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The Coffee Diterpene Kahweol Prevents Osteoclastogenesis via Impairment of NFATc1 Expression and Blocking of Erk Phosphorylation

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Abstract. Osteoclasts (OCLs) are multinucleated bone resorbing cells whose differentiation is regulated by receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). It is known that inflammatory cytokines and oxidative stress stimulate differentiation of OCLs. Here we evaluated the effects of kahweol, a coffee-specific diterpene, which has been reported to possess anti-oxidant and anti-inflammatory properties, on the differentiation of bone marrow–derived macrophages (BMMs) or murine monocytic cell line RAW-D cells into OCLs. Kahweol dose-dependently inhibited the formation of tartrate-resistant acid phosphatase staining–positive OCLs from both BMMs and RAW-D cells. In addition, kahweol prevented the bone resorbing activity of OCLs. Kahweol completely abolished RANKL-stimulated phosphorylation of extracellular signal-regulated kinase and impaired phosphorylation of Akt. Moreover, the protein levels of nuclear factor of activated T cells cytoplasmic-1 (NFATc1), a master regulator for OCL differentiation; and OCL markers transcriptionally regulated by NFATc1 such as Src and cathepsin K were down-regulated by kahweol treatment. As one of the molecular mechanisms for the inhibitory effects of kahweol, we also showed that kahweol up-regulated heme oxygenase-1 and inhibited high mobility group box 1 release. Thus, kahweol in coffee is a useful constituent for inhibition of OCL differentiation.

Keywords: osteoclast, kahweol, nuclear factor of activated T cells cytoplasmic-1 (NFATc1), extracellular signal-regulated kinase (Erk), receptor activator of nuclear factor kappa-B ligand (RANKL)

Introduction

Osteoclasts (OCLs) are multinucleated bone resorbing cells that are formed by the fusion of monocyte/macrophage precursor cells (1). The differentiation of OCLs is regulated by the 2 essential cytokines: receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). Association of RANKL with its receptor, receptor activator of nuclear factor kappa-B (RANK), leads to the activation of key signaling pathways in OCL differentiation: nuclear factor of activated T cells cytoplasmic-1 (NFATc1), nuclear factor kappa B (NF-κB), phosphatidylinositol 3-kinase (PI3K)/Akt, Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (Erk), and p38 mitogen-activated protein kinase (MAPK) (2 – 4). In addition to these signaling pathways, it has been revealed that reactive oxygen species (ROS) are essential for the OCL differentiation (5, 6). RANKL stimulation of macrophages transiently increased the intracellular level of ROS through a signaling cascade involving TRAF 6, Rac 1, and NADPH oxidase 1 (Nox 1). Addition of the reducing agent *N*-acetylcysteine (NAC) or diphenylene iodonium, an inhibitor of Nox, completely blocks ROS production associated with the differentiation of macrophages

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into OCLs via various signaling pathways, such as JNK, p38 MAPK, and Erk (6). Similarly, we have also demonstrated that OCL differentiation or survival is regulated by NAC, or the oxidation agent hydrogen peroxide, and this is accompanied by changes in the shape and sizes of the cells. Furthermore, we recently demonstrated that the RANKL-mediated suppression of anti-oxidant enzyme heme-oxigenase 1 (HO-1) promoted high mobility group box 1 (HMGB1) release and OCL differentiation (7). Therefore, the use of anti-oxidants may enable the development of therapeutic agents for bone disorders such as osteoporosis and periodontitis.

Kahweol is a diterpene molecule extracted from the beans of *Coffea arabica*. Kahweol has been shown to have a variety of pharmacological activities such as anti-oxidant, anti-inflammation, and anti-tumor (8). Recently, the protective effects of kahweol against oxidative stress have been reported. In these reports, kahweol inhibited hydrogen peroxide–induced oxidative stress and DNA damage in NIH3T3 cells (9), and kahweol protected neurons against oxidative stress by HO-1 up-regulation via PI3K and p38 MAPK pathways (10). These findings prompted us to investigate whether kahweol may inhibit osteoclastogenesis via anti-oxidative effects. In this study, we investigated effects of kahweol on OCL differentiation by using in vitro culture systems, which provide useful information about pharmacological effects (11, 12) and demonstrated that kahweol inhibits OCL differentiation.

