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Effect of (–)-epigallocatechin-3-gallate on anti-inflammatory response via heme oxygenase-1 induction during adipocyte–macrophage interactions

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Abstract In this study, we examined the effects of (–)-epigallocatechin-3-gallate (EGCG) on anti-inflammatory responses through the induction of heme oxygenase-1 (HO-1) in cocultured macrophages and adipocytes. EGCG significantly decreased the secretion of nitric oxide (NO) and monocyte chemoattractant protein-1 in the coculture of RAW 264.7 macrophages and differentiated 3T3-L1 adipocytes. In addition, EGCG inhibited the expression of inducible nitric oxide synthase in cocultured macrophages and peroxisome proliferator-activated receptor-gamma in cocultured adipocytes. Furthermore, the HO-1 expression showed an approximately 4-fold increase in cocultured adipocytes and an approximately 6-fold increase in cocultured macrophages. Lastly, HO-1 silencing induced NO generation in cocultured cells regardless of EGCG treatment. These results indicate that EGCG inhibited inflammatory responses by suppressing the production of proinflammatory cytokines through HO-1 induction during adipocyte–macrophage interaction.

Keywords: EGCG, macrophages, adipocytes, coculture, heme oxygenase-1

Introduction

Recently, several studies have suggested a close relationship between obesity and chronic low-grade inflammation in adipocyte (1,2). Adipocytes produce and secrete large amounts of adipokines, including monocyte chemoattractant protein-1 (MCP-1). Released adipokines recruit macrophages into the adipose tissue, and the infiltrated macrophages then enhance the inflammatory state. Macrophages promote inflammatory responses by releasing various types of inflammatory mediators such as nitric oxide (NO), prostaglandins, tumor necrosis factor-α (TNF-α), and interleukin-6 (3). Several studies showed that obesity and type-2 diabetes are related with inflammation of the adipose tissue (4,5). Therefore, anti-inflammatory compounds could contribute to improvement of obesity and its related disorders via suppression of the expression of proinflammatory factors in the adipose tissue.

Green tea contains various bioactive compounds. (–)-epigallocatechin-3-gallate (EGCG), in particular, is a major biological active component in green tea and has received abundant pharmacological attention because of its anti-inflammatory, anti-cancer, and anti-adipogenesis properties (6-8). Heme oxygenase-1 (HO-1), a rate-limiting enzyme, catalyzes heme to a biliverdin, carbon monoxide, and free iron. HO-1 and its catabolic heme products are known to regulate several biological responses such as oxidative stress and inflammation (9,10). EGCG can induce the expression of HO-1 in endothelial cells via p38 mitogen-activated protein kinases and nuclear factor erythroid 2-related factor 2 (11). In addition, the induction of HO-1 in adipocytes is associated with increased adiponectin levels and decreased proinflammatory cytokines (12,13). Therefore, we examined the anti-inflammatory responses of EGCG via the upregulation of HO-1 in cocultured adipocytes and macrophages.

Materials and Methods

Materials Dexamethasone, dimethyl sulfoxide (DMSO), EGCG, insulin, isobutylmethylxanthine (IBMX), 3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium (MTT), and Oil Red O (ORO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum (BS), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were purchased from Gibco BRL (Gaithersburg, MD, USA). An antibody to inducible

NO synthase (iNOS) was purchased from Cayman (Ann Arbor, MI, USA). Antibodies to β-actin and peroxisome proliferator-activated receptor γ (PPARγ) and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL™ reagents were purchased from GE Healthcare (Buckinghamshire, UK). All other reagents and solvents used were of analytical grade.

RAW 264.7 macrophage culture The RAW 264.7 mouse macrophage cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). RAW 264.7 macrophages cultured in DMEM contained 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Adipocyte differentiation and ORO staining 3T3-L1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The 3T3-L1 cells were cultured as previously described with minor modifications (14). In brief, 3T3-L1 cells were cultured in DMEM supplemented with 10% heat-inactivated BS to confluence. Adipocyte differentiation was induced by a mixture of IBMX (0.5 mM), dexamethasone (1 μM), and insulin (1 μg/mL) in DMEM containing 10% FBS on two days after cell confluence (referred to as day 0). On day 2nd, the medium was changed with DMEM containing 10% of FBS and insulin. On day 4th, the medium was replaced with DMEM supplemented with 10 % FBS only.

