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Peperomins as anti-inflammatory agents that inhibit the NF-κB signaling pathway

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ABSTRACT

The transcription factor nuclear factor κB (NF-κB) induces the expression of various inflammatory genes. In the common NF-κB signaling pathway, peperomin E and 2,6-didehydropeperomin B inhibited IκB degradation upon stimulation with TNF-α or interleukin-1. Consistent with these results, peperomin E and 2,6-didehydropeperomin B blocked the TNF-α-induced activation of IκB kinase, while they had no direct effect on the IκB kinase activity. Our present results clearly demonstrate that peperomins inhibit the NF-κB signaling pathway by blocking IκB kinase activation.

Inflammatory cytokines, such as tumor necrosis factor-α (TNF)-α and interleukin-1 (IL-1), play an essential role in inflammation and induce a variety of genes responsible for inflammatory responses, such as intercellular adhesion molecule-1 (ICAM-1; CD54).[1] During inflammation, the inducible expression of ICAM-1 on the vascular endothelium is regulated mainly by inflammatory cytokines, thereby facilitating the adhesion and subsequent transmigration of leukocytes.[2,3] The ICAM-1 expression is predominantly upregulated by the transcription factor nuclear factor κB (NF-κB).[1]

The NF-κB signaling pathway is activated by various stimuli including inflammatory cytokines. Upon TNF-α stimulation, TNF receptor 1 recruits several adaptor proteins to form a membrane-bound complex.[4] This complex is prerequisite to activating the IκB (inhibitor of κB) kinase (IKK) complex containing two catalytic subunits, IKKα and IKKβ, and a regulatory subunit IKKγ/NEMO.[5,6] On the other hand, IL-1 receptor recruits different sets of adaptor proteins to its cytoplasmic domain, leading to the activation of the IKK complex.[7] Once activated, the IKK complex phosphorylates IκB that interacts with the NF-κB heterodimer in the cytosol, and phosphorylated IκB is ubiquitinylated and immediately hydrolyzed by proteasome.[5–7] The NF-κB heterodimer becomes free and translocates to the nucleus where it activates a variety of genes responsible for inflammation as well as cancer development and progression.[8]

We have isolated 13 secolignans from the extract of *Peperomia dindygulensis* and investigated their SAR with focus on anti-inflammatory and anticancer activities.[9] Among these secolignans, peperomin E (Fig. 1A)[9] and its structural derivatives were found to inhibit cell-surface ICAM-1 expression induced by inflammatory cytokines.[9] Except for its anti-inflammatory activity,[9] multidrug-resistant reversal activity,[9] and anticancer activity,[9,10] the biological activities of peperomins are little understood. In this study, we further investigated the anti-inflammatory activities of peperomin E and its structural derivatives at the cellular and molecular levels.

Peperomin A (**1**),[9–12] peperomin B (**2**),[9–12] and peperomin E (**3**)[9–11] were isolated from the dried material of *P. dindygulensis* as previously described.[9] As 2,6-didehydropeperomin B (**4**) was not obtained from natural sources, **4** was synthesized from **2** as shown in Scheme 1 (experimental details in Supplementary data). The introduction of a double bond at C-2(6) position of **2** with a known method was unsuccessful, because the C-2 position of **2** is sterically hindered by the bulky substituent at C-3 of **2**. Enolsilylation of **2** with TMSOTf and Et_3N in CH_2Cl_2 and subsequent treatment of the resulting silyl enol ether with phenylselenyl chloride

