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Production and Cytophysiology Applications of 3-hydroxyalkanoic Acid Monomers

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Polyhydroxyalkanoates (PHAs) have been developed for use as polymeric materials to make bioplastics, fine chemicals, implant biomaterials, medicine, and biofuels. PHAs are produced by microorganisms grown under restricted conditions and they are comprised of structurally diverse monomers. Various 3-hydroxyalkanoic acids (3-HA) can be prepared by chemical synthesis, as well as by depolymerizing PHAs and microbial metabolic engineering. PHAs derived from 3-HA monomers are compatible with alcohol and carboxylic acids due to their easily modified hydroxyl and carboxyl functional groups. Therefore, an important application for 3-HA monomers is as a starting material for fine chemical synthesis, such as production of antibiotics, vitamins, aromatics and pheromones. 3-HA also has different physiological uses such and as such can be used for drug manufacture. Here, we focus on the production of 3-HA monomers, their relationship with the promotion of cell proliferation inhibition of apoptosis and suppression of oxidative and nitrosative stress. These discussions will provide a foundation for understanding the properties of 3-HA to optimize their applications in cytophysiology.

1. Introduction

Polyhydroxyalkanoates (PHAs) are microbial stored polyesters consisting of more than 150 types of 3-HA monomers. Common forms of 3-HA monomers are 3-hydroxybutyrate (3-HB), 3-hydroxyvalerate (3-HV), 3-hydroxyhexanoate (3-HHx), 3-hydroxyoctanoate (3-HO), 3-hydroxydecanoate (3-HD), and 3-hydroxydodecanoate (3-HDD) (Chen and Wu 2005 a). 3-HHx, 3-HO, 3-HD, and 3-HDD monomers are of medium chain length PHA (Zhang et al. 2009) and 3-HB is a short-chain PHA and a degradation product of PHB as well as the chief plasma and peripheral tissue ketone body in mammals, produced hepatically by long chain fatty acids (Massieu et al. 2003). Previously, diabetic ketoacidosis was considered to be a worrisome biomarker, especially when they reached 25 mM, as this depleted blood bicarbonate and led to hypovolemia due to urinary water, sodium, and potassium excretion from ketonuria (Gerhardt 1865). However, mild ketosis is now known to be therapeutic in various diseases (Chen and Wu 2005 b, Henderson 2008). In fact, 3-HB can be antimicrobial, insecticidal, and antiviral under certain conditions (Shiraki et al. 2006). Here we summarize the production and cytophysiology applications of 3-HA monomers, emphasizing 3-HB with respect to thepromotion of cell proliferation and inhibition of apoptosis and suppression of oxidative and nitrosative stress.

2. Production of 3-hydroxybutyric acid monomers

2.1 Chemical synthesis

(S)-3-hydroxytetradecanoic acid can be synthesized from (S)-epichlorohydrin (27% yield) as follows: (1) region- and chemo-selective epoxide opening of (S)-epichlorohydrin with a Grignard reagent under catalysis by Cu(I) followed by consecutive epoxide formation; (2) regioselective epoxide opening of (S)-1,2-epoxytridecane with cyanide anion under pH-controlled conditions followed by consecutive nitrile hydrolysis with alkaline H_2O_2 (to yield crude (S)-3-hydroxytetradecanoic acid); and (3) purification with N,N-dicyclohexylammonium salt (Matsuyama and Ikunaka 1999). Optically pure R and S enantiomers of 3-hydroxytetradecanoic acid and its methyl esters were synthesized by porcine pancreas lipase-catalyzed hydrolysis of racemic methyl 3-hydroxytetradecanoate in an aqueous medium (Kücük and Yusufoğlu 2013). A

