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Quantitative determination of isopentenyl diphosphate in cultured mammalian cells

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ABSTRACT

Isopentenyl diphosphate (IPP), an intermediate of the isoprenoid biosynthetic pathway (IBP), has several important biological functions, yet a method to determine its basal level has not been described. Here, we describe a nonradioactive and sensitive analytical method to isolate and specifically quantify IPP from cultured mammalian cells. This method applies an enzymatic coupling reaction to determine intracellular concentrations of IPP. In this reaction, geranylgeranyl diphosphate synthase catalyzes the formation of geranylgeranyl diphosphate (GGPP) from IPP and farnesyl diphosphate (FPP). Subsequently, geranylgeranyl protein transferase I conjugates GGPP with a fluorescently labeled peptide. The geranylgeranylated peptide can be quantified by high-performance liquid chromatography (HPLC) with a fluorescence detector, thereby allowing for IPP quantification. The detection lower limit of the fluorescence-labeled geranylgeranyl peptide is approximately 5 pg (∼0.017 pmol). This method was used to examine the effects of IBP inhibitors such as lovastatin and zoledronate on intracellular levels of IPP. Inhibition of hydroxymethylglutaryl coenzyme A reductase (HMGCR) by lovastatin (50 nM) decreases IPP levels by 78% and 53% in K562 and MCF-7 cells, respectively. Whereas zoledronic acid (10 μM) increased IPP levels 12.6-fold when compared with untreated cells in the K562 cell line, an astonishing 960-fold increase was observed in the MCF-7 cells.
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The isoprenoid biosynthetic pathway (IBP)[2] (Fig. 1) is responsible for the production of numerous molecules involved in diverse cellular functions. Isoprenoid-derived compounds are essential in maintaining membrane integrity (cholesterol), protein isoprenylation (farnesyl diphosphate [FPP] and geranylgeranyl diphosphate [GGPP]), and energy production (ubiquinone). The importance of the IBP is further emphasized by the number of diseases that are associated with its products. These diseases include hypercholesteremia, cancer, tuberculosis, Alzheimer's disease, and Parkinson's disease [1–4]. One common precursor of all isoprenoid-derived compounds is isopentenyl diphosphate (IPP), the first isoprenyl diphosphate synthesized in the IBP. Similar to other isoprenoids, IPP has biological activities beyond its role as a synthetic precursor. Although IPP is an essential IBP intermediate, a sensitive method to quantify basal levels of IPP in cultured cells has remained elusive.

The first recognized step of the IBP is the conversion of hydroxymethylglutaryl coenzyme A (HMG–CoA) to mevalonate by HMG-CoA reductase (HMGCR) (Fig. 1). Mevalonate is subsequently phosphorylated in consecutive steps and decarboxylated to yield IPP, a 5-carbon intermediate, which can be converted to its isomer dimethylallyl diphosphate (DMAPP) by isopentenyl diphosphate isomerase (IDI). Farnesyl diphosphate synthase (FDPS) uses DMAPP and IPP to produce the 10-carbon geranyl diphosphate (GPP) and the 15-carbon farnesyl diphosphate in successive steps. FPP and IPP are then used to produce the 20-carbon GGPP by GGPP synthase (GGDPS). Both FPP and GGPP are positioned at branch points leading to the synthesis of other longer chain isoprenoids. For instance, in the first committed step toward sterol synthesis, the enzyme squalene synthase (SQS) catalyzes the head-to-head condensation of two molecules of FPP into the 30-carbon lipid squalene [5]. In addition, FPP is used for synthesis of dolichols and the side chains of ubiquinone and heme A. The isoprenoid moieties of FPP and GGPP are used during protein farnesylation and geranylgeranylation (collectively isoprenylation), respectively. Farnesylation is catalyzed by farnesyl protein transferase (FTase), whereas geranylgeranyl protein transferases (GGTases) I and II mediate geranylgeranylation. Protein isoprenylation facilitates proper membrane localization and, thus, function of various