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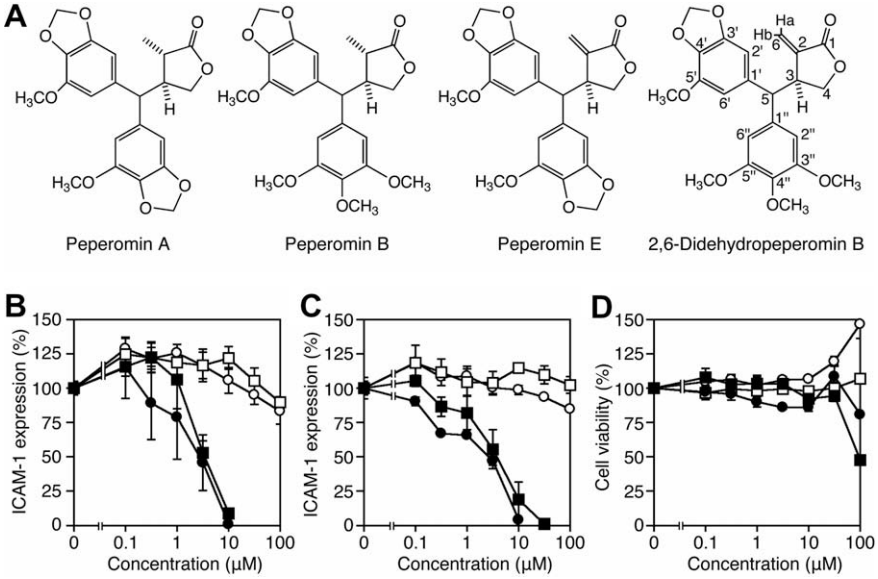
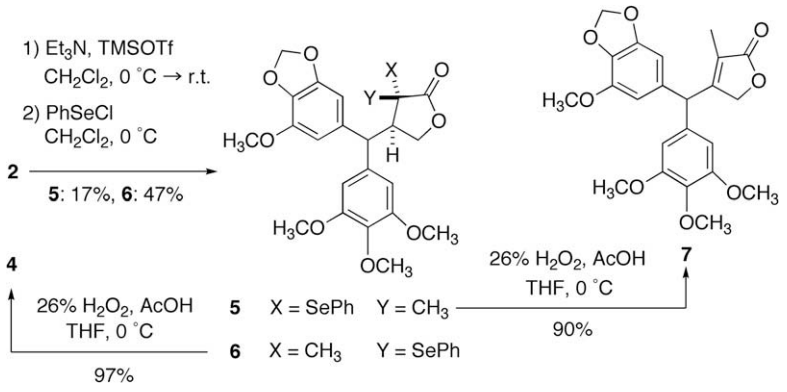


Figure 1. Peperomin E and 2,6-didehydropeperomin B inhibit cell-surface ICAM-1 expression induced by TNF-α or IL-1. (A) Structures of peperomins. (B and C) A549 cells were pretreated with various concentrations of peperomin A (open circles), peperomin B (open squares), peperomin E (filled circles), and 2,6-didehydropeperomin B (filled squares) for 1 h, and then incubated with TNF-α (2.5 ng/mL) (B) or IL-1 (0.25 ng/mL) (C) for 6 h in the presence or absence of peperomins. Cell-surface ICAM-1 expression was measured by the Cell ELISA assay. Data points represent means ± SD of triplicate cultures. (D) A549 cells were incubated with various concentrations of peperomin A (open circles), peperomin B (open squares), peperomin E (filled circles), and 2,6-didehydropeperomin B (filled squares) for 6 h. Cell viability (%) was measured by the MTT assay. Data points represent means ± SD of triplicate cultures.



Scheme 1. Synthesis of 2,6-didehydropeperomin B.

afforded a mixture of stereoisomers of phenylseleno lactones, the HPLC separation of which gave α-isomer **5** and β-isomer **6** in 17% and 47% yields, respectively. The structures of **5** and **6** were confirmed by the analyses of spectral data, including ^{1}H- and ^{13}C-NMR spectra and HREIMS. The oxidative syn elimination of **5** with 26% H_2O_2 gave undesired endo-unsaturated γ-lactone **7** in 90% yield. On the contrary, the oxidative syn elimination of **6** with 26% H_2O_2 gave desired **4** in 97%.