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versatile route for modular synthesis of 3-HA had been described (Jaipuri et al. 2004) and within this method, steps included a microwave-assisted catalytic transfer hydrogenation and a facile microwave-assisted hydrolysis of N-methoxy-N-methyl (Weinreb) amide, which enhanced the practicality of this protecting group for carboxylic acids. Moreover, efficient synthesis of (*R*)-3-hydroxytetradecanoic acid, a key component of bacterial endotoxins, included (*R*)-oxirane acetic acid ethyl ester as chiral source (Huang and Hollingsworth 1998). Various β-hydroxyalkanoic acid derivatives were prepared via palladium catalyzed aerobic oxidative-carbonylations of terminal olefins under normal carbon monoxide and oxygen mixed gas pressure (Urata 1988). Production of optically active 3-HB is possible by chemical synthesis but this is complex, expensive and inefficient and few reports describe chemosynthesis of optically pure 3-HB.

2.2 Production of 3-HB via depolymerization of PHA

An efficient technique for producing pure 3-HA and 3-HA methyl esters is acidic or basic hydrolysis of PHA isolated via solvent recovery and hydrolyzed by acid methanolysis. The obtained 3-HA methylester mixture is then distilled into several fractions (overall yield of 96.6% w/w) (De Roo et al. 2002). Abiotic hydrolysis of PHB has been investigated in acid and base media using native amorphous granules, precipitates and solvent-cast films of PHB matrix as raw materials, and the formation of two monomeric hydrolytic products, 3-HB and crotonic acid (CA) were depicted. Monomeric products were not released from PHB specimens in acidic solutions (0.1–4 N H^+), but were measured as the major hydrolytic products from alkaline hydrolysis (0.1–4 N OH^-) (Yu et al. 2005). 3-Hydroxybutyrate methyl ester (HBME) was prepared from hydrolysis of bacterial PHB using methanol as an esterification agent in the presence of sulfuric acid (Wang et al. 2010).

Another efficient method for pure 3-HA production is *in vitro* and *in vivo* enzymatic hydrolysis of PHA. PHB is degraded by various specific hydrolytic enzymes from microorganisms that can be broadly classified as intracellular and extracellular depolymerases (Calabia and Tokiwa 2004). An example is thermophilic *Streptomyces* sp. MG which has strong hydrolytic activity for depolymerization of PHB to produce 3-HB monomer. An advantage of this strain is high-temperature (50°C) stability which minimizes contamination not only during fermentation, but also during enzymatic degradation of PHB (Tokiwa and Ugwu 2007). 3-HB can be produced at a 96% yield in 30 min by *in vivo*depolymerization of PHB when cells have high intracellular PHA depolymerase activity and low (R)-(−)-3-hydroxybutyric acid dehydrogenase activity under suitable environmental conditions (Lee et al. 1999). Similar approaches have been used by other researchers to produce PHA monomers with microbial intracellular depolymerase (Saito and Saegusa 1994).

2.3 Microbial metabolic engineering

More efficient systems for 3-HA production have been developed by emplying PHA-producing bacteria and recombinant *E. coli.* To produce 3-HA, pathways of PHA production and degradation must be exploited. Taking PHB as an example, metabolic pathways and possible options for 3-HA production are shown in Fig. 1.

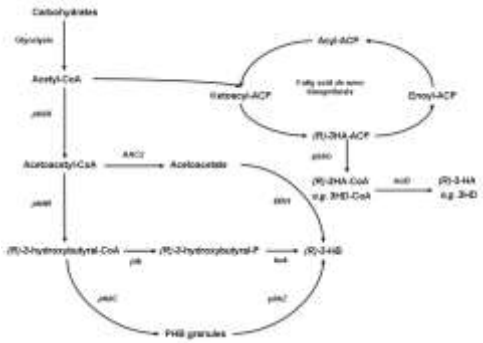

Figure 1: Metabolic pathway for microbial production of 3-HA from carbohydrates. phbA, phbB, phbC, phbZ, ptb, buk, AACS, BDH, phaG, and tesB represent genes of β-ketothiolase, acetoacetyl-CoA reductase, PHB polymerase, PHB depolymerase, phosphotransbutyrylase, butyrate kinase, acetoacetyl-CoA synthetase, 3-hydroxybutyrate dehydrogenase, (R)-3-hydroxydecanoyl-ACP: CoA transacylase, and thioesterase II, respectively.