Materials and Methods

Reagents

Kahweol was purchased from Wako Pure Chemicals (Osaka). M-CSF was purchased from Kyowa Hakko Kogyo (Tokyo). Recombinant RANKL was prepared as previously described (13). Antibodies were purchased from the following sources: β-actin (Sigma-Aldrich, St. Louis, MO, USA); Src (Upstate, Lake Placid, NY, USA); c-fms, RANK, and NFATc1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); HO-1 (Stressgen, Ann Arbor, MI, USA); HMGB1 (Abcam, Cambridge, UK); phospho-ERK1/2 (Thr202/Tyr204), phospho-JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-IκBα (Ser32), and phospho-Akt (Ser473) (Cell Signaling Technology, Danvers, MA, USA). Cathepsin K antibody was prepared as previously described (14). The Osteo Assay Stripwell Plate was purchased from Corning (Corning, NY, USA). All other reagents including PMSF and protease inhibitor cocktail, were obtained from Sigma-Aldrich.

Cell culture

Five-week-old male BALB/c mice were obtained from CLEA Japan, Inc. (Tokyo) and were maintained in an air-conditioned room with a 12-h light/dark cycle and given a basal diet, MF (Oriental Yeast, Co., Tokyo) and water ad libitum. All animals were handled in our facilities under the approved protocols of the Nagasaki University Animal Care Committee. To isolate BMMs, we cultured marrow cells from femurs and tibias of the mice overnight in α-minimal essential medium (α-MEM) (Wako Pure Chemicals) containing 10% FBS with 100 U/mL of penicillin and 100 μg/mL of streptomycin in the presence of M-CSF (50 ng/mL) at 37°C in 5% CO_2. By harvesting the non-adherent cells, stroma-free bone marrow cells were cultured in the presence of 50 ng/mL of M-CSF. After 3 days, the non-adherent cells were washed out, and the adherent cells were used as BMMs. The BMMs were replated and then further cultured with new medium containing M-CSF (30 ng/mL) and RANKL (50 ng/mL) for the times indicated. The cells were fixed with 4% paraformaldehyde and stained for TRAP activity using a previously described method (15). TRAP-positive cells with 3 or more nuclei were regarded as mature OCL. Murine monocytic cell line RAW-D cells were kindly provided by Prof. Toshio Kukita (Kyushu University, Fukuoka, Japan) and cultured in α-MEM containing 10% FBS with RANKL (50 ng/mL) (16). The bone-resorbing activity of OCLs was assayed using the Osteo Assay Stripwell Plate for 5 days of culture. The resorption area was determined using Image J software (http://rsbweb.nih.gov/ij/).

Cell viability assay

Cells seeded in 96-well cell culture plates were incubated with the Cell Counting Kit-8 (Dojindo, Kumamoto) for 1 h, and then the absorbance at 450 nm was measured with a microplate reader (Bio-Rad iMark™, Hercules, CA, USA).

Western blot analysis

BMMs were stimulated with or without RANKL in the presence of M-CSF for the indicated amount of time. Cells were rinsed twice with ice-cold PBS and lysed in a cell lysis buffer (50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM PMSF, and proteinase inhibitor cocktail). The protein concentration of each sample was measured with BCA Protein Assay Reagent (Thermo Pierce, Rockford, IL, USA). Five micrograms of lysate protein was applied to each lane. After SDS-PAGE, proteins were electroblotted onto a polyvinylidene difluoride membrane. The blots were blocked with 5% BSA/TBST for 1 h at room temperature, probed with various antibod-

ies overnight at 4°C, washed, incubated with HRP-conjugated secondary antibodies, and finally detected with ECL-Plus (GE Healthcare Life Sciences, Amersham Place, UK). The immunoreactive bands were analyzed by LAS1000 (Fuji Photo Film, Tokyo).

Immunofluorescence

BMMs were cultured with M-CSF (30 ng/mL) in the absence or presence of 50 μM kahweol. After 6 h of cell culture, media were changed to serum-free fresh media and further cultured for 2 h. After stimulation with RANKL (300 ng/mL) for 30 min, cells were fixed with 4% paraformaldehyde at 4°C for 1 h, permealized with 0.1% Triton X-100 for 10 min, and then blocked with 5% milk. For NF-κB localization, anti-p65 antibody was diluted 1:200 in 5% milk and incubated overnight at 4°C, followed by incubation for 1 h at room temperature with Alexa 488-conjugated anti-mouse immunoglobulin diluted in 1:200 in 5% milk. Microscope images of fluorescence were digitized using Zeiss Axioskop 2 microscope equipped with AxioCam HRc camera (Zeiss, Jena, Germany).