To measure the levels of intracellular lipids in differentiated adipocytes, ORO staining was performed on the differentiated 3T3-L1 adipocytes on day 6th as previously described (15). The differentiated 3T3-L1 adipocytes were rinsed with phosphate buffered saline (PBS) and fixed with 10% formaldehyde. The fixed adipocytes were then rinsed with distilled water. ORO staining solution (0.5% in isopropanol) was diluted with water to 60% (6 parts of ORO solution and 4 parts of water) and filtered before use. The cells were then incubated with ORO staining working solution for 20 min. Next, the plates were rinsed three or four times with distilled water. The staining dye in the adipocytes was eluted by adding isopropanol and its absorbance was measured at 500 nm.

MTT assay The cytotoxicity was evaluated using the MTT assay. RAW 264.7 cells (5×10^4 cells/well) were incubated in 96-well plates for 6 h, following which different concentrations of EGCG and lipopolysaccharide (LPS, 1 μg/mL) were added into the cells for 18 h. In addition, 3T3-L1 preadipocytes were differentiated in 48-well plates for 6 days with EGCG. After 18 h for macrophages and on day 6 for 3T3-L1 cells, MTT reagent in PBS (0.5 mg/mL) was added and incubated at 37°C. After 3 h, the culture medium was removed and the formazan was eluted with DMSO. Finally, the absorbance was measured at 550 nm.

Coculture of adipocytes and macrophages Adipocytes and macrophages were cocultured as previously described with minor modifications (3). 3T3-L1 cells were differentiated in 6-well plates, and the cells were incubated in serum-free DMEM for 24 h. Macrophages (8×10^5 cells) were then added onto the 3T3-L1 cells for 24 h with EGCG. We also used the transwell system (Costar Corning Inc., Corning, NY, USA) to obtain separately cocultured and differentiated 3T3-L1 cells and RAW 264.7 macrophages. After differentiation in the lower wells, 3T3-L1 adipocytes were rinsed with a serum-free medium and incubated with serum-free DMEM containing EGCG. Macrophages (8×10^5 cells) in serum-free medium containing EGCG were simultaneously added onto the insert wells with a pore size of 0.4 μm. After 24 h, the supernatants and each cell of the cocultures were harvested.

NO and MCP-1 assay The level of NO was determined by measuring the amount of nitrite. The culture medium (100 μL) was incubated with Griess reagent (50 μL) for 10 min. Then, the optical density was measured at 550 nm. Sodium nitrite was used as standard for NO concentration. MCP-1 concentration was determined using BD OptEIA kits for mouse MCP-1 (BD Biosciences Pharmingen, San Diego, CA, USA).

Western blot analysis Cocultured macrophages and adipocytes were harvested separately after 24 h. The collected cells were washed with PBS and resuspended in lysis buffer (iNtRON Biotech, Seongnam, Korea). The amount of protein was quantified using a bicinchoninic acid protein assay reagent (Thermo Scientific, Rockford, IL, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with a 10% separating gel. Then, the separated proteins were transferred onto nitrocellulose membranes. The membranes were incubated for 1 h in 5% skim milk solution and then incubated for 1 h in the primary antibodies diluted in the blocking buffer (1:1,000, v/v) at room temperature. The membranes were then rinsed with Tris-buffered saline with Tween-20 (TBST) and incubated in horseradish peroxidase-conjugated secondary antibodies diluted in the blocking buffer (1:1,000, v/v) for 1 h at room temperature. After washing the membranes with TBST, the protein bands were visualized using ECL™ detection reagents. The intensity of immunoblot bands was quantified using Image J software (NIH, Bethesda, MD USA).