Recently, a metabolic pathway for production and *in vivo* hydrolysis of PHB to release 3-HB in culture supernatant was investigated (Shirakiet al. 2006). High yields of 3-HB can be produced in PHA-producing bacteria by providing environmental conditions for high intracellular depolymeraseactivity (Lee et al. 1999). However, a significant drawback with this procedure is that the depolymerized products could be further cellularly metabolized to acetoacetate by 3-HB dehydrogenase (BDH). To overcome this obstacle, metabolically engineered *E. coli* strains (without the *BDH* gene) harboring heterologous PHA synthesis and

degradation pathways were established (Lee and Lee 2003). *W. eutropha* PHA biosynthesis genes were integrated into *E. coli* chromosomes to disrupte the *pta* (phosphotrans-acetylase) gene. This stable recombinant *E. coli* strain was constructed and when the *W. Eutropha* intracellular depolymerase gene was expressed, 3-HB was efficiently produced (Park et al. 2004).

New pathways for enhanced 3-HB production were constructed by simultaneous expression of genes for *phbA*, *phbB*, *ptb*, *buk* in *E. coli* DH5α (Liu and Steinbüchel 2000 a and b, Gao et al. 2002). With an isogenic *tesB*-negative knockout strain, *E. coli* CH01, expression of *tesB* and *phaG* were reported to up-regulate one other. In addition, 3-HD was synthesized from glucose or fructose by recombinant *E. coli* harboring*phaG* and *tesB*(Zheng et al. 2004 a). This study supports the hypothesis that the physiological role of *tesB* in *E. coli* is to prevent abnormal accumulation of intracellular acyl-CoA (Zheng et al. 2004 b). Recombinant *P. putida* harboring *tesB* also allowed the production of extracellular 3-HD from carbohydrates (Zheng et al. 2004 c). Zhao and the colleagues reported that addition of acrylic acid significantly increased production of 3-HB and mcl-3-HA consisting of 3-HB and 3-HD at a ratio of 1: 3 (Zhao et al. 2003). *E. coli* BW25113 (pSPB01) harboring *phbA*, *phbB*, and *tesB* genes produced approximately 4 g/L 3-HB in shake flask culture within 24 h with a glucose carbon source. Under anaerobic growth conditions, 3-HB production was more effective and 0.47 g 3-HB/g glucose was produced compared with 0.32 g 3-HB/g glucose obtained from an aerobic process (Liu et al. 2007).

Researchers have invested effort into identifying engineered organisms produce 3-HA. For example, engineered *cyanobacteria (PCC 6803)* photosynthetically produced (S)- and (R)-3-HB directly from sunlight and CO_2. Both 3-HB molecule types were produced and readily secreted from *Synechocystis* cells without transporter over-expression. Additional inactivation of the competing pathway by deleting *slr1829* and *slr1830* (encoding PHB polymerase) from the *Synechocystis* genome also promoted 3-HB production (Wang et al. 2013).

3. Promotion of cell proliferation and inhibition of apoptosis

To investigate cytotoxicity of oligo (3-hydroxyalkanoic acids; OHAs) to mouse fibroblast L929 cells, liposomes were employed to encapsulate OHAs and facilitate cytosolic transfer (Sun et al. 2007). OHAs (less than 20 mg/L) did not significantly affect cell viability, whereas OHAs exceeding 40 mg/L reduced cell viability as evidenced by apoptosis, cell cycle delays and reduced cell proliferation. Cytotoxicity decreased with increasing OHA side-chain length and the 3-HB monomer, a degradation product released from biopolymer PHA, is speculated to contribute to tissue regeneration. 3-HB stimulated cell cycle progression mediated by a calcium-dependent signaling pathway (Cheng et al. 2005) and 0.005–0.10 g/L 3-HB promoted cell proliferation of murine fibroblast L929 cells, human umbilical vein endothelial cells, and rabbit articular cartilage. In L929 cells, 0.02 g/L 3-HB stimulated a rapid increase cytosolic calcium and 3-HB promoted proliferation of L929 cells in high-density (1×10^5 cells/well) cultures (but not low density cultures) by preventing apoptotic and necrotic cell death induced by serum withdrawal (Cheng et al. 2006).

