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5-Aminolevulinic acid combined with ferrous iron enhances the expression of heme oxygenase-1

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ABSTRACT

5-Aminolevulinic acid (5-ALA) is the naturally occurring metabolic precursor of heme. Heme negatively regulates the Maf recognition element (MARE) binding- and repressing-activity of the Bach1 transcription factor through its direct binding to Bach1. Heme oxygenase (HO)-1 is an inducible enzyme that catalyzes the rate-limiting step in the oxidative degradation of heme to free iron, biliverdin and carbon monoxide. These metabolites of heme protect against apoptosis, inflammation and oxidative stress. Monocytes and macrophages play a critical role in the initiation, maintenance and resolution of inflammation. Therefore, the regulation of inflammation in macrophages is an important target under various pathophysiological conditions. In order to address the question of what is responsible for the anti-inflammatory effects of 5-ALA, the induction of HO-1 expression by 5-ALA and sodium ferrous citrate (SFC) was examined in macrophage cell line (RAW264 cells). HO-1 expression induced by 5-ALA combined with SFC (5-ALA/SFC) was partially inhibited by MEK/ERK and p38 MAPK inhibitor. The NF-E2-related factor 2 (Nrf2) was activated and translocated from the cytosol to the nucleus in response to 5-ALA/SFC. Nrf2-specific siRNA reduced the HO-1 expression. In addition, 5-ALA/SFC increased the intracellular levels of heme in cells. The increased heme indicated that the inactivation of Bach1 by heme supports the upregulation of HO-1 expression. Taken together, our data suggest that the exposure of 5-ALA/SFC to RAW264 cells enhances the HO-1 expression via MAPK activation along with the negative regulation of Bach1.

1. Introduction

5-Aminolevulinic acid (5-ALA), a natural amino acid, is synthesized through the condensation of glycine and succinyl–CoA by the catalytic effect of 5-ALA synthase. In the cytosol, 5-ALA sequentially generates porphobilinogen, hydroxymethylbilane, uroporphyrinogen III and finally coproporphyrinogen III. In the mitochondrion, coproporphyrinogen III is metabolized to coproporphyrinogen III, protoporphyrinogen IX and protoporphyrin IX, into which iron is inserted via a ferrochelatase-catalyzed reaction, the latter resulting in the formation of heme [1–3].

Recent studies reported that 5-ALA induced the upregulation of heme oxygenase (HO)-1 mRNA levels [4,5]. HO is the rate-limiting enzyme in heme catabolism. It catalyzes the degradation of heme, thereby producing iron, carbon monoxide (CO) and biliverdin. HO-1 is the inducible isoform of HO [6] and HO-1 expression is induced in a number of cell types by a range of stress stimuli [7–14], including heme [10,15] and other metalloporphyrins [16,17]. Exposure of HO-1-deficient mice to endotoxin leads to increased hepatocellular necrosis, the upregulation of splenic proinflammatory cytokine secretion and higher mortality from endotoxic shock compared with wild-type animals [18]. In addition, a recent study with humans with HO-1-deficiency has strengthened the above finding that HO-1 plays an important role in counteracting the deleterious increase in inflammation and oxidative injury [19]. Similarly, an in vitro study confirmed that there is a reduction of stress resistance in HO-1-deficient cells [20].

The signaling mechanisms that activate transcription of HO-1 are poorly defined. Most studies have focused on the activation of the mitogen-activated protein kinases (MAPKs) related to cell growth and the stress response. Extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 pathways appear to be involved to some extent in the upregulation of HO-1 expression in response to diverse stimuli [21–23]. The MAPK signaling leads to the translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) to the nucleus

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[23]. Nrf2 is one of the basic leucine zipper (bZip) transcription factor [24]. Additionally, recent work has indicated that under basal conditions, Bach1 which is also a bZip transcription factor [25] formed heterodimers with MafK, and these heterodimers repressed the transcription of HO-1 gene by binding to the MARE in the HO-1 promoter [26].

Induction of HO-1 expression by 5-ALA has been reported [4,5], whereas little is known about whether MAPKs and both of bZip transcription factors are involved in the induction of HO-1 expression by 5-ALA. Thus, in the present study, the mechanism underlying the 5-ALA-induced HO-1 expression was investigated using a mouse macrophage cell line, RAW264. Our results demonstrated that the 5-ALA combined with SFC (5-ALA/SFC) induced HO-1 protein expression, and was partially related to the activation of ERK. In addition, the silencing of Nrf2 by specific siRNA reduced the levels of HO-1 protein expression induced by 5-ALA/SFC. On the other hand, the treatment of 5-ALA/SFC increased the internal cellular heme levels.

2. Materials and methods

2.1. Cell culture and regents

The mouse macrophage cell line, RAW264, was obtained from the RIKEN Cell Bank (Ibaraki, Japan). The 5-ALA/HCl (COSMO ALA Co., Ltd., Tokyo, Japan) and Fe^{2+} (SFC, sodium ferrous citrate) (Eisai Food & Chemical Co., Ltd., Tokyo, Japan) were dissolved in distilled water, and the molar ratio of the 5-ALA: Fe^{2+} was 1:0.5. The Fe^{2+} was diluted in distilled water immediately before use. Hemin and SB203580 were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). PD98059 were purchased from LC Laboratories (Woburn, MA). Stealth RNAi Negative Control Medium GC Duplex #2 was purchased from Life Technologies. Phospho-p44/42 MAPK (Thr202/Tyr204), Phospho-p38 MAPK (Thr180/Tyr182), Phospho-stress-activated protein kinase (SAPK)/JNK (Thr183/Tyr185), p44/42 MAPK, p38 MAPK and SAPK/JNK antibodies were purchased from Cell Signaling Technology Japan K.K. (Tokyo, Japan). The Nrf2, Bach1 and α-tubulin antibodies were purchased from Santa Cruz (San Diego, CA). The HO-1 antibody was purchased from Abcam (Cambridge, UK).