HO-1 siRNA transfection HO-1 Stealth RNAi™ siRNA and negative control siRNA were obtained from Invitrogen (Carlsbad, CA, USA). The transfection mixtures of HO-1 Stealth RNAi™ siRNA or negative control siRNA were prepared using Lipofectamine 2000 (Invitrogen). The transfection mixtures of each siRNA were treated to adipocytes and macrophages, respectively. The supernatants were discarded and replaced with serum-free DMEM after 24 h of siRNA transfection. HO-1 siRNA-transfected macrophages and adipocytes were cocultured with EGCG for 24 h. The immunoblot analysis was conducted to evaluate silencing efficiency of HO-1.

Statistical analysis All data are representative of three independent experiments. All numerical data are presented as the means± standard errors. Statistical significance was assessed by Duncan's multiple range tests, according to which *p*-values<0.05 were considered significant.

Results and Discussion

The effects of EGCG on lipid accumulation in adipocytes and NO production by macrophages The MTT assay was performed to evaluate the cytotoxicity of EGCG in 3T3-L1 cells. No cytotoxicity was observed at any concentration of EGCG (Fig. 1A). Lipid accumulation is shown in Fig. 1B, and was measured by ORO staining of the differentiated 3T3-L1 adipocytes. EGCG significantly decreased lipid accumulation in the differentiated adipocytes. Thereafter, to examine whether EGCG inhibited NO production in LPS-stimulated RAW 264.7 macrophages, the cells were incubated for 18 h with various concentrations of EGCG and LPS (1 μg/mL). As shown in Fig. 1C, cytotoxicity of EGCG was not observed at any concentrations tested on RAW 264.7 macrophages. LPS-induced NO production was significantly inhibited by EGCG (Fig. 1D). In previous studies, it was shown that EGCG inhibited adipogenesis and stimulated fat cell apoptosis in 3T3-L1 cells (16,17). In addition, EGCG was shown to decrease NO production by reducing the expression of iNOS mRNA (18). NO is associated with inflammatory responses, and thus, the inhibition of NO production has become a target to ameliorate inflammatory disorders (19). Therefore, we investigated the effects of EGCG or inflammation in cocultured adipocytes and macrophages.

Anti-inflammatory responses of EGCG in cocultured adipocytes and macrophages The production levels of NO and MCP-1 were measured in coculture supernatants. The production of NO was very low when adipocytes and macrophages were cultured separately. NO production was elevated by the coculture of these cells; however, the treatment of EGCG significantly decreased NO production in the coculture system (Fig. 2A). Similarly, MCP-1 secretion was elevated in cocultures; however, treatment with EGCG decreased MCP-1 levels (Fig. 2B). The major sources of MCP-1 are the monocyte and macrophage; however, adipocytes have also been reported to produce MCP-1 (20). Adipocyte-derived free fatty acids (FFA), macrophage-derived TNF-α, and MCP-1 induce a vicious cycle of inflammation and insulin resistance in obese adipose tissue (3). In addition, inflammatory stress enhances lipid accumulation in hepatic cells (21). Macrophages infiltrate into obese adipose tissues through the induction of FFA and MCP-1, which are secreted from mature adipocytes (22). Therefore, these results suggest that EGCG has anti-inflammatory properties in obesity.