Effects of 3-HB on murine osteoblast MC3T3-E1 cell differentiation *in vitro* and on anti-osteoporosis *in vivo* were evaluated as well (Zhao et al. 2007). The intensity of *in vitro* cell differentiation directly proportional to the concentration of 3-HB when it was less than 0.01 g/L. Calcium deposition, a strong indication of cell differentiation, also increased with increasing 3-HB concentration (from 0 to 0.1 g/L). RT-PCR indicated higher expression of osteocalcin (OCN) mRNA in MC3T3-E1 cells after 3-HB administration. *In vivo* work indicated that 3-HB enhanced femur maximal load and bone deformation resistance and improved trabecular bone volume. Animal experiments suggest that 3-HB increased serum ALP activity and calcium deposition, decreased serum OCN, and prevented bone mineral density reductions in ovariectomized animals.

In vitro effects of 3-HB and 3-HBME on cell apoptosis and cytosolic Ca^{2+} in mouse glial cells were evaluated and 3-HB derivatives were found to inhibit cell apoptosis mediated by signaling pathways related to cytosolic Ca^{2+}elevation (Xiao et al. 2007). Cells undergoing apoptosis decreased significantly in the presence of 3-HB and 3-HBME and 3-HB derivatives dramatically elevated cytosolic Ca^{2+}. The effect of 3-HBME on cytosolic Ca^{2+} was reduced by nifedipine, an L-type voltage-dependent calcium channel antagonist. In comparison, 3-HBME permeated cells better than D-3-HB and DL-3-HB. Next, 3-HB, 3-HBME and 3-HBEE (3-hydroxybutyrate ethyl ester) were evaluated for their ability to stimulate metabolic activity of neuroglial cells (Zou et al. 2009). After 1–3 days in culture, 3-HB (0.5–10 mg/L) stimulated neuroglial cell metabolism significantly more than controls and maximal stimulation was observed after three days of treatment with 5 mg/L 3-HBEE, and after two days of treatment with 5 mg/L 3-HB or 3-HBME.

4. Suppression of oxidative and nitrosative stress

Oxidative stress is involved in neuron apoptosis, specifically via damage by reactive oxygen species (ROS) such as H_2O_2, superoxide, and free radicals. Oxidative stress is implicated in neurodegenerative diseases and cerebral ischemic injury. 3-HB was studied (Cheng et al. 2013) in PC12 cells exposed to different

