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# 3-Bromopyruvate inhibits calcium uptake by sarcoplasmic reticulum vesicles but not SERCA ATP hydrolysis activity

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ABSTRACT

3-Bromopyruvate (3BrPA) is an antitumor agent that alkylates the thiol groups of enzymes and has been proposed as a treatment for neoplasias because of its specific reactivity with metabolic energy transducing enzymes in tumor cells. In this study, we show that the sarco/endoplasmic reticulum calcium ($Ca^{2+}$) ATPase (SERCA) type 1 is one of the target enzymes of 3BrPA activity. Sarco/endoplasmic reticulum vesicles (SRV) were incubated in the presence of 1 mM 3BrPA, which was unable to inhibit the ATPase activity of SERCA. However, $Ca^{2+}$-uptake activity was significantly inhibited by 80% with 150 μM 3BrPA. These results indicate that 3BrPA has the ability to uncouple the ATP hydrolysis from the calcium transport activities. In addition, we observed that the inclusion of 2 mM reduced glutathione (GSH) in the reaction medium with different 3BrPA concentrations promoted an increase in 40% in ATPase activity and protects the inhibition promoted by 3BrPA in calcium uptake activity. This derivatization is accompanied by a decrease of reduced cysteine (Cys), suggesting that GSH and 3BrPA increases SERCA activity and transport by pyruvylation and/or S-glutathiolation mediated by GSH at a critical Cys residues of the SERCA.



## 1. Introduction

The antitumor drug 3-bromopyruvate (3BrPA) is an alkylating agent that reacts with thiol (—SH) and hydroxyl (—OH) groups of proteins (Sanborn et al., 1971). This drug inhibits glucose metabolism and has been proposed as a new drug class that is capable of eradicating hepatocellular carcinomas (Geschwind et al., 2002). Our group has demonstrated unequivocally that glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, monocarboxylate transporter and succinate dehydrogenase are irreversibly inhibited in human hepatocarcinoma HepG2 cells (Pereira da Silva et al., 2009). These enzymes present —SH groups that are essential for catalysis and the stabilization of their protein structures, which are susceptible to alkylation. Since 3BrPA inhibits energy metabolism, ATP production is impaired, which results in cell death by apoptosis (Aft et al., 2002). Transformed cells demand high amounts of nutrients for their energy needs, and this demand results in a fermentative metabolism with high rates of lactate production (Warburg, 1956). 3BrPA is an analog of lactate and passes through the plasma membrane via lactate transporters, thus further inhibiting the energy metabolism of tumor cells (Ko et al., 2004). In skeletal muscle, in particular, there are lactate transporters that permit the entry of 3BrPA, but the main targets of 3BrPA alkylation are unknown.

Cytoplasmic $Ca^{2+}$ levels are crucial for signaling in different cell types. The imbalance between calcium entry and clearance from the cytosol by $Ca^{2+}$-pump activity raises the $Ca^{2+}$ concentration, leading to a cascade of events that culminate in cell death by apoptosis (Wojda et al., 2008). The sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) is a P-type enzyme that transports $Ca^{2+}$ from the cytosol to the lumen of the reticulum at the expense of ATP hydrolysis (de Meis and Vianna, 1979). The $Ca^{2+}$ uptake by SERCA regulates muscle relaxation (de Meis and Vianna, 1979), apoptosis and proliferation (Misquitta et al., 1999). In skeletal muscle, SERCA 1a, the prevalent isoform of fast-twitch muscle (Lytton et al., 1992; Møller et al., 1996), and SERCA 2a, the predominant resident in slow-twitch muscle (Møller et al., 1996), present Cys residues that can be modulated by reactive oxygen species (Kukreja et al., 1988) like peroxynitrite (ONOO—), a strong and selective oxidant that regulates SERCA activity by thiol modifications (Adachi et al., 2004).

Previously, it was demonstrated that in smooth muscle cells, SERCA 2 is pathologically inhibited by peroxynitrite, and in the presence of reduced glutathione (GSH), this enzyme is reversibly activated by an S-glutathiolation mechanism. However, the oxidation of thiol groups that occurs during oxidative stress, which is observed in chronic diseases, impairs the S-glutathiolation of SERCA 2 and its subsequent activation (Adachi et al., 2004; Lokuta

*Abbreviations:* SRV, sarcoplasmic reticulum vesicles; SERCA, sarco/endoplasmic reticulum calcium $Ca^{2+}$ ATPase; 3BrPA, 3-bromopyruvate; GSH, reduced glutathione; IAA, iodoacetamide.

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et al., 2005; Vinner et al., 1999; Li et al., 2006; Matsunaga et al., 2003). SERCA 1 is also inactivated by peroxynitrite, but the presence of added GSH fails to completely reverse this effect when it is used at high concentrations as an oxidizing agent (Viner et al., 1996). Gutiérrez-Martín et al. (2004) showed that this inhibition is due to oxidation of thiol groups and tyrosine nitration and the target of peroxynitrite is the globular domain of SERCA. This inhibition of SERCA1 promoted by peroxinitrite might be protected by skeletal muscles intracellular reductants.

The purpose of the present study was to evaluate the effect of 3-BrPA on SERCA 1a activity in sarcoplasmic reticulum vesicles (SRV) from rabbit skeletal white muscle. The data indicate that 3BrPA inhibits calcium uptake but not SERCA ATP hydrolysis. Other studies demonstrate that SERCA inhibition maybe a possible therapeutic target for cancer treatment (Jakobsen et al., 2001; Denmeade and Isaacs, 2005), and the implications of these findings for healthy tissues and tumor cells are discussed. Curiously, we verified that the presence of reduced glutathione (GSH) not only prevents the inhibitory effect of 3BrPA but also increases SERCA 1a activity possibly by S-glutathiolation.

