FEBS Letters 588 (2014) 138–142

FEBS Letters

journal homepage: www.FEBSLetters.org

Skeletal muscle glycogen phosphorylase is irreversibly inhibited by mercury: Molecular, cellular and kinetic aspects

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ARTICLE INFO

Article history:
Received 12 September 2013
Revised 31 October 2013
Accepted 14 November 2013
Available online 21 November 2013

Edited by Judit Ovádi

Keywords:
Enzyme inhibition
Mechanistic toxicology
Mercury
Glycogen metabolism

ABSTRACT

Muscle glycogen phosphorylase (GP) plays an important role in muscle functions. Mercury has toxic effects in skeletal muscle leading to muscle weakness or cramps. However, the mechanisms underlying these toxic effects are poorly understood. We report that GP is irreversibly inhibited by inorganic (Hg^{2+}) and organic (CH_3Hg^+) mercury (IC_{50} = 380 nM and k_{inact} = 600 M^{-1} s^{-1} for Hg^{2+} and IC_{50} = 43 μM and k_{inact} = 13 M^{-1} s^{-1} for CH_3Hg^+) through reaction of these compounds with cysteine residues of the enzyme. Our data suggest that the irreversible inhibition of GP could represent one of the mechanisms that contribute to mercury-dependent muscle toxicity.
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1. Introduction

Muscle glycogen phosphorylase (GP, EC 2.4.1.1) is the rate-limiting enzyme of glycogen breakdown in skeletal muscle [1]. Mutations that impair GP activity leads to McArdle's disease which is characterized by exercise intolerance, muscle fatigue, and cramps [2,3].

Mercury is a widespread occupational and environmental pollutant [4]. Inorganic (Hg^{2+}) and organic mercury (CH_3Hg^+) exerts a wide range of toxic effects through different mechanism. The toxicity of inorganic and organic mercury is thought to be caused primarily by binding and subsequent loss of protein functions [5]. In addition to lungs, kidney and nervous system, deposition of mercury in skeletal muscle also occurs [4]. Importantly, mercury has been shown to contribute to skeletal muscle symptoms such as muscle weakness and cramps and to induce myopathic changes in rats [6,7]. Although the underlying toxicological mechanisms are poorly understood, inhibition of key muscle enzymes such as sarcoplasmic reticulum ATPases by mercury have been reported [8]. Moreover, exposure to mercury of rats has been shown to alter the metabolism of glycogen (elevation of glycogen levels) in the liver and kidneys of rat and zebrafish [9–11].

We show here that mercury impairs the glycogenolytic activity of muscle GP. Mercury was able to inhibit endogenous GP enzyme in skeletal muscle cells and in mouse extensor digitorum longus (EDL) muscle extracts. The inhibition of GP by these chemicals is a irreversible biomolecular process that relies on the formation of a thiol–Hg bond involving cysteine residues. The irreversible inhibition of GP could represent one of the mechanisms that contribute to mercury-dependent muscle toxicity.

2. Materials and methods

2.1. Materials

Glycogen phosphorylase (GP) in its b form was prepared from rabbit skeletal muscle according to Fischer and Krebs using β-mercaptoethanol instead of cysteine for crystallization [12]. Glucose-6-phosphate dehydrogenase (G6P-DH) and monoclonal anti-fluorescein were purchased from Roche. Otherwise noted, all other compounds were from Sigma.

2.2. GP activity assay

The measurement of GP activity was carried out in the direction of glycogen breakdown in presence of AMP, as previously

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http://dx.doi.org/10.1016/j.febslet.2013.11.021

described [13]. The formation of glucose-1-P was measured in a coupled assay system containing phosphoglucomutase, G6P-DH and NADP, by reading NADPH formation at 340 nm. The phosphorylase activity assay was carried out at 37 °C in 50 mM glycine–glycine (gly–gly) buffer, pH 6.8. Briefly, the reaction mixtures consisted of pure GP (or cell/tissue extracts), 2 mg/ml glycogen, 16 mM Pi, 0.8 mM AMP, 8 mM magnesium acetate, 0.64 mM NADP, 5.88 μM glucose 1,6-diP, 0.128 units of phosphoglucomutase and 0.0875 units of G6P-DH in a final volume of 250 μl. Each reaction was performed in triplicate.

2.3. Effects of mercury on GP activity

Prior to any experiment, GP enzyme was reduced by 10 mM dithiothreitol (DTT) at 0 °C for 30 min and then dialysed overnight at 4 °C against 50 mM gly–gly buffer. Protein concentration was determined using a Bradford assay with BSA (Bovine serum albumin) as standard (Bio-rad). Mercury and GP (0.5 μM final concentration) reactions were carried out in 50 mM gly–gly buffer (pH 6.8) at 37 °C for 30 min followed by dilution (50 times) with gly–gly buffer prior to enzyme assay.

To test whether the reaction of GP with mercury was irreversible, purified enzyme (0.5 μM final) was first incubated in presence or absence of mercury for 30 min at 37 °C. The samples were then dialysed overnight at 4 °C against gly–gly buffer prior to enzyme assay. Controls were carried out with non dialysed samples.

To test whether the mercury-dependent inhibition of GP could be reversed by reducing or chelating agents, GP (0.5 μM final) was first inhibited by mercury for 30 min at 37 °C. Samples were then diluted (50 times) with gly–gly buffer containing different concentrations of reduced glutathione (GSH) or ethylenediaminetetraacetic acid (EDTA) and further incubated for 15 min at 37 °C prior to enzyme assay.