concentrations of H_2O_2 over different periods after 3-HB pretreatment and 3-HB was shown to slow loss of cell viability induced by H_2O_2. Also 3-HB decreased apoptosis induced by H_2O_2. Changes in intracellular ROS, total glutathione (GSH), mitochondrial membrane potential (MMP) and caspase-3 activity due to H_2O_2 exposure were partially reversed in PC12 cells with 3-HB. Thus, 3-HB inhibited oxidative stress in PC12 cells.
3-HB is an endogenous and specific inhibitor of class I histone deacetylases (HDACs). Inhibition of HDAC by 3-HB was correlated with global changes in transcription, including that of genes encoding oxidative stress resistance factors FOXO3A and MT2 (Shimazu et al. 2013). Treatment of cells with 3-HB increased histone acetylation at the Foxo3a and Mt2 promoters and both genes were activated by selective depletion of HDAC1 and HDAC2. Data show that treatment of mice with 3-HB was accompanied by increased FOXO3A and MT2 activity, and that this conferred substantial protection against oxidative stress.
The *in vitro* effect of normal (0.01 to 1 mM) and subketotic (1 to 2.5 mM) doses of 3-HB on respiratory burst activity of bovine polymorphonuclear leucocytes (PMNL) was studied with chemiluminescence (CL) (Hoeben et al. 1997). In a cell-free assay, consisting of sonicated cells and H_2O_2, no activity changes were observed. Myeloperoxidase activity was not significantly altered as shown by an ortho-dianisidine-oxidation assay. Also, O^{2-} production was not affected by different doses of 3-HB which did not scavenge hypochlorite. Subketotic concentrations of 3-HB significantly inhibited CL, and decreased production of H_2O_2. This inhibitory effect on respiratory burst activity in PMNL suggests that elevated 3-HB after parturition in high yielding cows may be partly responsible for greater susceptibility to local and systemic infections during the postpartum period and during subclinical and clinical ketosis (Klucinski et al. 1988).
Recent work indicates that 3-HB and acetoacetate (1 mM each) decreased cell death in acutely dissociated rat neocortical neurons subjected to 10 μM glutamate excitotoxicity (Maalouf et al. 2007). These compounds also prevented changes in neuronal membrane properties induced by glutamate. Ketones significantly decreased mitochondrial production of ROS and associated excitotoxic changes by increasing NADH oxidation in the mitochondrial respiratory chain. However, neither compound changed endogenous antioxidant GSH. Data show that ketones reduce glutamate-induced free radical formation by increasing the NAD^+/NADH ratio and enhancing mitochondrial respiration in oxidatively stressed neocortical neurons. Other studies confirm that 3-HB prevented hippocampal neuron death after exposure to $A\beta_{1–42}$, and protected cultured mesencephalic dopaminergic neurons from toxic effects of 1-methyl-4-phenylpyridinium (MPP^+, an inhibitor of NADH dehydrogenase that increases oxygen radical formation), and reduced brain injury in rodents (Suzuki et al. 2002). 3-HB and Vitamin E significantly reduced striatal lesions and lipid peroxidation, suggesting that glycolytic impairment favors a pro-oxidant condition, and that oxidative damage is an important mediator of *in vivo* induced excitotoxicity. Data indicate a neuroprotective potential for 3-HB against *in vivo* excitotoxic oxidative damage (Mejía-Toiber et al. 1979).
Ketone bodies can oxidize coenzyme Q and a major source of mitochondrial free radicals is the half-reduced semiquinone of coenzyme Q (Chance et al. 1979). Q semiquinone reacts directly with O_2 to form superoxide radical O^{2-}. By decreasing reduced coenzyme Q forms, mitochondrial production of free radicals is decreased. Next, ketone body metabolism can reduce mitochondrial NAD and cytoplasmic free NADP, favoring GSH reduction, which is in near equilibrium through the action of GSH reductase (Krebs and Veech 1969). This then favors destruction of H_2O_2 by GSH peroxidase reactions (Veech et al. 2001).
Mitochondrial dysfunction leading to increased ROS is associated with neurodegenerative disorders. Rotenone, a mitochondrial stressor, induces caspase-9 and -3 activation and leads to proteolytic cleavage of substrate nuclear poly (ADP-ribose) polymerase (PARP). Cleavage of PARP is directly related to apoptotic cell death (Kabiraj et al. 2012). Na-D-β-hydroxybutyrate (NaβHB) markedly reduces the incidence of synphilin-1 (a rotenone-induced parkinsonin-onset biomarker) aggregation. Furthermore, a metabolic byproduct of NaβHB also prevents rotenone-induced caspase-activated apoptotic cell death in dopaminergic SH-SY5Y cells. These data suggest that NaβHB is neuroprotective, attenuates effects originating from mitochondrial insult, and can serve as a scaffold for the design and development of sporadic neuropathies.

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