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Sesamin increases heme oxygenase-1 protein in RAW 264.7 macrophages through inhibiting its ubiquitination process

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ABSTRACT

Sesamin is a major component in lignans of sesame seed oil, known to possess potent anti-oxidative capacity. In this study, the variation of heme oxygenase (HO)-1, a kind of anti-oxidative enzyme, by sesamin in murine macrophage cell line RAW 264.7 cells was investigated. Lipopolysaccharide (LPS; 10 μg/ml) exposure tended to increase HO-1 protein expression. Co-treatment with 100 μM sesamin for 12 h up-regulated the HO-1 protein level increased by LPS; however, HO-1 mRNA was unaffected. Sesamin delayed the reversal, by the protein synthesis inhibitor cycloheximide (1 μM), of the LPS-induced increase of HO-1 protein level. Meanwhile, sesamin suppressed LPS-induced expression of inducible nitric oxide (NO) synthase (iNOS) protein and associated NO release. LPS-induced increase of iNOS protein expression was also reversed by cycloheximide, which was not affected by sesamin, unlike HO-1. To clarify the mechanisms that underlie the up-regulation of HO-1 protein level by sesamin, the human embryonic kidney (HEK) 293 T cell line transfected with Flag-tagged HO-1 was used. A proteasome inhibitor, MG-132 (10 μM), stabilized HO-1 protein in HEK 293T cells. Co-treatment with sesamin decreased ubiquitinated HO-1 protein accumulation by MG-132. However, sesamin did not affect the proteasome activity. These findings suggest that sesamin disturbs the degradation of HO-1 protein through inhibiting its ubiquitination, resulting in HO-1 protein up-regulation.

1. Introduction

Lignans in sesame seed oil have been focused on due to emerging evidence of their potent anti-oxidative capacity. Among these lignans, it has been reported that co-ingestion of sesamin with α-tocopherol reduced blood cholesterol level in a rat hyperlipidemia model (Rogi et al., 2011). Moreover, sesamin decreased kinic acid-induced cell death by suppressing lipid peroxidation in adrenal pheochromocytoma cell line PC 12 cells (Hsieh et al., 2011).

Heme oxygenase (HO) is known as a rate-limiting enzyme that degrades heme to iron, carbon monoxide and biliverdin. Biliverdin is then immediately converted into bilirubin by biliverdin reductase (Tenhunen et al., 1968). HO is classified as an anti-oxidative enzyme since the products from heme degradation, such as bilirubin, have anti-oxidative capacity (Kirkby and Adin, 2006). Some isoforms of HO have been identified, for example, inducible HO-1, and constitutive HO-2 and -3 (Maines, 1997). HO-1 is induced against various stresses, resulting in self-protection (Keyse et al., 1990; Otterbein and Choi, 2000; Gozzelino et al., 2010). We previously demonstrated that thrombin evokes delayed HO-1 expression in microglia, a population resembling macrophages in the central nervous system, in organotypic corticostriatal slice cultures of rats (Ohnishi et al., 2010a). On the other hand, inducible nitric oxide (NO) synthase (iNOS), representing the activation of microglia/macrophages (Lyons et al., 1992; Ohnishi et al., 2013), induced by lipopolysaccharide (LPS) undergoes negative feedback by HO-1 (Ashino et al., 2008). Activated microglia/macrophages produce various cytokines, such as tumor necrosis factor-α, leading to the initiation of inflammatory events in peripheral tissues (Hecker et al., 1996; Ohnishi et al., 2010b). Our recent study has revealed that sesamin attenuates microglial activation, resulting in neuroprotection in in vivo rat intracerebral hemorrhage (Ohnishi et al., 2013). Taking these findings together,

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sesamin likely induces HO-1 in macrophages and shows anti-inflammatory activity because macrophages and microglia share a monocytic origin.

Here, we examined the presence or absence of HO-1 variation in the anti-inflammatory effect of sesamin and furthermore attempted to clarify the mechanism in detail using murine macrophage cell line RAW 264.7 cells and the human embryonic kidney (HEK) 293T cell line.

2. Materials and methods

2.1. Materials

Drugs and chemicals were obtained from Nacalai Tesque (Kyoto, Japan), unless otherwise indicated. LPS was obtained from Sigma-Aldrich (St. Louis, MO, USA). MG-132 and zinc (II) protoporphyrin IX (ZnPP IX) were obtained from Calbiochem (San Diego, CA, USA).

2.2. Cell cultures and drug treatment

Cells of the murine macrophage cell line RAW 264.7 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Japan Bioserum, Hiroshima, Japan), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Tokyo, Japan) and 3.5 g/L glucose at 37 °C in a humidified atmosphere containing 5% CO_2. DMEM without added glucose was used for HEK 293T cells. RAW 264.7 cells and HEK 293T cells were plated on 35 mm dishes at a concentration of 2×10^6 cells/ml or 2×10^5 cells/ml, respectively. The supplemented media were replaced with FBS-free media, and the cells were then treated with drugs for the indicated periods.