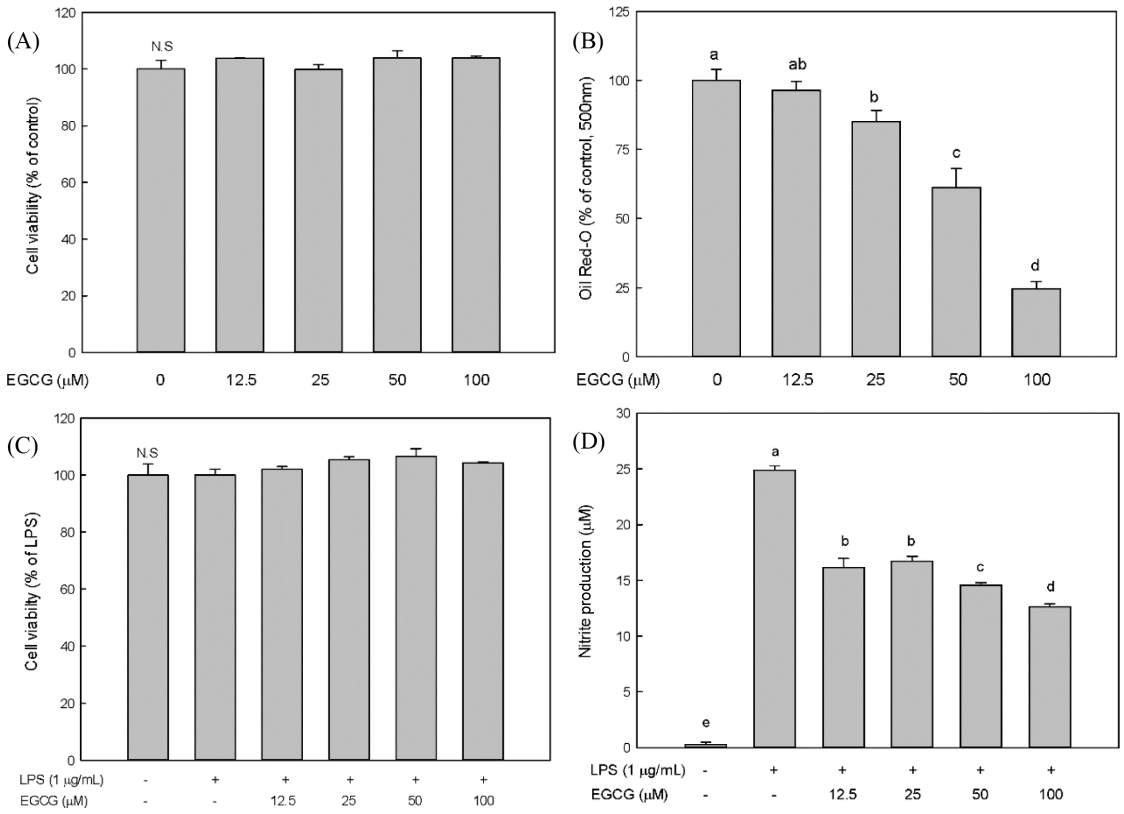

Fig. 1. Cell viability (A) and lipid accumulation (B) of EGCG treatment of 3T3-L1 adipocytes; and cell viability (C) and nitrite production (D) with EGCG treatment of LPS-stimulated RAW 264.7 macrophages. Each value is expressed as the mean±standard error (*n*=3). [a–e]Duncan's multiple range tests showed that the means corresponding to the different letters were significantly different compared to that of the control ($p<0.05$).