Statistical analysis

All values were expressed as means ± standard deviations (S.D.). The Tukey-Kramer method was used to identify differences between concentrations when ANOVA indicated that a significant difference (*$P < 0.05$ or **$P < 0.01$) existed.

Results

Kahweol inhibits osteoclastogenesis in vitro

Figure 1 shows the structure of the coffee-derived diterpene kahweol. To investigate the effects of kahweol on RANKL-induced osteoclastogenesis, we first examined the effects of kahweol on OCL differentiation of murine monocytic cell line RAW-D cells with RANKL (50 ng/mL). As shown in Fig. 2A, TRAP activity staining showed that 25 μM of kahweol completely inhibited the formation of mononuclear and multinuclear OCLs. The numbers of TRAP-positive, multinucleated OCLs after kahweol treatment were decreased in a dose-dependent manner (Fig. 2B). The cell viability was not inhibited at up to 10 μM of kahweol, although at higher concentrations (25 or 50 μM), kahweol significantly inhibited cell viability (Fig. 2C).

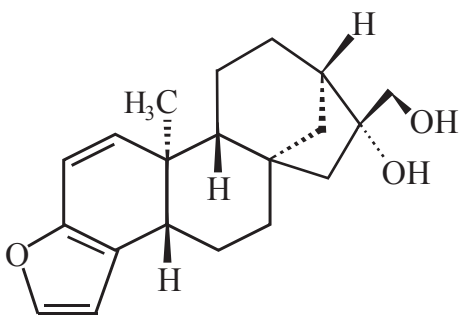

Fig. 1. Structure of kahweol.

To confirm whether similar effects were also observed in native cells, we evaluated the effects of kahweol on RANKL-induced osteoclastogenesis of BMMs. The formation of TRAP-positive BMM-derived OCLs was diminished in a dose-dependent manner by kahweol (Fig. 2: D and E). Similarly, the cell viability of OCLs treated with 0.1 – 10 μM kahweol was indistinguishable from that of untreated cells (Fig. 2F). These results indicate that kahweol inhibits osteoclastogenesis in vitro in the RANKL-induced culture system.

Effects of kahweol on the bone resorbing activity of OCLs

To further investigate whether kahweol decreases the bone resorbing activity of OCLs, we performed a pit formation assay with BMM-derived OCLs after the induction of M-CSF and RANKL. As shown in Fig. 3A, kahweol significantly inhibited bone resorbing activity. The calculated resorption area of kahweol-treated OCLs was reduced in a dose-dependent manner compared with that of untreated OCLs (Fig. 3B). Thus, kahweol clearly inhibits the physiological bone resorbing activity of OCLs.

Effects of kahweol on intracellular signaling and expression levels of marker proteins of OCLs

We next examined the effects of kahweol on RANKL-induced intracellular signaling during the OCL differentiation of BMMs. For this purpose, we evaluated the effects of kahweol as phosphorylation of p38 MAPK, JNK, IκBα, Erk, and Akt by western blotting, since these signaling cascades are important for osteoclastogenesis (17). The cells were treated with a high concentration of RANKL (300 ng/mL) in order to obtain a signal clearly detectable by western blotting. BMMs were pre-incubated with 10 μM of kahweol for 12 h and subsequently stimulated with RANKL. Phosphorylation of Erk was strongly inhibited by kahweol treatment (Fig. 4A). Similarly, kahweol inhibited phosphorylation of Akt, while kahweol had no effect on other MAPK signal cascades, p38 MAPK and JNK. In addition, phosphorylation of IκBα at 5 min after RANKL stimulation was delayed to 15 min by kahweol treatment. Since the phosphorylation of IκBα promotes its degradation and nuclear transport of NF-κB as a transcription factor, we next examined the effect of kahweol on NF-κB activation by immunofluorescence