2.4. Kinetic analysis of inhibition of GP by mercury

In order to determine the second-order rate constant for inhibition of GP, equimolar concentrations of GP and mercury were incubated in 50 mM gly–gly buffer (pH 6.8) at 25 °C as described previously [14]. Aliquots were withdrawn every 6 min, diluted with gly–gly buffer (50 times) and assayed for residual enzyme. Controls were carried out in absence of mercury. The k_{inact} value was determined by fitting data to the following equation: $E_0/E = 1 + k_{inact} \cdot E_0 \cdot t$, where E is the active enzyme concentration (residual activity), E_0 is the initial enzyme concentration (initial activity) and t is time.

2.5. Effects of mercury on GP cysteine residues

Free cysteine residues in GP (before and after treatment with mercury) were analysed in two ways. First, free cysteines were detected by irreversible labeling with fluorescein-conjugated iodoacetamide. To this end, GP (0.5 μM) was incubated with or without different concentrations of mercury in 50 mM gly–gly buffer (pH 6.8) at 37 °C for 30 min. Samples were then diluted in gly–gly buffer containing 5-IAF (20 μM final) and incubated for 30 min at 37 °C. Samples were then dot-blotted on nitrocellulose using a Bio-rad dot-blot apparatus according to manufacturer's instructions. Detection of labeled-free cysteines and GP protein were carried out using anti-fluorescein antibody and Ponceau S stain, respectively, as described previously [15].

DTNB (5,5′-dithiobis-(2-nitrobenzoic acid) was used to quantify the number of cysteine residues in GP that reacts with mercury as previously described [16,17]. To this end, reduced GP (2.0 μM final) was incubated with mercury at a concentration that gives 100% inhibition (4.0 μM final for $HgCl_2$) in 50 mM gly–gly buffer (pH 6.8) at 37 °C for 60 min. DTNB (1.0 mM in 3.0 M guanidinium chloride final) was added and the mixture further incubated for 60 min at room temperature. Absorbance of TNB^- anion (2-nitro-5-thiobenzoate) was measured at 412 nM. Reaction of DTNB with free cysteines is stoichiometric so that TNB^- concentration is equal to free cysteine concentration. The number of free thiols was calculated using a molar extinction coefficient of 13 700 M^{-1} cm^{-1}.

2.6. Effects of mercury on the endogenous GP activity of cultured myotubes

C2C12 mouse myoblast cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum (FBS) at 37 °C in 100 mm petri dishes. C2C12 cells were differentiated into myotubes by growing cells in 2% FBS. The resulting cultures were determined to be >95% differentiated myotubes by visual inspection. Myotube monolayers (90% confluence) were washed with PBS and exposed to different concentrations of mercury in PBS for 45 min at 37 °C. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) assays were carried out to ensure that the concentrations of mercury were subtoxic in the conditions used. After treatment, cells were scraped in lysis buffer (PBS with 0.1% Triton X-100 supplemented with protease/phosphatase inhibitors). Cell lysates were briefly sonicated, centrifuged (13 000×g, for 15 min) and GP activity measured in supernatants.

2.7. Effects of mercury on the endogenous GP activity of mouse extensor digitorium longus (EDL) muscle

EDL muscles from a 3-months old male Swiss mice were dissected, cut in pieces and homogenized in PBS 0.1% Triton X-100 supplemented with protease/phosphatase inhibitors using a tissue glass Dounce homogenizer. After brief sonication, the homogenate was centrifuged (13 000×g for 15 min). The supernatant was incubated with different concentrations of mercury for 45 min at 37 °C and residual GP activity measured.

3. Results and discussion

3.1. Mercury inhibit the activity of purified and endogenous GP enzyme

Mercury has been shown to deposit in skeletal muscle and to exert symptoms such as muscle weakness and cramps. Genetically-based impairment of muscle GP enzyme is known to be the cause of metabolic myopathy with different symptoms including muscle fatigue and cramps [6,7]. Moreover, it has been shown that exposure to mercury can alter the metabolism of glycogen in liver and kidney of rats [9,10]. To test whether mercury could impact the functions of muscle GP, we analyzed the effects of different levels of Hg^{2+} ($HgCl_2$) and CH_3Hg^+ (CH_3HgCl) on the phosphorolysis of glycogen by purified rabbit GP. Mammalian muscle GP enzymes are highly conserved (amino acid identity >95%) and most studies on muscle GP have been done with the rabbit enzyme, which shares 98% identity with the human and mouse ortholog. As shown in Fig. 1, exposure of GP to Hg^{2+} ($HgCl_2$) or CH_3Hg^+ led to the dose-dependent inhibition of the enzyme with half-maximal inhibitory concentration (IC_{50}) values of 0.38 ± 0.08 (Fig. 1A) and 43 ± 3 μM (Fig. 1B), respectively. Hg^{2+} was found to be a more potent inhibitor than CH_3Hg^+ with nearly 90% of the enzyme inhibited at a concentration of 800 and 100 μM, respectively. Such a trend has been shown for other enzymes inhibited by mercury [14,18]. We investigated the ability of mercury to inhibit the activity of endogenous GP in cultured mouse myotubes. To this end, C2C12 myotubes (which are a well-known model of differentiated skeletal muscle cells) were exposed to subtoxic doses of Hg^{2+}

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