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Role of polyhydroxybutyrate in mitochondrial calcium uptake

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ABSTRACT

Polyhydroxybutyrate (PHB) is a biological polymer which belongs to the class of polyesters and is ubiquitously present in all living organisms. Mammalian mitochondrial membranes contain PHB consisting of up to 120 hydroxybutyrate residues. Roles played by PHB in mammalian mitochondria remain obscure. It was previously demonstrated that PHB of the size similar to one found in mitochondria mediates calcium transport in lipid bilayer membranes. We hypothesized that the presence of PHB in mitochondrial membrane might play a significant role in mitochondrial calcium transport. To test this, we investigated how the induction of PHB hydrolysis affects mitochondrial calcium transport. Mitochondrial PHB was altered enzymatically by targeted expression of bacterial PHB hydrolyzing enzyme (PhaZ7) in mitochondria of mammalian cultured cells. The expression of PhaZ7 induced changes in mitochondrial metabolism resulting in decreased mitochondrial membrane potential in HepG2 but not in U87 and HeLa cells. Furthermore, it significantly inhibited mitochondrial calcium uptake in intact HepG2, U87 and HeLa cells stimulated by the ATP or by the application of increased concentrations of calcium to the digitonin permeabilized cells. Calcium uptake in PhaZ7 expressing cells was restored by mimicking calcium uniporter properties with natural electrogenic calcium ionophore – ferutinin. We propose that PHB is a previously unrecognized important component of the mitochondrial calcium uptake system.

1. Introduction

Poly-3-hydroxybutyrate (PHB) is a polyester made of monomers of 3-hydroxybutyric acid (HB). PHB is ubiquitously present in all living organisms ranging from bacteria to humans. In mammalian organisms PHB is found in the form of a short chain or "complexed" PHB (cPHB), consisting of polymers with chain lengths ranging from 2 to 120 monomers [1–3]. cPHB has been found in all living organisms examined, suggesting its important biological function [2]. While physiological roles of PHB of higher eukaryotes, including mammals, are in general poorly understood, several studies suggest that it might be involved in the regulation of membrane transport. Specifically, it has been demonstrated that cPHB is directly involved in the formation of bacterial cation selective channels through the formation of a polyphosphate (polyP) polyP/Ca^{2+}/PHB complex [4] and is closely associated with such protein channels as KcsA [5], OmpA [6] and mammalian TRPM8 [7].

In mammalian cells most of the cPHB is found in the mitochondria and is primarily localized in the mitochondrial membrane fraction [3]. It was previously demonstrated that cPHB isolated from rat liver mitochondria is complexed with calcium and polyP and can form stable ion channels when reconstituted into artificial lipid bilayers [8]. The properties of this channel were similar to the properties of mitochondrial permeability transition pore (mPTP) [9,10], suggesting that mitochondrial cPHB might be an important target for the polyP – a potent activator of mPTP [11–13]. Furthermore, earlier studies have shown that synthetic PHB of a size similar to that found in mitochondria can induce cation selective ion transport in model lipid membranes. Importantly, in these experiments ion transport can occur by both channel [14] and carrier [15] mechanisms and does not require the presence of polyP. Specifically, it was demonstrated that liposomes containing small amount of PHB can accumulate calcium and that the kinetics of this process are similar to the action of calcium ionophores [2,16,17]. Taking this into account we hypothesized that cPHB may play an important role in mitochondrial ion transport. To test this hypothesis, we investigated the activity of the calcium uptake system of mammalian mitochondria expressing bacterial PHB depolymeraze (PhaZ7), an enzyme that specifically hydrolyses cPHB [18]. We found that the expression of this enzyme significantly inhibits

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mitochondrial calcium uptake. Our data suggest that cPHB is a previously unrecognized important player in mitochondrial calcium uptake.

2. Materials and methods

2.1. Generation of mitochondrial PhaZ7 (mPhaZ7) construct

pAcGFP1-Mito cDNA (MTS-GFP), which encodes a fusion protein consisting of a mitochondrial targeting sequence, derived from the precursor of subunit VIII of human cytochrome c oxidase, and the GFP from Aequorea coerulescens, was purchased (Clontech, Mountain View, CA) and modified by the removal of the GFP stop codon and its replacement with an in-frame XhoI/NotI linker. A PHB depolymeraze cDNA, PhaZ (kindly provided by Dieter Jendrossek), was amplified by high-fidelity PCR (Phusion polymeraze, NEB), with primers containing 5′ XhoI and 3′ NotI restriction sites, and subcloned into the modified pAcGFP1-Mito vector by standard techniques. The DNA sequences of all constructs were determined to be correct before transfection experiments. All plasmid DNAs to be transfected into mammalian cells we prepared using the Endo-Free Plasmid Maxi Kit (Qiagen).

2.2. Cell culture

Human hepatocellular carcinoma HepG2 cell line, human glioblastoma U87 cell line and HeLa cervical cancer cells were cultured in high glucose Dulbecco's modified Eagle's medium (Gibco) containing 10% FBS (Invitrogen) and 1% Pen/Strep (Invitrogen) at 37 °C with 5% CO_2. Cells were transfected with a standard calcium–phosphate procedure.