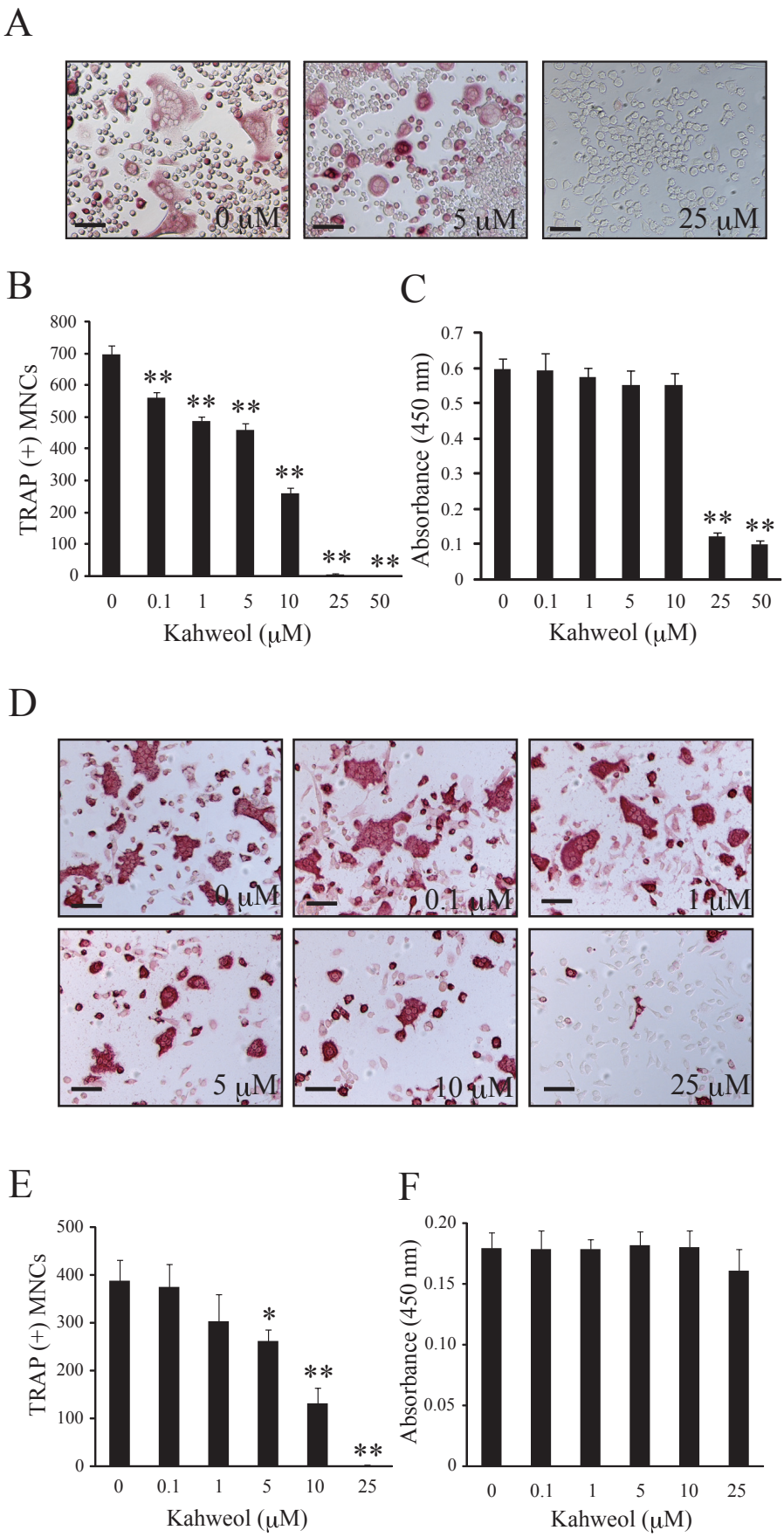

Fig. 2. Effects of kahweol on OCL differentiation from RAW-D cells or BMMs cultured for 72 h. A) RAW-D cells were treated with kahweol at the indicated concentrations together with RANKL (50 ng/mL). TRAP staining was performed. Bars, 50 μm. B) The number of TRAP-positive OCLs was counted. C) Cell proliferation of the RAW-D-derived OCLs was analyzed using the Cell Counting Kit. The data are shown as the mean ± S.D. (significance compared with RANKL. $^{**}P < 0.01$). Results are representative of 3 independent experiments. D) BMMs were cultured for 72 h with M-CSF (30 ng/mL), RANKL (50 ng/mL), and kahweol at the indicated concentrations. TRAP staining was performed. Bars, 50 μm. E) The number of TRAP-positive OCLs was counted. F) Cell proliferation of the BMM-derived OCLs was analyzed using the Cell Counting Kit. The data are shown as the mean ± S.D. (significance compared with M-CSF and RANKL. $^{*}P < 0.05$, $^{**}P < 0.01$). Results are representative of 3 independent experiments. MNCs: multi-nucleated cells.

