Biochemistry and Biophysics Reports 11 (2017) 105–111

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Dynamics of absorption, metabolism, and excretion of 5-aminolevulinic acid in human intestinal Caco-2 cells

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ARTICLE INFO

Keywords:
5-Aminolevulinic acid
Caco-2 cell
Heme oxygenase 1
Bach1

ABSTRACT

5-Aminolevulinic acid (ALA) is a precursor for the biosynthesis of porphyrins and heme. Although the oral administration of ALA has been widely applied in clinical settings, the dynamics of its absorption, metabolism, and excretion within enterocytes remain unknown. In this study, after enterocytic differentiation, Caco-2 cells were incubated with 200 μM ALA and/or 100 μM sodium ferrous citrate (SFC) for up to 72 h. Both ALA and the combination of ALA and SFC promoted the synthesis of heme, without affecting the expression of genes involved in intestinal iron transport, such as *DMT1* and *FPN*. The enhanced heme synthesis in Caco-2 cells was more pronounced under the effect of the combination of ALA and SFC than under the effect of ALA alone, as reflected by the induced expression of heme oxygenase 1 (HO-1), as well as a reduced protein level of the transcriptional corepressor Bach1. Chromatin immunoprecipitation analysis confirmed Bach1 chromatin occupancy at the enhancer regions of HO-1, which were significantly decreased by the addition of ALA and SFC. Finally, Transwell culture of Caco-2 cells suggested that the administered ALA to the intestinal lumen was partially transported into vasolateral space. These findings enhance our understanding of the absorption and metabolism of ALA in enterocytes, which could aid in the development of a treatment strategy for various conditions such as anemia.

1. Introduction

5-Aminolevulinic acid (ALA) is an important precursor of heme. This compound is synthesized from glycine and succinyl-CoA in mitochondria; this process is catalyzed by two different ALA synthases (ALAS): one expressed ubiquitously (ALAS1) and the other expressed only by erythroid precursors (ALAS2) [1]. During synthesis, ALA is converted to protoporphyrin IX, and heme is generated by the insertion of ferrous iron into protoporphyrin IX. The oral administration of ALA has recently been widely used in various clinical settings. For example, because porphyrin is known to be a strong photosensitizer, ALA has been used to diagnose and treat various cancers [2]. Furthermore, ALA was expected to have therapeutic effects on some types of anemia. It has been suggested that ALA may represent a novel therapeutic option for congenital sideroblastic anemia (CSA), attributable to the mutation of ALAS2, which converts glycine and acetyl-coenzyme A to generate ALA [3]. In addition to ALA, some studies have suggested the efficacy of orally administering the combination of ALA and iron for pre-diabetic subjects in reducing serum glucose levels [4,5]. The increase in the heme synthesis in the body by the combination of ALA and iron, rather than ALA alone, was estimated to have a beneficial effect on the serum glucose levels [4,5]. Whereas pharmacokinetic studies of ALA have demonstrated good oral bioavailability [6–8], other reports have suggested that the administration could cause an excessive accumulation of ALA in enterocytes [9,10]. In addition, the detailed differences of the combination of ALA and iron versus ALA only on the enterocytes remain unknown. Thus, there is a need to clarify the dynamics of absorption, metabolism, and excretion of ALA and/or iron in enterocytes.

Herein, based on a model of enterocytic differentiation using human intestinal Caco-2 cells, we evaluated the effects of ALA on enterocytes.

Abbreviations: ALA, 5-aminolevulinic acid; ALAS2, 5-aminolevulinic acid synthase 2; ChIP, chromatin immunoprecipitation; CSA, congenital sideroblastic anemia; DMT1, divalent metal transporter 1; FPN, ferroportin; HO-1, heme oxygenase 1; PP IX, protoporphyrin IX; RT-PCR, reverse transcription polymerase chain reaction; SFC, sodium ferrous citrate

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http://dx.doi.org/10.1016/j.bbrep.2017.07.006
Received 1 March 2017; Received in revised form 6 July 2017; Accepted 11 July 2017
Available online 13 July 2017

2. Materials and methods

2.1. Cell culture and reagents

Caco-2 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were grown in a humidified incubator at 37 °C with 5% carbon dioxide, and maintained in a DMEM medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (Biowest, Miami, FL). ALA hydrochloride and sodium ferrous citrate (SFC) (SBI Pharmaceuticals Co., Ltd., Tokyo, Japan) were prepared using distilled water. Cells were seeded at a density of 1×10^4 cells/cm^2 onto 60-mm plastic flasks or 6-well Transwell permeable support plates (Costar, Corning Inc., New York, USA), and cultured for 21 days. The medium was refreshed every 2–3 days. Cells were incubated with a medium containing 200 μM ALA and/or 100 μM SFC for a culture period of up to 72 h. ALA and/or SFC were added to the upper chamber to the Transwell inserts, corresponding to the intestinal lumen. The cells used for all experiments are described here in passages 24–35.

