased the lactate production rate by tumor tissues (85% inhibition) without affecting the other measurements made, i.e. lactate production by control tissues or glucose consumption by both, control and tumor tissues. This result directly correlates with the inhibition promoted by CTZ on the activity of the major regulatory glycolytic enzyme 6-phosphofructo-1-kinase (PFK) that was observed in tumor tissues (84% inhibition) but not in control tissues. Fractionation of the tissues revealed that this inhibition does not occur in the soluble fraction of the enzyme, but is exclusive of a particulate fraction. It has been previously shown that the particulate fraction of PFK activity in tumors is associated to actin filaments (f-actin). Thus, we investigated whether CTZ would affect the association between PFK and f-actin and we found that the drug directly induces the dissociation of the two proteins in the same extent that it inhibits lactate production, total PFK activity and the particulate PFK activity. We concluded that CTZ disrupts glycolysis on human breast tumor tissues, inhibiting PFK activity by dissociating the enzyme from f-actin.

1. Introduction

Clotrimazole (CTZ[2]) is an antifungal imidazole derivative, which has been proposed to present anti-tumor effects [1–8]. This drug is described to decrease several cancer cell lineages viability by altering their glucose metabolism and energy production [1,4,6,7]. Glucose is the primary source of energy for tumor cells and its metabolism has been used as a target for the development of novel drugs and therapeutic approaches [9–16]. Cancer presents the unique characteristic of a high fermentative glycolytic flux even in the presence of high supply of oxygen, the so-called "Warburg effect"[9,13]. As a consequence of the Warburg effect, tumors produce large amounts of lactate, which confers several advantages for their growth and invasion [9,13,14,16–18]. The elevated production of lactate has been correlated to the activation of 6-phosphofructo-1-kinase (PFK, phosphofructokinase), the major regulatory glycolytic enzyme [14,19,20]. PFK activity has been correlated with the whole glycolytic flux rate and the lactate production rate [14,19,20]. We have previously shown that CTZ directly inhibits PFK activity, altering the enzyme quaternary structure and affinity for its substrates [3,8]. However, it is not clear whether this inhibition could occur in cellular systems or tissues. Moreover, tumor tissues are described to present a highly activated PFK, which presents an altered intracellular distribution and regulation [4,19–21]. Therefore, the aim of the present work was to evaluate the effects of CTZ on glucose metabolism, PFK activity and distribution in human breast cancer tissues in order to strengthen the potential use of CTZ on cancer treatment.

2. Materials and methods

2.1. Materials

ATP, CTZ and fructose-6-phosphate (F6P) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ^{32}Pi was purchased from Instituto de Pesquisas Energéticas e Nucleares (São Paulo, Brazil). [γ-^{32}P] ATP was prepared according to Maia et al. [22]. Purified PFK and f-actin were prepared from rabbit skeletal muscle, as previously described [23]. All protein content measurements were performed as described by Lowry et al. [24].

Abbreviations: PFK, 6-phosphofructo-1-kinase phosphofructokinase; CTZ, clotrimazole; F6P, fructose-6-phosphate; f-actin, filamentous actin; TH, total homogenate.

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2.2. Volunteer tissues and data collection

All tissues were obtained from female donors along with the written consent of the patients undergoing mastectomy at Hospital Universitário Clementino Fraga Filho (HUCFF-UFRJ), Rio de Janeiro, Brazil. Tumor and control tissues from the same donors were removed during surgery. After histological analysis to classification and grading of tumors, samples were immediately frozen in liquid N_2 and stored until use. This project was developed after the approval of the National Ethical Committee (CONEP—approval protocol 1897.0.000.197-06).

2.3. Tissue fractionation

Tissues fractionation was performed as previously described [21]. After N_2 withdrawal, tissues were homogenized in a polytron with a buffer containing 50 mM Tris–HCl, 0.25 M sucrose, 20 mM KF, 0.2 mM 2-mercaptoetanol and 0.5 mM phenylmethanesulfonyl fluoride (PMSF) at pH 7.4. Homogenized tissues were centrifuged for 5 min at 100*g* (4 °C) for separation of cellular debris and non-digested tissues. The resultant supernatant, called total homogenate (TH), represents total enzyme activity. TH was centrifuged for 15 min at 27.000*g* (4 °C) and the supernatant was centrifuged again for 45 min at 120.000*g* (4 °C). The resulting high-speed supernatant was used to evaluate the soluble fraction enzyme activity, while the respective pellet was resuspended in the same original volume with the same buffer and used to analyze the particulate fraction enzyme activity.

2.4. PFK activity

PFK activity was measured by the method described by Sola-Penna et al. [25] with the modifications introduced by Zancan and Sola-Penna [26,27]. The reaction medium contained 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP (4 μCi/nmol), 1 mM fructose-6-phosphate and 50 μg/ml of the tissues extracts, considering their protein content. The reaction was stopped after increasing reaction times by the addition of a suspension of activated charcoal in 0.1 M HCl and 0.5 M mannitol, and after centrifugation, the supernatant containing [1-^{32}P]fructose-1,6-bisphosphate was analyzed in a liquid scintillation counter. The signals from appropriate blanks in the absence of fructose-6-phosphate were measured and subtracted from all measurements to account for ATP hydrolysis. The catalytic rate was calculated by linear regression of the amount of F1,6BP formed vs. reaction time. The enzyme activity was expressed as mU of PFK activity, where 1 mU was taken to be the formation of 1 nmol of fructose-1,6-bisphosphate per minute of reaction.