2.3. Lactate dehydrogenase (LDH) release assay

Released LDH was measured using a commercially available MTX-LDH kit (Kyokuto Seiyaku, Tokyo, Japan), according to the instructions of the manufacturer. Briefly, 75 μl of chromogenic component was applied to a 96-well plate, and then 25 μl of culture medium was added to each well. Incubation was carried out under dark conditions for 10 min at room temperature (RT). The reaction was stopped using 1 N HCl and absorbance at 540 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA).

2.4. Western blotting

The cells were washed with phosphate-buffered saline (PBS) and then dissolved in lysis buffer (20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 50 nM okadaic acid (for detection of phosphorylated protein only), 1% Nonidet P-40 and 10% glycerol). The lysates were centrifuged at 15,000*g* for 10 min at 4 °C. Next, the supernatants were boiled with Laemmli's sample buffer (124 mM Tris/HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 10% glycerol, 0.02% bromophenol blue and 4% 2-mercaptoethanol) for 5 min. The samples were then electrophoresed on 8% SDS-polyacrylamide gels and transferred to nitrocellulose or polyvinylidene fluoride membranes. These membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.2% Tween 20 (TBST) for 1 h at RT or overnight at 4 °C. After rinsing with TBST, the membranes were incubated with anti-HO-1 (1:5000; Stressgen Bioreagents, St. Louis, MO, USA), anti-iNOS (1:1000; BD Biosciences, San Diego, CA, USA), anti-p38 MAPK (1:1000; Cell Signaling, Beverly, MA, USA), anti-phospho-p38 MAPK (1:1000; Cell Signaling), anti-β-actin (1:5000; Sigma-Aldrich), anti-ubiquitin (1:500; Santa Cruz, CA, USA) and anti-Flag (1:1000; Sigma-Aldrich) as primary antibodies in TBST for 1 or 2 h at RT or overnight at 4 °C. After rinsing with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:5000; GE Healthcare, Buckinghamshire, UK) or anti-rabbit IgG (1:5000; GE Healthcare) as secondary antibodies for 1 h at RT. After rinsing with TBST, antigen–antibody complexes were detected with an enhanced chemiluminescence (ECL) system (GE Healthcare).

2.5. Quantitative reverse transcription-polymerase chain reaction (PCR)

Total RNA was prepared from RAW 264.7 cells according to the acid guanidinium thiocyanate-phenol-chloroform method. Complementary DNA (cDNA) was produced using MuLV reverse transcriptase (Applied Biosystems, Tokyo, Japan) and 50 pmol random primers and was applied to real-time PCR with a LightCycler 2.0 (Roche, Tokyo, Japan). Briefly, the given amount of reverse-transcribed cDNA corresponding to 0.1 μg of the original total RNA was used per reaction along with the LightCycler FastStart DNA Master SYBER Green I (Roche) with 1 μM primers shown in Table 1. The amounts of sample mRNA were determined from data relative to the control or the LPS treatment group (100%) according to the LightCycler software (Roche) and are expressed as values normalized by GAPDH.

2.6. Immunocytochemistry and nuclear staining

RAW 264.7 cells were plated on 18×18 mm^2 cover glasses (Matsunami, Osaka, Japan). Specimens were fixed with 4% paraformaldehyde (PFA), and blocked and permeabilized with 0.5% Triton X-100 in PBS containing 5% goat serum for 1 h at room temperature. Specimens were then incubated with anti-NF-κB (p65) antibody (1:100; Santa Cruz) overnight at 4 °C. After washing with PBS, specimens were incubated with Alexa Fluor 546 anti-mouse IgG (1:200; Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. In addition, specimens were incubated with 1 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Dojindo, Kumamoto, Japan) for 15 min at room temperature, followed by rinsing with PBS. Fluorescence signals were acquired through a confocal fluorescence microscope (Leica, Wetzlar, Germany).

2.7. Nitrite quantification

NO release was quantified as the concentration of nitrite in culture medium. Culture media were supplemented with an equal volume of Griess reagent (1% sulfanilamide and 0.1% N-(1-naphtyl)-ethylenediamine dihydrochloride in 2.5% phosphoric acid) and kept for 10 min at RT. The absorbance of produced diazonium compound was measured at 540 nm on a microplate reader (Bio-Rad). The amount of nitrite was calculated by reference to a standard curve obtained from the defined concentrations of sodium nitrite.

Table 1
List of primers used for PCR.

Gene	Primer sequence	
	Forward	Reverse
HO-1	5′-gctcgaatcaacactctg-3′	5′-ggtctttgtgttcctctg-3′
iNOS	5′-cagctgggctgtacaaacctt-3′	5′-cattggaagtgaagcgtttcg-3′
GAPDH	5′-agcccagaacatcatcatccctg-3′	5′-caccaccttcttgatgtcatc-3′