## 2. Materials and methods

### 2.1. Sarcoplasmic reticulum vesicles

During all of the experiments, white male rabbits were treated in accordance with published regulations for animal laboratorial use. White muscles were dissected from rabbit hind limbs. Vesicles derived from the longitudinal sarcoplasmic reticulum of the two types of muscle were prepared as described previously (Reis et al., 2002) and stored at −80 °C.

### 2.2. ATP hydrolysis

ATP hydrolysis in the samples was evaluated by two different methods: (a) measuring free Pi according to the method described by Fiske and Subbarow (1925). The reaction medium consists of the following: 50 mM MOPS/Tris pH 7.0, 50 μM $CaCl_2$, 10 mM $MgCl_2$, 1 mM ATP and 100 mM KCl. The reaction was measured at 37 °C at pH 7.0. The reaction was initiated by the addition of protein to the reaction medium in a final concentration of 0.04 mg/mL and was stopped with the addition of 5% (w/v) TCA. Or (b) ATP hydrolysis by [γ-$^{32}$P]ATP was determined as previously described and corrected for non-specific binding (Grubmeyer and Penefsky, 1981). The reaction medium consists of 50 mM MOPS/Tris pH 7.0, 1 mM ATP, 10 mM $MgCl_2$, 50 μM $Ca^{2+}$, 100 mM KCl, 10 mM Pi and trace amounts of radioactivity ([γ-$^{32}$P]ATP). The reactions were quenched with 1 vol. of a mixture of 200 (w/v) trichloroacetic acid. The filters were washed with 15× 5 mL of an ice-cold solution containing 0.1 M HCl and counted for radioactivity in a liquid-scintillation counter. [γ-$^{32}$P]ATP was prepared as described by Glynn and Chappell.

### 2.3. Calcium uptake

$Ca^{2+}$ uptake was measured by the filtration method (Chiesi and Inesi, 1979). The reaction medium was the same used for ATP hydrolisys described above (50 mM MOPS/Tris pH 7.0, 1 mM ATP, 10 mM $MgCl_2$, 50 μM $Ca^{2+}$, 100 mM KCl) and trace amounts of radioactivity ($^{45}CaCl_2$). 10 mM Pi was used as precipitant agent. The reaction was measured at 37 °C at pH 7.0. The reaction was initiated with the addition of protein to the reaction medium in final concentration of 0.04 mg/mL and was stopped by the filtering of the samples (0.4 mL of reaction medium) through nitrocellulose filters (medium pore 0.45 μm) at different times. After filtration, the filters were washed five times with 5 mL of 3 mM $La(NO_3)_3$, and the radioactivity remaining on the filters was quantified using a liquid scintillation counter.

### 2.4. Protein determination

The protein concentration in the samples was determined as described by Lowry et al. (1951).

### 2.5. Thiol-disulfide status quantification

The thiol-disulfide state was measured as described previously by Hansen et al. (2009).

### 2.6. Oxidation reactions followed by oxygen consumption rate measurements

Oxygen consumption was measured by high-resolution respirometry using the OROBOROS Oxygraph 2k at 37 °C with chamber volumes set at 2 mL. DatLab software (Oroboros Instruments, Innsbruck, Austria) was used for data acquisition and analysis. The SRV (0.01 mg/mL) were incubated with 2 mL of incubation medium containing Mops–Tris pH 7.0. Other additions are indicated in the figure legends.

### 2.7. Statistical analysis

Statistical analyses were performed using Origin® 7.5 (OriginLab). All results are expressed as means ± S.E.M. for $n$ independent experiments. Statistical significance was determined using a Student's $t$-test. Differences were considered statistically significant for $P < 0.05$.

## 3. Results

### 3.1. Preincubation of SRV with 3BrPA inhibits ATPase activity of SERCA

To evaluate the effect of 3BrPA on SERCA ATPase activity, the $Ca^{2+}$-ATPase activity was measured in SRV. The SRV were added to the reaction medium containing increasing amounts of 3-BrPA (from 0.5 to 3 mM). Under these conditions, 3BrPA was unable to inhibit ATP hydrolysis by SERCA (Fig. 1A). However, when the vesicles were preincubated with different concentrations of 3-BrPA in the absence of either ATP or $Ca^{2+}$, approximately 500 μM of 3BrPA inhibited SERCA ATPase activity by 60% after 30 min of preincubation (Fig. 1B, closed circles). After 60 min of preincubation, greater than 80% inhibition was attained with 500 μM of 3BrPA (Fig. 1B, closed triangles). The increase in the preincubation time implied that a higher fraction of the enzyme activity was reduced. No inhibition of SERCA ATPase activity was observed by pre-incubating the enzyme with 1 mM sodium bromide (NaBr) (Fig. 1C, open triangles), pyruvate or lactate (Fig. 1C, closed squares and closed triangles), indicating that the decrease is not promoted by the presence of the bromide group or carbon-skeletal analogs of 3BrPA. These data indicate that the inhibitory effect of 3BrPA is mainly observed when the enzyme is free of ligands, such as ATP/$Mg^{2+}$ and $Ca^{2+}$.

### 3.2. Glutathione protects the loss of SERCA ATPase activity promoted by 3BrPA

We hypothesized that the 3BrPA reaction leads to an inactivation of SERCA ATPase activity in a mechanism that includes alterations, such as oxidations of —SH groups in Cys residues. To investigate whether the reduction in SERCA activity that was promoted by preincubation with 3BrPA could be prevented by GSH,