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[2] *Abbreviations used:* IBP, isoprenoid biosynthetic pathway; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; HMG–CoA, hydroxymethylglutaryl coenzyme A; HMGCR, HMG-CoA reductase; DMAPP, dimethylallyl diphosphate; IDI, isopentenyl diphosphate isomerase; FDPS, farnesyl diphosphate synthase; GPP, geranyl diphosphate; GGDPS, GGPP synthase; SQS, squalene synthase; FTase, farnesyl protein transferase; GGTase, geranylgeranyl protein transferase; NBP, nitrogenous bisphosphonate; tRNA, transfer RNA; HPLC, high-performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; LC, liquid chromatography; D*-GCVLS, dansyl Gly-Cys-Val-Leu-Ser; D*-GCVLL, dansyl Gly-Cys-Val-Leu-Leu; F-D*-GCVLS, farnesylated D*-GCVLS; GG-D*-GCVLL, geranylgeranylated D*-GCVLL; PBS, phosphate-buffered saline.

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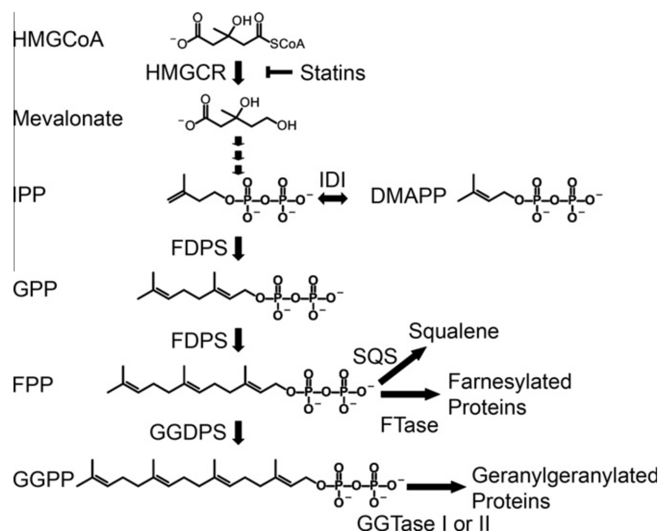

Fig.1. Isoprenoid biosynthetic pathway. IPP, FPP, and GGPP are key intermediates in the IBP. See abbreviations note for abbreviations.

proteins, including members of the Ras GTPase superfamily [6]. Many of these isoprenylated small GTPases play important roles in signal transduction pathways [7].

The association between human disease and various isoprenoid-derived compounds has encouraged the design and development of inhibitors for specific enzymes of the IBP. Indeed, the statins, which inhibit HMGCR, are used clinically in treatment of hypercholesteremia. By targeting HMGCR, statins deplete mevalonate and other downstream isoprenoid products, including cholesterol [8]. Nitrogenous bisphosphonates (NBPs) are used in treatment of osteoporosis and other bone-associated diseases. These agents are thought to inhibit FDPS, thereby depleting FPP and FPP-derived products [9]. Other efforts have focused on targeting other isoprenoid biosynthetic enzymes such as SQS, FTase, and the GGTases [4,10]. Our laboratory has recently developed a series of GGDPS inhibitors [11–13] and evaluated the cellular consequences of GGPP depletion [14–17]. Although the focus with these inhibitors has been largely on depletion of downstream products, numerous threads of evidence suggest that accumulation of upstream substrates is also relevant [18–20]. For example, treatment of mice with inhibitors of SQS leads to large increases in urinary excretion of FPP derivatives [21]. In addition, we have shown that SQS inhibitors dramatically increase intracellular levels of FPP [22]. This is important because FPP and its derivatives, such as farnesol, are known to induce apoptosis when elevated [23]. As more agents target isoprenoid biosynthesis, it will be necessary to understand the effects on upstream isoprenoid levels and how they relate to the resulting cellular consequences.