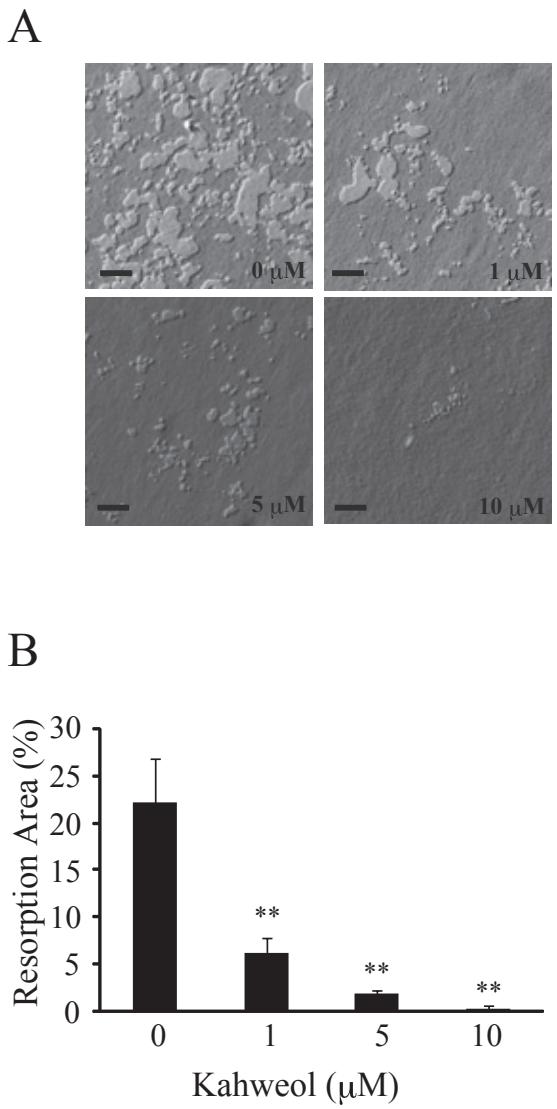

Fig. 3. Effects of kahweol on the bone resorbing activity of OCLs. BMMs were cultured with M-CSF (30 ng/mL) and RANKL (50 ng/mL), and kahweol at the indicated concentrations for 5 days. A) Photograph of the bone resorbing activity of OCLs. Bars, 50 μm. B) The resorption area was determined using Image J software (significance compared with 0 μM kahweol. $^{**}P < 0.01$).