Peperomin E and 2,6-didehydropeperomin B are structural analogues of peperomin A and peperomin B in which α-methylene γ-lactone groups are replaced by α-methyl γ-lactone groups, respectively (Fig. 1A). Human lung carcinoma A549 cells were pretreated with various concentrations of peperomins for 1 h and then incubated with TNF-α or IL-1 for 6 h in the presence of peperomins. Cell-surface expression of ICAM-1 was measured by the Cell ELISA assay.[13] Peperomin A and peperomin B did not reduce cell-surface ICAM-1 expression up to a concentration of 100 μM (Fig. 1B and C). By contrast, peperomin E and 2,6-didehydropeperomin B inhibited the cell-surface ICAM-1 expression induced by TNF-α or IL-1 in a dose-dependent manner and at almost equivalent concentrations (Fig. 1B and C). In human breast adenocarcinoma MCF7 cells, peperomin E and 2,6-didehydropeperomin B also inhibited the cell-surface expression induced by TNF-α or IL-1 as strongly as in A549 cells, while peperomin A and peperomin B exerted approximately 10-fold weaker inhibitory effects (Supplementary Fig. 1A and B). Therefore, it seems that the α-methylene γ-lactone moiety in peperomin E and 2,6-didehydropeperomin B is important for their inhibitory activities. Indeed, the inhibitory concentration of peperomin E and 2,6-didehydropeperomin B on the IL-1-induced ICAM-1 expression was comparable with that of santonin-related compound 2 harboring the α-methylene-γ-lactone moiety.[14] As judged by the MTT assay,[13] while peperomin E and 2,6-didehydropeperomin B did not reduce cell viability of MCF7 cells up to 100 μM (Supplementary Fig. 1C), they partially decreased cell viability of A549 cells (Fig. 1D). Therefore, peperomin E and 2,6-didehydropeperomin B may exert some nonspecific cytotoxic effects at concentrations higher than 100 μM.

It has been shown that ICAM-1 expression is highly NF-κB-dependent in A549 cells.[15] The amounts of cell-surface and intracellular proteins expressed in A549 cells were measured by Western blotting.[16] The expression of ICAM-1 was barely detectable in unstimulated A549 cells, while stimulation with TNF-α or

IL-1 induced a marked increase in ICAM-1 expression. Peperomin E and 2,6-didehydropeperomin B substantially decreased ICAM-1 expression at concentrations higher than 10 μM and almost completely decreased the expression at 100 μM, whereas peperomin A and peperomin B were inactive up to 100 μM (Fig. 2 and Supplementary Fig. 2). It is known that cyclooxygenase-2 (Cox-2) expression is induced by inflammatory cytokines in an NF-κB-dependent manner.[8] The expression of Cox-2 was markedly increased when A549 cells were stimulated with TNF-α or IL-1 (Fig. 2). Likewise, peperomin E and 2,6-didehydropeperomin B, but neither peperomin A nor peperomin B, reduced the expression of Cox-2 to background levels at 100 μM (Fig. 2 and Supplementary Fig. 2). These results indicate that peperomin E and 2,6-didehydropeperomin B generally inhibit NF-κB-dependent gene expression induced by TNF-α or IL-1. It should be noted that the inhibitory effects of peperomin E and 2,6-didehydropeperomin B on ICAM-1 expression are approximately 10-fold stronger than those on Cox-2 expression. Since ICAM-1 is a cell-surface glycoprotein, it might be possible that these compounds influence the ICAM-1 processing and/or its transport from the endoplasmic reticulum to the cell-surface.

TNF-α and IL-1 induce rapid phosphorylation of IκBα, and phosphorylated IκBα immediately undergoes proteasomal degradation.[5–7] A549 cells constitutively expressed IκBα, and stimulation with TNF-α or IL-1 induced rapid IκBα degradation. Peperomin E and 2,6-didehydropeperomin B, but neither peperomin A nor peperomin B, inhibited the IκBα degradation induced by TNF-α or IL-1 (Fig. 3 and Supplementary Fig. 3). Time-course experiments revealed that IκBα is phosphorylated within 5 min following stimulation with TNF-α or IL-1, and then degraded to barely detectable levels within 10 min. Peperomin E prevented IκBα phosphorylation as well as its subsequent degradation upon stimulation with TNF-α or IL-1 (Fig. 3 and Supplementary Fig. 4). The expression of two IKK catalytic subunits, IKKα and IKKβ, was not obviously influenced by peperomin E during TNF-α and IL-1 stimulation (Fig. 3 and Supplementary Fig. 4).