IPP and its isomer DMAPP not only serve as the 5-carbon building block for the biosynthesis of GPP, FPP, GGPP, and longer chain isoprenoids but also are directly involved in cellular activities. For instance, a study showed that IPP may serve as an antinociceptive substance that inhibits TRPA1 and TRPV3 ion channels [24]. In addition, the isopentenylation of adenosine in certain transfer RNAs (tRNAs) can be required for translational efficiency and fidelity [25]. Furthermore, the isopentenylation of adenosine 37 is also necessary for the seleneocysteine tRNA [26]. Seleneocysteine is the 21st amino acid that is necessary for the synthesis of seleneoproteins, many of which are involved in cellular antioxidant functions [26]. Cellular inhibition of FDPS by the NBPs leads to accumulation of IPP and subsequent activation of $CD56^+$ γδ-T lymphocytes that display potent anticancer activity [27]. The formation of an IPP and/or DMAPP adduct with AMP has been reported when MCF-7 cells were treated with zoledronate [28]. This ATP analogue is thought to be pro-apoptotic.

Recently, we reported a sensitive and nonradioactive analytical method to quantify FPP or GGPP in biological samples by high-performance liquid chromatography (HPLC) [22,29]. The method is based on the incorporation of FPP or GGPP into fluorescent peptides catalyzed by either FTase or GGTase I, respectively. This method has allowed for successful determination of FPP and GGPP levels in cultured cells and animal tissues [22,29]. The sensitivity of this method allows for detection of changes in FPP and GGPP levels in cultured cells in the presence or absence of IBP inhibitors [11,29–32].

The current IPP quantification methods from the literature rely on radioactive tracing or are measured as the alcohol form, isopentenol, by gas chromatography–mass spectrometry (GC–MS) or are measured directly by liquid chromatography–tandem MS (LC–MS/MS) [33,34]. These methods have been used to describe the intracellular IPP concentration after incubation with NBPs, but basal levels of IPP have not been reported [35,36]. The main challenge in developing a routine quantification method for IPP analysis in biological samples is that the concentration of IPP in mammalian cells or tissues is very low. In addition, IPP may be rapidly converted to DMAPP or FPP. Furthermore, the instability of IPP is also a problem during sample preparation. To our knowledge, an established method for measuring basal levels of IPP from mammalian cells has yet to be reported. In the current article, we describe a newly developed analytical procedure (Fig. 2) that can be used to measure IPP levels from cultured cells in the presence or absence of IBP inhibitors.

Materials and methods

Cell culture and reagents

MCF-7 and K562 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The K562 cell line is a human erythroleukemia line established from a patient with chronic myelogenous leukemia, whereas the MCF-7 cells are a breast cancer cell line [37,38]. K562 and MCF-7 cells were grown in RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM), respectively. Both cell lines were supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, amphotericin (2.5 μg/ml), and glutamine (2 mM) and were cultured at 37 °C in the presence of 5% carbon dioxide. IPP, FPP, GGPP, *n*-octyl β-glucopyranoside, and lovastatin were purchased from Sigma (St. Louis, MO,

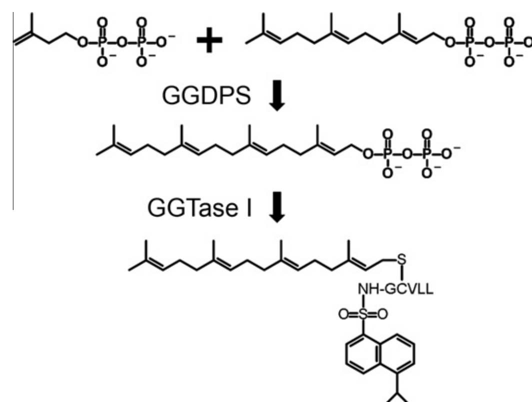

Fig.2. Assay scheme for quantification of IPP by conversion to GGPP and transfer to a fluorescent peptide. Under the catalysis of GGDPS, IPP reacts with FPP (great access) to form GGPP. Then, GGPP and D*-DCVLL are coupled by GGTase I to form a fluorescence product, which could be analyzed by HPLC.

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