2.2. Protein preparation and Western blot analysis

Protein preparation and western blot analysis were performed as described previously with modification [27]. In brief, cell lysates were prepared by RIPA buffer (Wako) and total proteins (20 μg) were separated on 4–10% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membranes were incubated overnight with primary antibodies at room temperature with following dilutions; 1:4000 for the phospho-p44/42 MAPK (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), phospho-SAPK/JNK (Thr183/Tyr185), p44/42 MAPK, p38 MAPK, SAPK/JNK and HO-1 antibodies, and 1:2000 for the α-tubulin antibody. The ImageQuant LAS 4000 (GE Healthcare, Little Chalfont, UK) was used to measure the relative optical density of each specific band obtained after the Western blotting.

2.3. Cellular immunostaining

After fixation with 4% paraformaldehyde in PBS for 10 min at room temperature, cells were permeated with 0.5% Triton X-100 in PBS for 1 h. The cells were incubated with primary antibody for 2 h at room temperature, followed by incubation with a secondary antibody conjugated to FITC for 1 h at room temperature, and subsequent counterstaining with 0.2 μg/ml propidium iodide (PI) for 2 min at room temperature. Images were obtained using a confocal laser scan microscope (Olympus, Tokyo, Japan).

2.4. siRNA preparation and transfection

The siRNA specific for Nrf2 (Stealth siRNA MSS207018) and Bach1 (Stealth siRNA MSS202323) were purchased from Life Technologies. RAW264 cells were plated the day before transfections and grown to 30–40% confluence in 60-mm dishes. Transfections were carried

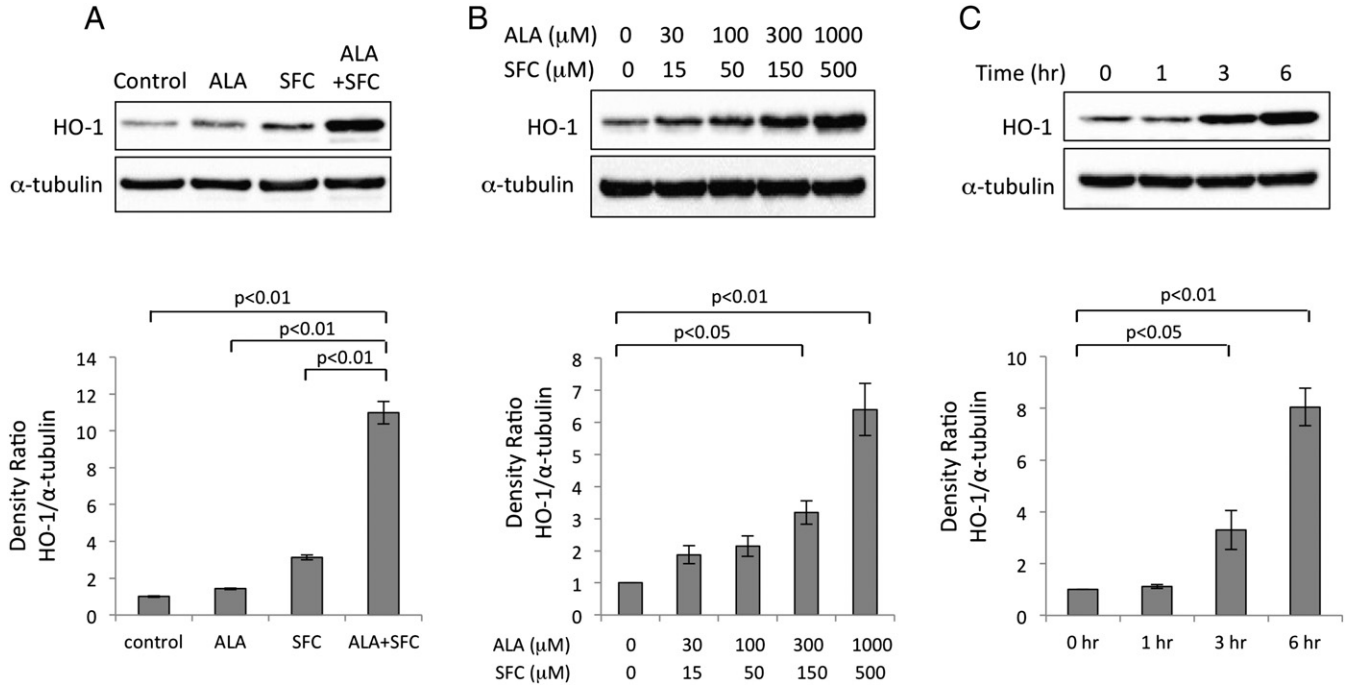

Fig. 1. *5-ALA/SFC induced HO-1 expression in RAW264 cells.* A) RAW264 cells were treated with or without 1000 μM 5-ALA and/or 500 μM SFC for 6 h. B) RAW264 cells were treated with 0–1000 μM 5-ALA and 0–500 μM SFC, as shown, and were cultured for 6 h. C) RAW264 cells were treated with 1000 μM 5-ALA and 500 μM SFC for the indicated periods. After treatment, the cells were harvested, total proteins were prepared and the levels of HO-1 expression were quantified by a Western blot analysis, as described in Materials and methods. The data are presented as the means ± SEM from three independent experiments.