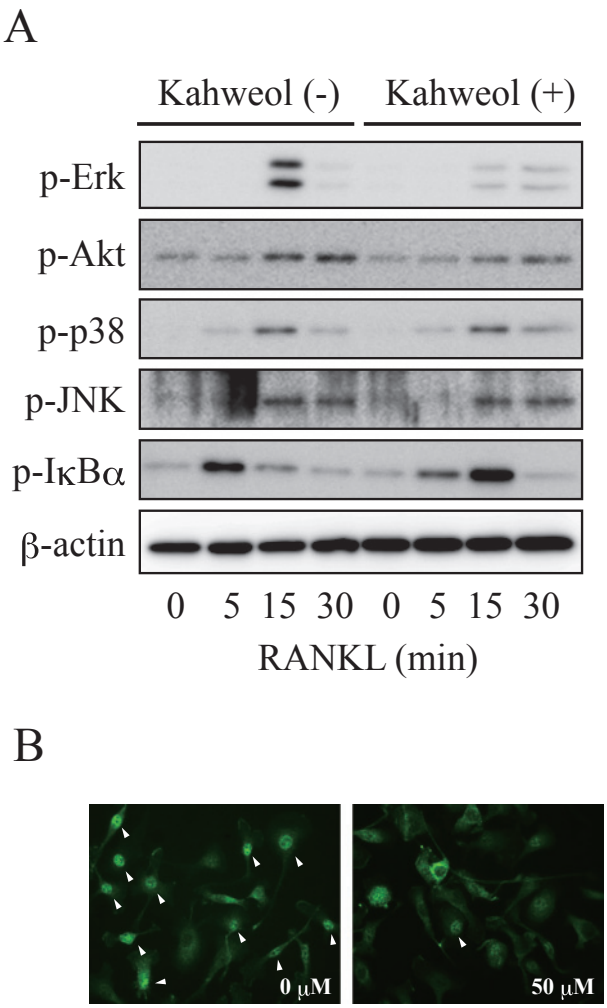

Fig. 4. Effects of kahweol on the essential signaling of OCL differentiation. A) BMMs were cultured with M-CSF (30 ng/mL) for 12 h in the presence of vehicle or 10 μM kahweol. The cells were subsequently stimulated with RANKL (300 ng/mL) for the indicated times (0, 5, 15, and 30 min). The cell lysates were subjected to SDS-PAGE, followed by western blotting with antibodies to p-Erk, p-Akt, p-p38 MAPK, p-JNK, p-IκBα, and β-actin. The results are representative of 3 independent experiments. B) BMMs were cultured with M-CSF (30 ng/mL) for 6 h in the absence or presence of 50 μM kahweol. The cells were subsequently stimulated with RANKL (300 ng/mL) for 30 min. For NF-κB localization, anti-p65 antibody was used as the primary antibody, followed by Alexa 488-conjugated second antibody. Microscope images of fluorescence were digitized using Zeiss Axioskop 2 microscope. Arrowhead indicates the nuclear localization of p65.

microscopy. With vehicle treatment, RANKL stimulated nuclear localization of NF-κB p65 (Fig. 4B, left). Pretreatment with 50 μM of kahweol inhibited the nuclear accumulation of p65 (Fig. 4B, right), but 10 μM of kahweol had no effect on nuclear localization of p65 (data not shown). These results indicate that kahweol strongly blocks an Erk-dependent pathway and partially interferes with IκBα- and Akt-dependent pathways.

To further examine the effects of differences in the OCLs, we analyzed the expression levels of marker proteins of OCLs by western blotting. RANK is a type I membrane protein that is expressed on the cell surface of OCLs and has been implicated in OCL activation (18). NFATc1 is a master regulator for OCL differentiation through Ca^{2+}/calmodulin-dependent calcineurin (19). In addition, c-fms is an M-CSF receptor, while c-Src is a non-receptor-type tyrosine kinase that regulates the formation of actin-rich podosomes in the OCLs (20).

Cathepsin K is a lysosomal cysteine proteinase specifically expressed in OCLs (21). As shown in Fig. 5A, the expression levels of RANK remained at 10 μM of kahweol in RAW-D derived OCLs. However, upon kahweol treatment, the protein levels of c-fms, NFATc1, and Src were decreased at 10 μM of kahweol. It is known that NFATc1 binds directly to the promoter regions of its target genes such as Src and cathepsin K and induces expression (22). To confirm the inhibition effects of kahweol on the expression of NFATc1, we further examined the expression levels of Src and cathepsin K in BMM-derived OCLs. The results indicated that kahweol significantly down-regulated NFATc1 in BMMs-derived OCLs as seen in RAW-D derived OCLs. In addition, the expression levels of Src and cathepsin K were also decreased by kahweol treatment in BMMs-derived OCLs (Fig. 5B). Thus, kahweol blocks the protein expression of NFATc1 and its regulated proteins, such as Src and cathepsin K.

Kahweol inhibits OCL differentiation via up-regulation of HO-1 and down-regulation of HMGB1

Lastly, to explore the molecular mechanisms by which kahweol inhibits OCL differentiation, we investigated the effects of kahweol on the expression levels of HO-1 and HMGB1 extracellular release. Previously, HMGB1 was identified as one of the OCL differentiation cytokines (23). Recently, we demonstrated that RANKL-induced suppression of HO-1 promotes the HMGB1 release during osteoclastogenesis (7). Therefore, we investigated whether kahweol inhibits OCL formation via the HO-1- and HMGB1-dependent pathways. As shown in Fig. 6A, the expression of HO-1 in OCLs treated with 10 μM kahweol was increased in a time-dependent manner compared with that of untreated controls. Conversely, the extracellular release of HMGB1 was decreased in a dose-dependent manner (Fig. 6B). Thus, kahweol inhibits OCL differentiation through the HO-1 induction and inhibition of HMGB1 release.

