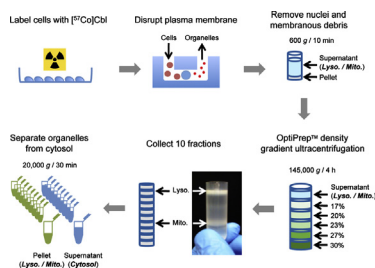Basic Neuroscience

Analysis of subcellular [57Co] cobalamin distribution in SH-SY5Y neurons and brain tissue

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HIGHLIGHTS

- Methods were developed that can be used to accurately measure [57Co] Cbl levels in lysosomes, mitochondria and cytosol obtained from neuronal and fibroblast cell lines and mouse brain tissue.
- The relative distribution of [57Co] Cbl in neurons and fibroblasts was 6% in the lysosomes, 14% in the mitochondria and 80% in the cytosol.
- This technique was also used to fractionate organelles obtained from mouse brain, and the relative distribution of [57Co] Cbl was 12% in the lysosomes, 15% in the mitochondria and 73% in the cytosol.
- The establishment of subcellular fractionation methods herein provides a useful tool for the investigation of intracellular Cbl trafficking. This method can be adapted to study the effects of age- or neuropathology-related lysosomal dysfunction on intracellular [57Co] Cbl transport in cells and animal brain tissues.

GRAPHICAL ABSTRACT

ARTICLE INFO

ABSTRACT

Cobalamin (Cbl) utilization as a cofactor for methionine synthase and methylmalonyl-CoA mutase is dependent on the transport of Cbl through lysosomes and its subsequent delivery to the cytosol and mitochondria. We speculated that neuropathological conditions that impair lysosomal function (e.g., age-related lipofuscinosis and specific neurodegenerative diseases) might impair lysosomal Cbl transport. To address this question, an appropriate method to quantify intracellular Cbl transport in neuronal cell types and brain tissue is required. Thus, we developed methods to measure [57Co] Cbl levels in lysosomes, mitochondria and cytosol obtained from in vitro and in vivo sources. Human SH-SY5Y neurons or HT1080 fibroblasts were labeled with [57Co] Cbl and homogenized using a ball-bearing homogenizer, and the lysates were separated into 10 fractions using ultracentrifugation in an OptiPrep density gradient. Lysosomes were recovered from the top of the gradient (fractions 1–5), which were clearly separated from mitochondria (fractions 7–9) on the basis of the expression of the marker proteins, LAMP2

and VDAC1. The isolated lysosomes were intact based on their colocalization with acid phosphatase activity. The lysosomal and mitochondrial fractions were free of the cytosolic markers beta-actin and methionine synthase. The relative distribution of [57Co] Cbl in both neurons and fibroblasts was as follows: 6% in the lysosomes, 14% in the mitochondria and 80% in the cytosol. This technique was also used to fractionate organelles from mouse brain, where marker proteins were detected in the gradient at positions similar to those observed for the cell lines, and the relative distribution of [57Co] Cbl was as follows: 12% in the lysosomes, 15% in the mitochondria and 73% in the cytosol. These methods provide a useful tool for the investigation of intracellular Cbl trafficking in a neurobiological setting.

1. Introduction

Cobalamin (Cbl) is required for erythrocyte formation and DNA synthesis and plays a crucial role in the maintenance of neurological function. Methyl Cbl (MeCbl) and adenosyl Cbl (AdoCbl) are the forms that are active in human metabolism. These two forms of Cbl differ only in the functional group that is attached to the Co atom at the center of the Cbl corrin ring. MeCbl is used to transform homocysteine (Hcy) into methionine (Met) via cytosolic methionine synthase (MS). This reaction requires the donation of a methyl group derived from the transformation of 5-methyltetrahydrofolate (5-methyl-THF) into tetrahydrofolate (THF). Via the action of methionine adenosyltransferase, Met is utilized in the formation of S-adenosylmethionine (SAM), a universal methyl donor for numerous substrates, including DNA, RNA, hormones, proteins, and lipids. AdoCbl is required for the conversion of methylmalonyl-coenzyme A (Mm-CoA) to succinyl-coenzyme A (Succ-CoA) via mitochondrial Mm-CoA mutase (MMCM). Succ-CoA then enters the Krebs cycle, after which it may be utilized in many pathways, including the conversion to succinate, which may be used as an electron donor or in the synthesis of porphyrins such as heme.