2.5. Cosedimentation of PFK and f-actin

In absence of nucleotide, f-actin (50 μg) was mixed with 5 μM PFK at room temperature under the same buffer contained 50 mM Tris–HCl (pH 7.0), 50 mM KCl and 5 mM $MgCl_2$ for 2 h in the absence and the presence of 500 μM CTZ. The samples were centrifuged at 100.000*g* for 45 min at 4 °C and the pellets were assayed for PFK activity, which, in the absence of f-actin, was negligible regardless of the presence of CTZ (data not shown).

2.6. Glucose consumption and lactate production

Tissues were sliced, weighed and incubated in a buffer containing 50 mM Tris–HCl [pH 7.4], 0.25 M sucrose, 20 mM KF, 0.2 mM 2-mercaptoetanol and 5 mM glucose, in the absence or in the presence of 500 μM CTZ. The rates of glucose consumption and lactate production were assessed as described previously [20]. Glucose consumption was recorded by evaluating the glucose content in the incubation medium after distinct incubation periods. Glucose content was evaluated by an enzymatic assay for glucose based on glucose oxidase and catalase, using a commercial evaluation kit (Doles, Brazil). Lactate production was recorded evaluating the lactate content in the incubation medium after distinct incubation periods. Lactate content was evaluated adding 50 μL of the incubation medium to 100 μL reaction medium containing 50 mM Tris–HCl (pH 9.2), 100 mM KCl, 1 mM $MgCl_2$, 0.3 mM NAD^+ and 20 U lactate dehydrogenase. The formation of NADH was evaluated spectrometrically (340 nm) and the total amount calculated using a standard curve performed under the same conditions.

3. Results and discussion

The present work analyzed breast tissues samples from 19 female volunteers submitted to mastectomy at the Hospital of the Federal University of Rio de Janeiro (Hospital Universitário Clementino Fraga Filho, HUCFF-UFRJ). This protocol has been approved by the national ethics committee (CONEP 1897.0.000.197-06). The samples used were diagnosed as invasive ductal carcinoma and the non-tumor samples were collected from the same patients as the common protocol for the mastectomy.

The effects of CTZ on glucose metabolism of human breast tissues were evaluated by analyzing the patterns of glucose consumption and lactate production by these tissues. As expected, both parameters are augmented in tumor tissues as compared to non-tumor tissues. The glucose consumption recorded in tumor tissues is 25 ± 7% higher as compared to non-tumor tissues, while lactate production is 3.3-fold higher by the same comparison (Fig. 1). The much higher increase of lactate production as compared to glucose consumption is due to the Warburg effect, which is a characteristic of tumor cells and confers to

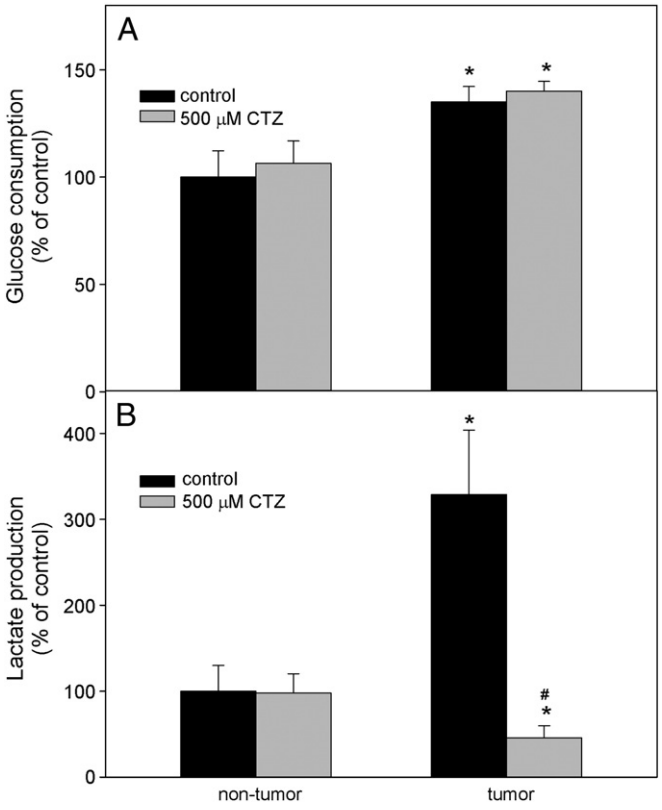

Fig. 1. Glucose consumption and lactate production by non-tumor and tumor tissues from human breast. Glucose consumption and lactate production rates were evaluated as described under Materials and methods. Panel A: glucose consumption. Panel B: lactate production. Values were normalized considering as 100% the rate for glucose consumption or lactate production assessed with control tissues in the absence of CTZ. $^*P<0.05$ comparing with the same condition for non-tumor tissues. $^\#P<0.05$ comparing with the control (without CTZ) of the same tissue.