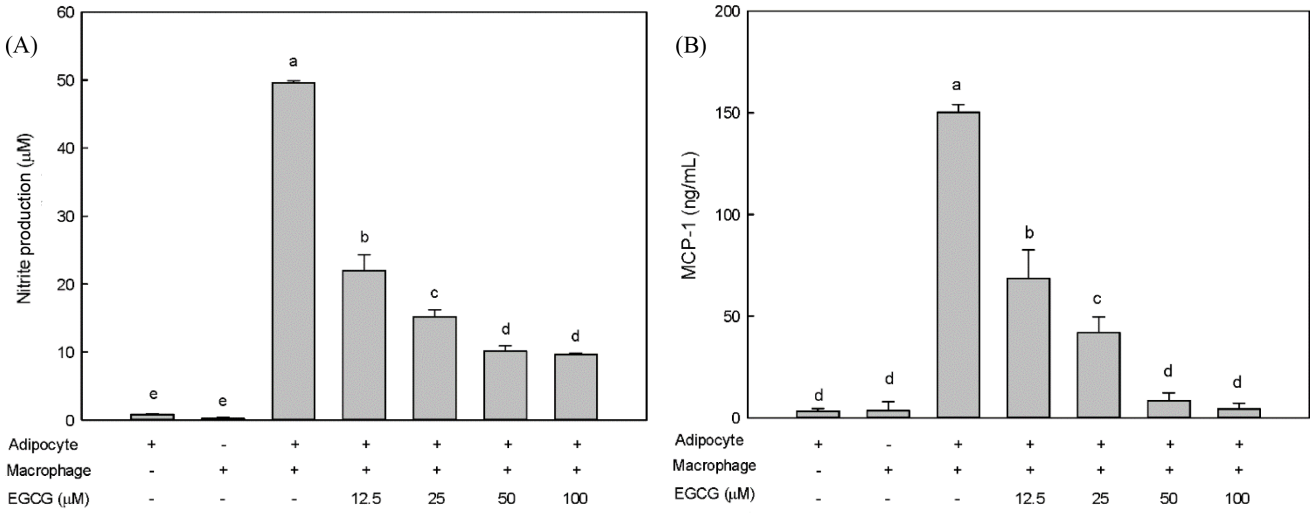

Fig. 2. Inhibition of nitrite production (A) and MCP-1 (B) production by EGCG in cocultured RAW 264.7 macrophages and differentiated 3T3-L1 adipocytes. Each value is expressed as the mean±standard error (n=3). $^{a-e}$Duncan's multiple range tests showed that the means corresponding to the different letters were significantly different compared to that of the control (p<0.05).

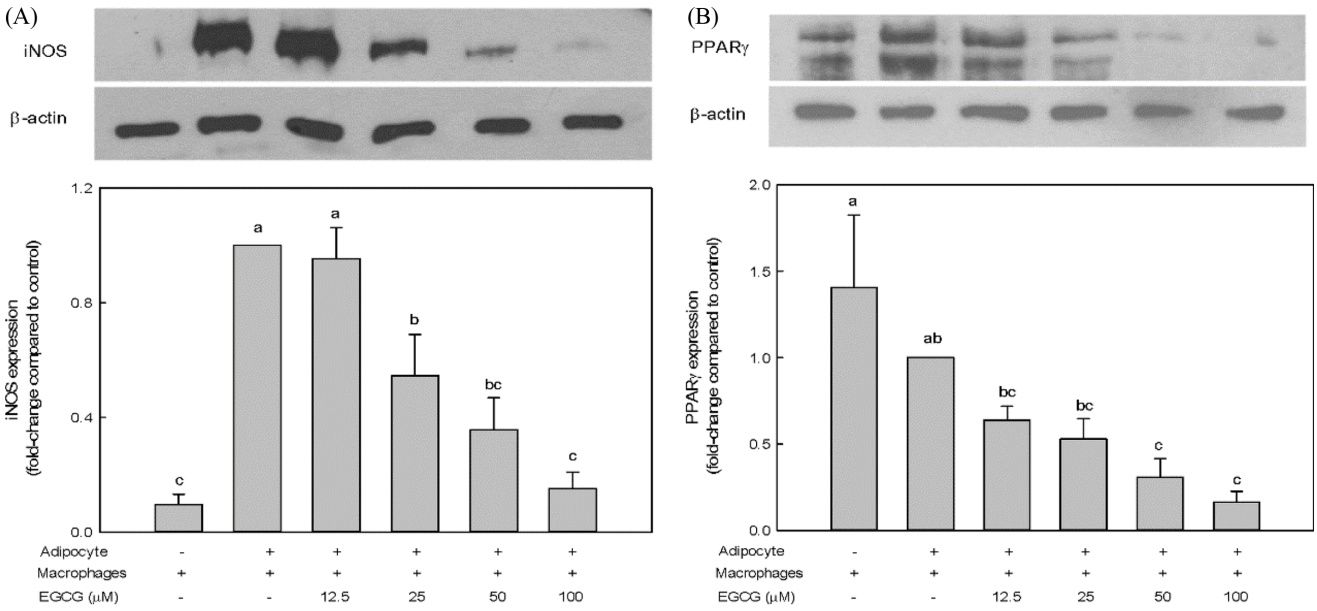

Fig. 3. Protein expression of iNOS in cocultured RAW 264.7 macrophages (A) and PPARγ in cocultured differentiated 3T3-L1 adipocytes. Each value is expressed as the mean±standard error (n=3). $^{a-c}$Duncan's multiple range tests showed that the means corresponding to the different letters were significantly different compared to that of the control (p<0.05).