MS and MMCM activities are reduced in human Cbl-deficiency states, causing increased tissue and plasma Hcy levels and, subsequent to the conversion of Mm-CoA to methylmalonic acid (MMA), an increase in tissue and plasma MMA levels. Because the roles of both MS and MMCM in methylation reactions and multiple pathways are related to, for example, amino acid and lipid metabolism, the clinical Cbl-deficiency phenotypes are multifaceted (Baik and Russell, 1999). In addition to the "loss of function" caused by impaired MS and MMCM activities, the accumulation of Hcy and MMA is neurotoxic and is thought to contribute to neurodegeneration and a loss of cognitive capacity (Kolker et al., 2000; Morris, 2003).

Cbl utilization is dependent on its transit via the intracellular lysosomal compartment (Banerjee et al., 2009; Gailus et al., 2010; Zhao et al., 2011). We have recently proposed that pathophysiological impairment of lysosomal functions that occurs in various conditions, such as Alzheimer's disease, lysosomal storage disorders and age-related neuronal lipofuscinosis, may form a "road block" for efficient Cbl utilization in cells. We predicted that these conditions may impair the release of Cbl from its carrier protein transcobalamin-II (TCII) and inhibit the transport of lysosomal Cbl to both MS and MMCM (Zhao et al., 2011). In addition, it is known that the acidic pH of the lysosome also influences the conversion of Cbl from the "base-on" to "base-off" state, which refers to the interaction of the dimethylbenzimidazole moiety of the Cbl molecule with the central Co atom (Banerjee, 2006). The Cbl base-off state is thought to be important for subsequent interactions with cytosolic cargo proteins.

To accurately assess the trafficking of Cbl via the lysosomal compartment, it is necessary to establish subcellular fractionation methods that clearly separate lysosomes from the intracellular compartments that contain MS and MMCM, i.e., the cytosol and mitochondria, respectively. Thus, the aim of the current experiments was to develop subcellular fractionation methods whereby cells and brain tissues could be metabolically labeled with [57Co] Cbl to quantify Cbl distribution between lysosomes, mitochondria and the cytosol.

2. Materials and methods

2.1. Cell culture

Experiments were performed using human neuroblastoma cells (SH-SY5Y, ATCC #CRL-2266) and human fibrosarcoma cells (HT1080, ATCC #CCL-121), which were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 1% glutamine at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were grown in four 175 cm² plastic flasks until the cells were approximately 70% confluent. The cells were then incubated with [57Co] cyanoCbl (0.025 μCi/ml; Cat. No. 06B-430002, MP Biomedicals, USA) in DMEM with 10% human serum, 1% penicillin/streptomycin and 1% glutamine for 48 h. The cells were then rinsed with cold (10 °C) PBS, harvested with 1% trypsin, and centrifuged at $600 \times g$ for 5 min at 4 °C. A small portion of the cells was stained with 0.5% trypan blue to determine the number of viable cells.

2.2. Cell homogenization

A lysosome enrichment kit (Pierce, Cat #89839, USA) was applied to perform subcellular fractionation. An 800 μl aliquot of extraction buffer Lysosome Enrichment Reagent (LER) "A" containing 1% protease inhibitors was added to the cell pellets. The pellets were gently re-suspended and incubated on ice for no more than 2 min The cell suspension was transferred to a ball-bearing cell homogenizer (Isobiotec, Germany) and homogenized on ice. To confirm lysis efficiency, 10 μl of cell lysate was stained with 0.5% trypan blue and viewed under a light microscope. Homogenization was continued until 95% cell membrane breakage was achieved (typically 10–15 passages through the homogenizer). Next, the lysed cells were transferred into a 2 ml microcentrifuge tube, and 800 μl of LER "B" containing 1% protease inhibitors was mixed with the lysed cells. The mixed cell lysates were then centrifuged at $600 \times g$ for 10 min at 4 °C to remove nuclei and membranous debris, and the supernatant (1500 μl) containing lysosomes, mitochondria and cytosol was collected.

2.3. Density gradient ultracentrifugation

To prepare a discontinuous density gradient, five gradient solutions were prepared by mixing gradient dilution buffer (a 1:1 mixture of LER A and LER B) with the OptiPrep medium (which is supplied as a 60% solution in H_2O) as described in Table 1. The diluted OptiPrep density gradient solutions were carefully overlaid in descending concentration order (i.e., 30% OptiPrep solution first and then 27%, 23%, 20% and 17%) in a 7 ml ultracentrifuge