The IKK complex directly phosphorylates IκBα at N-terminal two serine residues (Ser-32 and Ser-36).[6] The IKK complex was pulled down from cell lysates by immunoprecipitation using anti-IKKα antibody and its kinase activity toward IκBα (1–54) fused to glutathione S-transferase (GST) was measured as described in supplementary data. A549 cells were pretreated with peperomins or not pretreated for 1 h, and stimulated with TNF-α for 15 min in the presence or absence of peperomins. The IKK complex exhibited strong activity toward GST-IκBα (1–54) in an ATP-dependent manner, as well as only when A549 cells were stimulated with TNF-α (Fig. 4A and Supplementary Fig. 5A). The IKK activity was completely blocked when A549 cells were pretreated with peperomin E or 2,6-didehydropeperomin B prior to TNF-α stimulation (Fig. 4A and Supplementary Fig. 5A). It seems that peperomin E and 2,6-didehydropeperomin B do not primarily disrupt the interaction of IKKβ with IKKα, as IKKβ was co-immunoprecipitated with IKKα even in the presence of peperomin E and 2,6-dide-

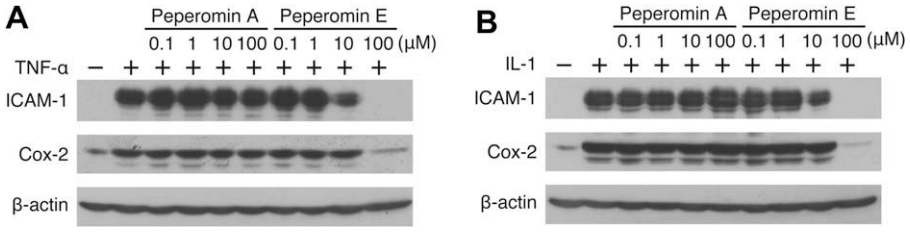


Figure 2. Peperomin E inhibits expression of ICAM-1 and Cox-2 upon stimulation with TNF-α or IL-1. A549 cells were pretreated with various concentrations of peperomin A or peperomin E for 1 h, and then incubated with (+) or without (−) TNF-α (2.5 ng/mL) (A) or IL-1 (0.25 ng/mL) (B) for 6 h in the presence (+) or absence (−) of peperomin A or peperomin E. Expression of ICAM-1, Cox-2, and β-actin was analyzed by Western blotting.