2.2. Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was purified using TRIzol (Invitrogen) and 1 μg of the purified total RNA was used to synthesize complementary DNA (cDNA) with ReverTra Ace qPCR RT Master Mix (Toyobo). Reaction mixtures (20 μL) for real-time quantitative RT-PCR comprised 2 μL of cDNA, 10 μL of Quantitect SYBR Green PCR Master Mix (Qiagen), and 8 μL of the appropriate primers. Product accumulation was monitored by measuring SYBR Green fluorescence and normalized relative to GAPDH messenger RNA (mRNA). We used the following primers: DMT1, AGCAGGCCTTTAGAGATGCTTA and ATTATATGTGGTGGCTGCTGTG; FPN, CCTGTTAACAAGCACCTCAGC and TTGCAGAGGTCAGGTAGTCG; ALAS1, GGCAGCACAGATGAATCAGA and CCTCCATCGGTTTTCACACT; heme oxygenase 1 (HO-1), ATGAACTCCCTGGAGATGACTC and CCTTGGTGTCATGGGTCAG; GAPDH, GAAGGTCGGAGTCAACGGATTT and GAATTTGCCATGGGTGGAAT; solute carrier family 36 (proton/amino acid symporter), member (SLC36A1), GACTACCACGACTACAGCTCCA and CCTTTTAACAGGTGGATCAAGG; solute carrier family 15 (oligopeptide transporter), member 1 (SLC15A1), ATACGTTTGTGGCTCTGTGCTA and TTACTGAGGTGACTGCTTGTCC. To evaluate absolute expression levels of human ALA transporters (SLC36A1 and SLC15A1), an amplified cDNA fragment of each gene was cloned into the pGEM™-T Easy Vector (Promega, Madison, WI), and was used as an internal standard in quantitative RT-PCR. The plasmid copy number was calculated as follows: copy number (copy/μL) = $6.02 \times 10^{23} \times$ [plasmid DNA concentration (μg/μL)] $\times 10^{-6}$/[total plasmid size (base pair)] $\times$ 660, as described previously [3].

2.3. Intracellular heme and protoporphyrin IX content

Intracellular heme content was determined fluorometrically, as described previously [11]. In brief, cell pellets were suspended in 2 M oxalic acid and boiled (100 °C) for 30 min to dissociate protoporphyrin IX and iron from heme. The fluorescence for protoporphyrin IX was then measured at 400 nm (excitation) and 662 nm (emission). To exclude endogenous levels of protoporphyrin IX, the fluorescence of unboiled samples was subtracted.

2.4. Measurement of ALA, total porphyrin, and heme

Heme content in medium was determined using a chemiluminescence assay as described previously [12]. In brief, the same amounts of medium and chemiluminescence detection reagents (Pierce western blotting substrate plus; ThermoFisher, USA) were mixed and incubated at room temperature for 5 min. Chemiluminescence intensities were then measured using a luminometer (GloMax 20/20; Luminometer, Promega, USA).

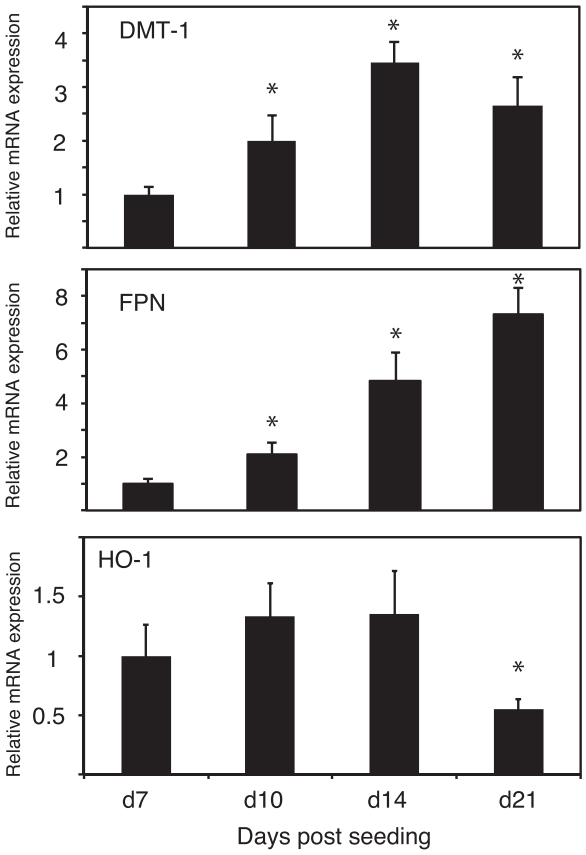

Fig. 1. Differentiation of human intestinal Caco-2 cells. Quantitative RT-PCR analysis of DMT1, FPN, and HO-1 in Caco-2 cells during days 7–21 after seeding. Data are expressed as mean ± standard error (SE). n = 3; *, $p < 0.05$. Asterisks indicate levels that are statistically significantly different from those on day 7.

ALA levels in medium were analyzed by synthesizing a fluorescent ALA derivative and measuring its concentration with a fluorometric HPLC system, as described previously [13]. Porphyrin levels in medium were also measured with the HPLC system, as described previously [14].

2.5. Western blotting

Western blotting was conducted as described previously [15,16]. Antibodies for ferritin (ab75973) and HO-1 (ab 13248) were purchased from Abcam (Cambridge, UK). The antibody for Bach1 was a generous gift from Prof. Kazuhiko Igarashi (Tohoku University, Japan). A densitrometric analysis of Western blot was conducted with ImageJ software (http://rsbweb.nih.gov/ij/). For calculating relative intensity, control samples were set to 1.

2.6. Quantitative ChIP analysis

Real-time PCR-based quantitative chromatin immunoprecipitation (ChIP) analysis was conducted as described previously [17]. We used the following primers: HO-1 E1, CATTTCTGCTGCGTCATGTT and GAGGCTTCTGCCGTTTTCTA; and HO-1 E2, CCCTGCTGAGTAATCCTTTCC and GGCGGTGACTTAGCGAAAAT. Primer sequences for the *RPII215* and *NECDIN* promoters were as previously reported [16].

2.7. Statistics

Statistical significance was assessed by one-way ANOVA followed by Tukey's post hoc test. In all analyses, differences were considered significant at $p < 0.05$.