2.3. Confocal experiments

For measurements of mitochondrial membrane potential ($\Delta\psi_m$), cells were loaded with 25 nM tetramethylrhodamine methylester (TMRM) for 30 min at room temperature and the dye was present during the experiment. TMRM is used in the redistribution mode to assess $\Delta\psi_m$, and therefore a reduction in TMRM fluorescence represents mitochondrial depolarization.

Cells were loaded for 30 min at room temperature with 5 μM X-rhod-1 AM or Fluo-3 AM, (Molecular Probes, Invitrogen) prior to imaging in HEPES-buffered salt solution (HBSS) composed of (mM): 156 NaCl, 3 KCl, 2$MgSO_4$, 1.25 KH_2PO_4, 2 $CaCl_2$, 10 glucose and 10 HEPES, pH adjusted to 7.35 with NaOH. For cell permeabilization experiments HBSS was replaced with pseudo intracellular buffer solution composed of (mM) 120 KCl, 10 NaCl, 1 KH_2PO_4, 2 $MgCl_2$, 20 HEPES–KOH pH 7.4 containing 5 mM succinate and 1 μM rotenone and supplemented with 1 mM EGTA and 20 μM digitonin. Media was then replaced by the same pseudo intracellular buffer without digitonin and containing increasing concentrations of calcium. Concentration of free calcium was estimated using the Ca^{2+}–EGTA Calculator v1.3 [19], software available at: http://www.stanford.edu/~cpatton/CaEGTA-TS.htm. Confocal images were obtained using a 510 CLSM (Zeiss, Thornwood, NY) equipped with a META detection system and a X40 oil-immersion objective. The 488 nm Argon laser line was used to excite fluo-3 fluorescence, which was measured using a bandpass filter from 505 to 550 nm. Illumination intensity was kept to a minimum (at 0.1–0.2% of laser output) to avoid phototoxicity and the pinhole was set to give an optical slice of ~2 μm. TMRM and X-rhod-1 were excited using the 543 nm laser line and fluorescence measured using a 560 nm longpass filter.

Ferutinin (Enzo Life Sciences) was added to a final concentration of 10–50 μM and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (CCCP) was added to a final concentration of 1 μM.

All the imaging data presented in the paper are representative of at least 3 experiments.

2.4. Cell survival experiments

HeLa and HepG2 cells were transfected with either the mito-GFP or the mPhaZ7 expression construct by a standard calcium phosphate procedure. The number of green cells in ten fields per dish was counted at 24 and 48 h post transfection and the percentage of green cells at 48 h was expressed as a percentage of the 24 h count. Cells were stained with propidium iodide (5 μM) and only cells that failed to stain were counted as viable.

2.5. Statistical analyses

Statistical analysis was performed using Origin 8.5 software (Microcal Software, Northampton, MA). Results are expressed as means ± SEM.

3. Results

3.1. Targeted expression of PHB-depolymeraze (PhaZ7) in mitochondria of mammalian cells

The endogenous enzymes responsible for mitochondrial PHB metabolism are currently unknown. For that reason, we chose to address the potential role of PHB in mitochondrial function by expressing a bacterial enzyme that is known to specifically hydrolyze the PHB polymer. For these experiments we used the bacterial PHB-depolymeraze, PhaZ7. Although a number of bacterial PHB-depolymerazes have been identified, PhaZ7 is a unique enzyme which specifically hydrolyzes short-chain or "complexed" cPHB present in mammalian mitochondria, but not storage sPHB [18,20]. Here, we generated a DNA construct through fusion of PhaZ7 coding DNA with DNA coding a mitochondrially targeted GFP protein. Targeted expression of mPhaZ7 was confirmed by the appearance of the green fluorescent signal in the mitochondria of transfected cells (Fig. 1). As can be seen from the images of HeLa cells, the GFP signal is co-localized with the red fluorescent signal originating from the potential sensitive mitochondrial probe, TMRM. A similar mitochondrial expression pattern was found in all cell types used in the present study. In our experiments we used a mitochondrial targeting sequence specific to the matrix targeted proteins, which suggests that the expressed enzyme was likely localized in the matrix of mitochondria.

3.2. Effect of mPhaZ7 overexpression on mitochondrial membrane potential

Next we investigated how the expression of mPhaZ7 affects $\Delta\psi_m$. We found that the expression of mPhaZ7 caused a significant reduction of mitochondrial membrane potential in HepG2 cells, as evidenced by the decrease of the TMRM fluorescence by 65 ± 6.5% of control (n = 55 control cells; n = 55 mPhaZ7 cells; $p < 0.001$; see Fig. 2A). However, we did not detect any significant effect of mPhaZ7 expression on mitochondrial membrane potential in HeLa (n = 25 control cells; n = 25 mPhaz7 cells; see Fig. 2B) or U87 cells (n = 35 control cells; n = 35 mPhaZ7 cells, see Fig. 2C). Thus, the expression of PHB depolymerazing enzyme in mitochondria alters mitochondrial metabolism in HepG2 cells, but not in U87 or HeLa cells. Notably, in control experiments where HepG2 cells were transfected with a point mutant (Ser136Ala) of mPhaZ7 (mPhaZ7-PM), which is known to lack PHB depolymeraze activity [18], the membrane potential was not changed (Fig. 2A). This suggests that

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