Discussion

In this study, we demonstrated that kahweol inhibited OCL differentiation from BMMs or RAW-D cells into mature OCLs in vitro. In addition, kahweol markedly inhibited the bone resorbing activity of OCLs. Importantly, kahweol-treated OCLs exhibited markedly abolished phosphorylation of Erk, and there were slight effects on IκBα- and Akt-dependent pathways. Moreover, upon kahweol treatment, NFATc1 and its target proteins, such as Src and cathepsin K, were down-regulated in OCLs. As one of the molecular mechanisms, kahweol inhibits OCL differentiation via up-regulation of HO-1 and the inhibition of HMGB1 release. Thus, this study is the first report, to our knowledge, that kahweol has an inhibitory effect on osteoclastogenesis.

Several studies have shown that RANK activates 6 key signaling pathways in OCL differentiation: NFATc1, NF-κB, PI3K/Akt, JNK, Erk, and p38 MAPK, although all of the precise signaling pathways have not been fully elucidated (17). In this study, kahweol inhibited OCL differentiation through down-regulation of NFATc1 and abolishment of Erk phosphorylation. In addition, kahweol had partial effects on IκBα and Akt-dependent pathways. It is likely that the inhibition of NFATc1 and Erk pathways by kahweol is characteristic in OCLs. For example, in human leukemia U937 cells, kahweol induced apoptosis via down-regulation of Akt and the activation of JNK

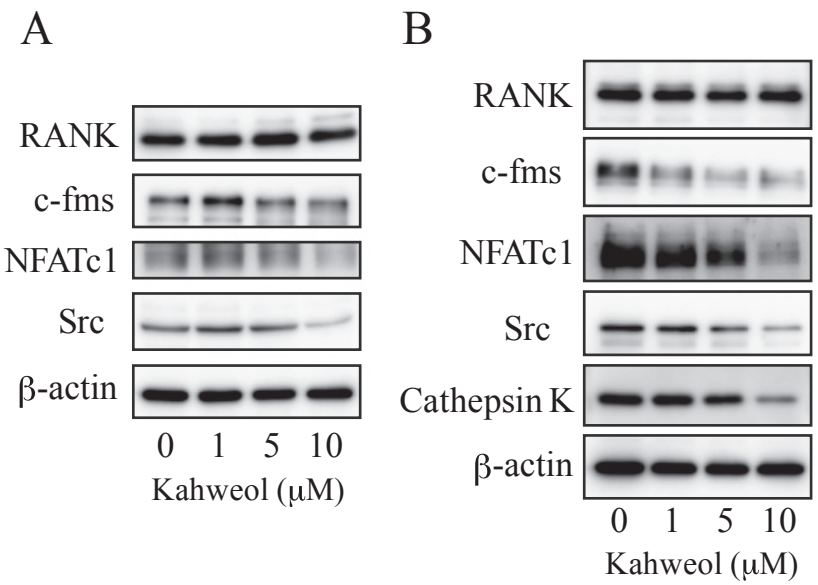

Fig. 5. Effects of kahweol on protein expression of OCL marker proteins. A) RAW-D cells were cultured with RANKL (50 ng/mL) for 48 h in the absence or presence of kahweol at the indicated concentrations (0, 1, 5, and 10 μM). The cell lysates were subjected to SDS-PAGE, followed by western blotting with specific antibodies to RANK, c-fms, NFATc1, Src, and β-actin. The results are representative of 3 independent experiments. B) BMMs were cultured with M-CSF (30 ng/mL) and RANKL (50 ng/mL) for 60 h in the presence of kahweol at the indicated concentrations (0, 1, 5, and 10 μM). The cell lysates were subjected to SDS-PAGE, followed by western blotting with antibodies specific to RANK, c-fms, NFATc1, Src, cathepsin K, and β-actin.