Adipogenic and inflammatory protein expression in cocultured adipocytes and macrophages As the secretion of inflammatory mediators was induced by the coculture of adipocytes and macrophages, the effects of EGCG on protein expression in cocultured macrophages and adipocytes were evaluated. Increased iNOS protein expression was found in cocultured RAW 264.7 macrophages; however, treatment with EGCG significantly inhibited the expression of iNOS in a concentration-dependent manner (Fig. 3A). In particular, EGCG markedly suppressed iNOS expression at its highest concentration (100 μM). The large amounts of NO and the elevated expression of iNOS are considered as contributing factors to the pathogenesis of inflammatory diseases (23). Moreover, iNOS is well known to be associated with the overproduction of NO (24). Therefore, the results suggest that EGCG suppresses the inflammatory response through inhibition of iNOS expression in cocultured macrophages.

The expression of PPARγ by EGCG was evaluated in cocultured adipocytes. Treatment with EGCG significantly decreased PPARγ expression in cocultured adipocytes (Fig. 3B). PPARγ is a nuclear receptor that responds as a transcription factor and regulates the expression and transcription of adipokines in adipogenesis (25).

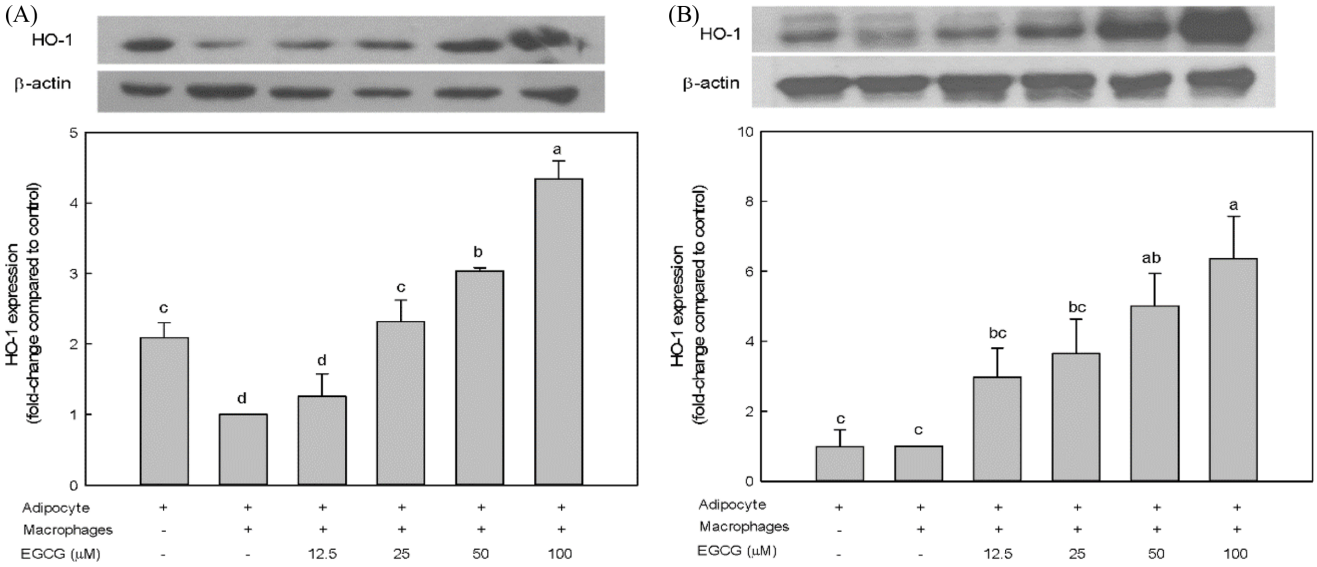

Fig. 4. HO-1 expression induced by EGCG in cocultured differentiated 3T3-L1 adipocytes (A) and in cocultured RAW 264.7 macrophages (B) using transwell system. Each value is expressed as the mean±standard error (n=3). $^{a-d}$Duncan's multiple range tests showed that the means corresponding to the different letters were significantly different compared to that of the control (p<0.05).