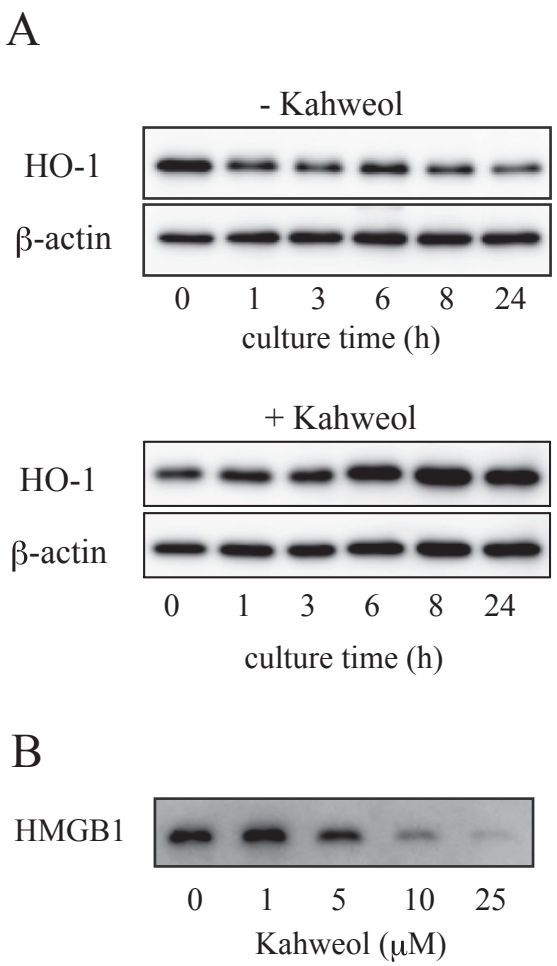

Fig. 6. Effects of kahweol on the protein expression of HO-1 and HMGB1 release. A) BMMs were cultured with M-CSF (30 ng/mL) and RANKL (50 ng/mL) for the indicated time in the absence or presence of 10 μM kahweol. The cell lysates were subjected to SDS-PAGE followed by western blotting with antibodies to HO-1 and β-actin. B) BMMs were cultured with M-CSF (30 ng/mL) and RANKL (50 ng/mL) for 72 h in the presence of kahweol at the indicated concentration (0, 1, 5, 10, and 25 μM). The same protein amounts of culture media were subjected to SDS-PAGE, followed by western blotting with antibodies against HMGB1.

(24). In lipopolysaccharide-activated murine macrophage RAW264.7 cells, kahweol suppressed inflammation via STAT-1- and NF-κB-dependent pathways (25). In human neuronal cells, kahweol induced cell death via PI3K- and p38 MAPK-dependent pathways after induction by the neurotoxin 6-hydroxydopamine (10). Thus, kahweol has unique inhibitory effects on OCL differentiation through the down-regulation of NFATc1 and abolishment of Erk phosphorylation in OCLs.

Since NFATc1 is a crucial transcription factor in RANKL-induced osteoclastogenesis, the down-regulation of NFATc1 by kahweol is probably effective for the direct suppression of OCL differentiation. The importance of NFATc1 on osteoclastogenesis was supported by in vitro experiments where NFATc1-deficient stem cells failed to differentiate into OCLs and where the expression of NFATc1 promoted OCL differentiation in the absence of RANKL (19). The expression of c-Src and cathepsin K is known to be regulated by NFATc1 (22). OCLs that are c-Src-deficient display reduced motility and abnormal organization of the ruffled border and cytoskeleton necessary for bone resorption (26). Cathepsin K is a lysosomal cysteine proteinase specifically expressed in OCLs (21) and has a strong potency for digesting native collagen fibers (27). In our study, kahweol significantly suppressed NFATc1 up-regulation by RANKL. We suggest that the down-regulation of NFATc1 by kahweol may be a major factor of suppression of c-Src and cathepsin K and the inhibition of bone resorption.

A previous study has demonstrated that kahweol up-regulated HO-1 expression in dopaminergic neurons (10). In addition, Zwerina et al. revealed that the induction of HO-1 is a negative regulator of osteoclastogenesis (28). In this study, our results demonstrated that kahweol significantly up-regulated HO-1 expression in OCLs. Recently, we have shown that suppression of HO-1 by pharmacological and genetic inhibition causes enhanced HMGB1 release during osteoclastogenesis (7). Extracellular HMGB1, which acts as a proinflammatory cytokine, can bind to cell-surface receptors such as the receptor for advanced glycation end products and Toll-like receptors 2 and 4 and modulates differentiation of OCL precursors in the presence of RANKL in vitro and in vivo (23, 29, 30). In this study, kahweol induced up-regulation of HO-1 and inhibited extracellular release of HMGB1 during osteoclastogenesis. Although the precise molecular mechanisms by which HO-1 and HMGB-1 function in OCLs remain unknown, it is important to determine the molecular target of kahweol in OCLs.

In conclusion, kahweol inhibited OCL differentiation through blocking phosphorylation of Erk and down-regulation of NFATc1. In addition, kahweol inhibits OCL differentiation via up-regulation of HO-1 and inhibition of HMGB1 release. Taken together, it is likely that kahweol inhibits OCL differentiation via multiple pathways, including Erk-, NFATc1-, HO-1-, and HMGB1-dependent pathways.

Acknowledgments

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