Recently, Pinent *et al.* (26) reported that isoflavones suppress the induction of several genes related to lipid and cholesterol metabolism as well as transcription factors linked to adipogenesis, including PPARγ in inflammation-induced 3T3-L1 adipocytes. Moreover, a previous study has highlighted the therapeutic potential of PPARγ in the prevention and treatment of obesity-associated inflammation and insulin resistance (27). Therefore, the results indicate that the down-regulation of PPARγ in cocultured adipocytes by EGCG may relate to the anti-inflammatory responses of adipocytes.

HO-1 expression and its role in cocultured adipocytes and macrophages As the upregulation of HO-1 represents an anti-inflammatory response, HO-1 expression levels were evaluated in EGCG-treated cocultured adipocytes and macrophages. The protein levels of HO-1 by EGCG were increased in a dose-dependent manner in cocultured adipocytes and macrophages (Fig. 4A and 4B). HO-1 is an anti-oxidative protein that plays a key role in heme catabolism and modulates various aspects of cellular responses to oxidative, inflammatory, and metabolic stresses (28). In adipocytes, the natural plant flavonoid 7,8-dihydroxyflavone increased the expression of HO-1 and exhibited an anti-obesity property through its antioxidant activity (29). Therefore, we examined the role of HO-1 in the coculture of adipocyte and macrophage using HO-1 silencing as EGCG increased HO-1 expression in both cocultured cell types. HO-1 siRNA was transfected into adipocytes and macrophages, respectively, and both cell types were then cocultured for 24 h with or without EGCG. The silencing of HO-1 was effectively induced by HO-1 siRNA transfection in both adipocytes (Fig. 5A) and macrophages (Fig. 5B). In addition, siRNA-mediated HO-1 silencing elevated the NO production in cocultured supernatants despite the presence of EGCG (Fig. 5C), whereas negative control siRNA transfection with EGCG treatment inhibited the secretion of NO. A previous study reported that HO-1 induction reduces adipogenesis and improved insulin sensitivity and glucose tolerance (30). Furthermore, a human clinical study reported that intramuscular HO-1 mRNA levels decreased in type-2 diabetic patients (31). Recently, treatment with the HO-1 inducer, cobalt protoporphyrin, was found to reduce adipose tissue volume and improve obesity-induced insulin resistance in mice (30). These results suggest that the induction of HO-1 is related with EGCG-mediated suppression of inflammatory responses in adipocyte–macrophage cocultures. Therefore, EGCG may be useful for the prevention and treatment of obesity.

In this study, we demonstrated the effects of EGCG against inflammation in cocultured adipocytes and macrophages. EGCG showed anti-inflammatory properties by inhibiting the production of proinflammatory mediators in cocultured adipocytes and macrophages. In addition, we demonstrated that EGCG induced HO-1 protein expression in cocultured adipocytes and macrophages, which was associated with the decreased production of NO. In conclusion, EGCG exerted its anti-inflammatory properties in adipocyte–macrophage cocultures via HO-1 induction. This study provides information on the anti-inflammatory properties of EGCG in adipocytes, which may be targeted to improve chronic inflammation in obesity.

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Disclosure The authors declare no conflict of interest.

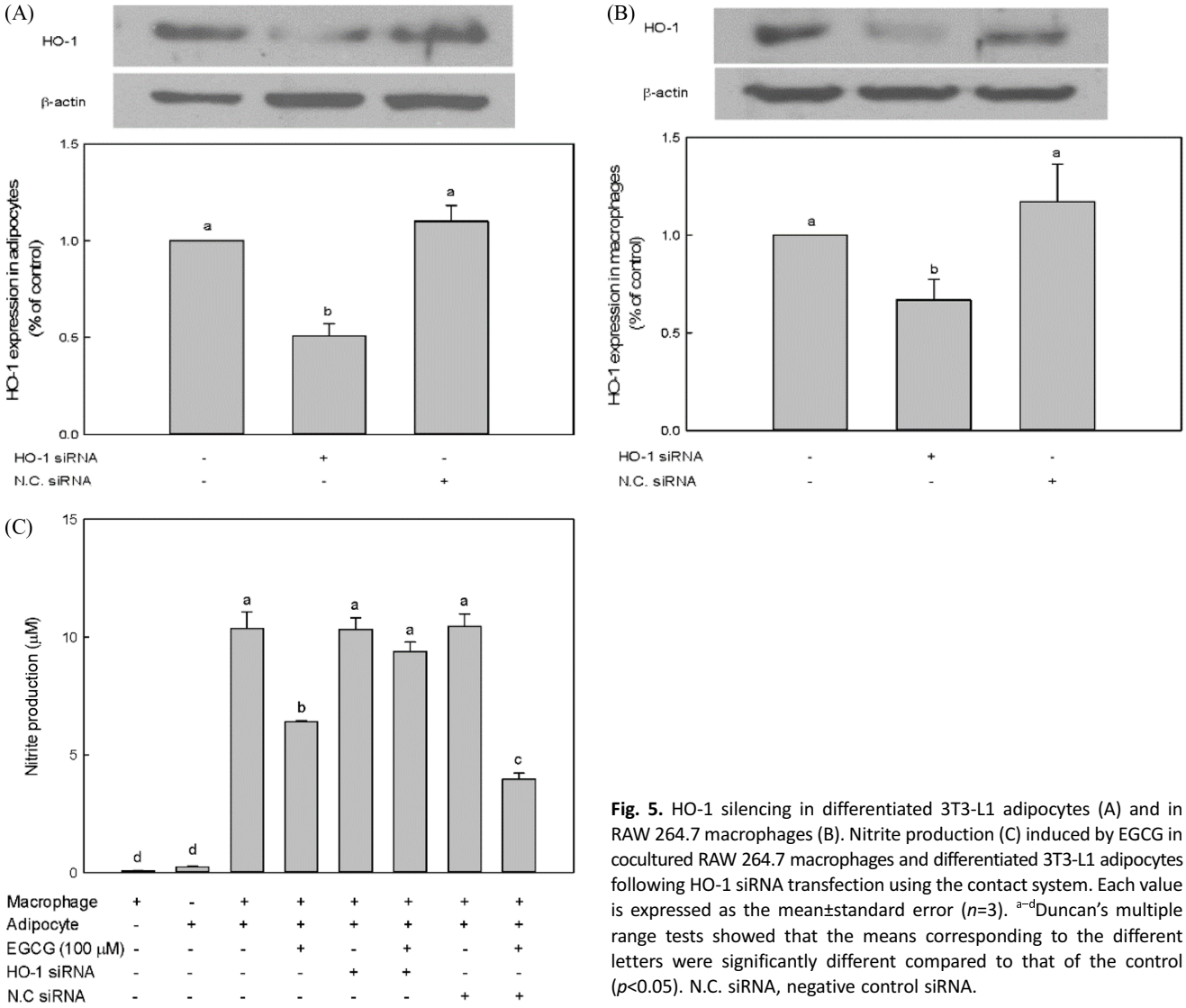

Fig. 5. HO-1 silencing in differentiated 3T3-L1 adipocytes (A) and in RAW 264.7 macrophages (B). Nitrite production (C) induced by EGCG in cocultured RAW 264.7 macrophages and differentiated 3T3-L1 adipocytes following HO-1 siRNA transfection using the contact system. Each value is expressed as the mean±standard error (n=3). $^{a-d}$Duncan's multiple range tests showed that the means corresponding to the different letters were significantly different compared to that of the control (p<0.05). N.C. siRNA, negative control siRNA.

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