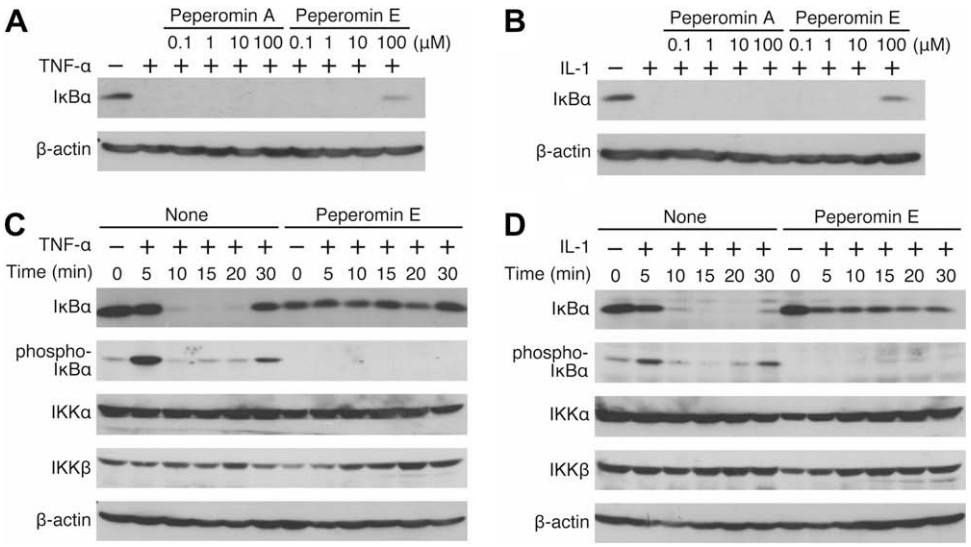


Figure 3. Peperomin E inhibits IκBα phosphorylation and its subsequent degradation upon stimulation with TNF-α or IL-1. (A and B) A549 cells were pretreated with various concentrations of peperomin A or peperomin E for 1 h, and then incubated with (+) or without (−) TNF-α (2.5 ng/mL) (A) or IL-1 (0.25 ng/mL) (B) for 15 min in the presence (+) or absence (−) of peperomin A or peperomin E. IκBα and β-actin expression was analyzed by Western blotting. (C and D) A549 cells were pretreated with peperomin E (100 μM) or not pretreated for 1 h, and then incubated with (+) or without (−) TNF-α (2.5 ng/mL) (C) or IL-1 (0.25 ng/mL) (D) for the indicated times in the presence (+) or absence (−) of peperomin E. The expression of IκBα, phospho-IκBα, IKKα, IKKβ, and β-actin was analyzed by Western blotting. The amounts of protein bands (C and D) were quantified by the ImageJ image processing program, and relative intensity compared with β-actin was shown in Supplementary Figure 4A and B, respectively.

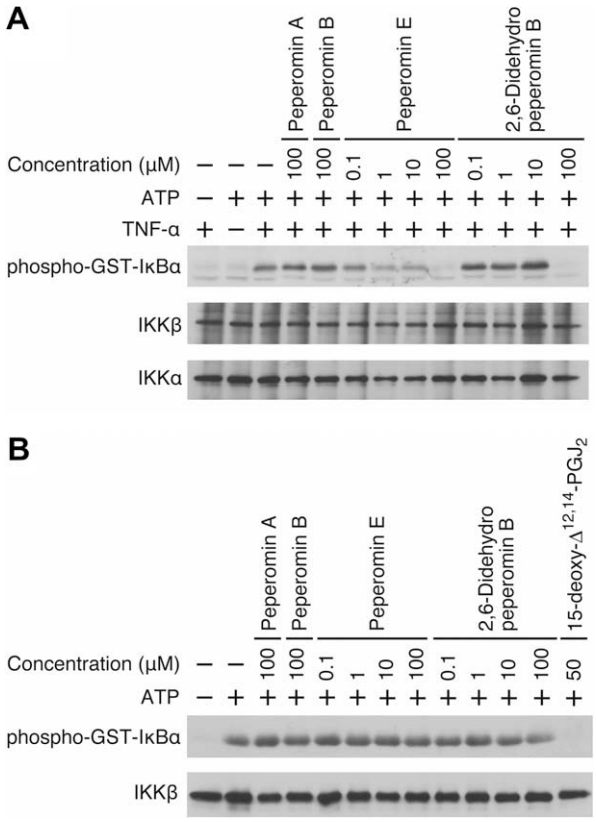


Figure 4. Peperomin E and 2,6-didehydropeperomin B inhibit TNF-α-induced activation of the IKK complex. (A) A549 cells were pretreated with various concentrations of peperomins for 1 h, and then incubated with (+) or without (−) TNF-α (2.5 ng/mL) for 15 min in the presence of peperomins. The IKK complex was immunoprecipitated with anti-IKKα antibody and the resultant immunoprecipitates were used for the kinase reaction using GST-IκBα (1–54) as substrate in the presence (+) or absence (−) of ATP (10 μM). Phospho-GST-IκBα (1–54) was detected by Western blotting using anti-phospho-IκB antibody. The amounts of IKKα and IKKβ in the immunoprecipitates were determined by Western blotting. The amounts of protein bands were quantified, and relative intensity of phospho-IκBα (1–54) and IKKβ compared with IKKα was shown in Supplementary Figure 5A. (B) FLAG-IKKβ was transiently overexpressed in HEK293 cells for 24 h and then immunoprecipitated with anti-FLAG antibody. The resultant immunoprecipitates containing FLAG-IKKβ were incubated with (+) or without (−) peperomins, 15-deoxy-$\Delta^{12,14}$-PGJ_2 (50 μM) or ATP (10 μM) in the presence of GST-IκBα (1–54). Phospho-GST-IκBα (1–54) was detected by Western blotting using anti-phospho-IκB antibody. The amounts of FLAG-IKKβ in the reaction mixtures were determined by Western blotting. The amounts of protein bands were quantified, and relative intensity of phospho-GST-IκBα (1–54) compared with FLAG-IKKβ was shown in Supplementary Figure 5B.

hydropeperomin B (Fig. 4A and Supplementary Fig. 5A). IKKβ is the major kinase responsible for IκBα phosphorylation in the classical NF-κB signaling pathway.[5,6] To obtain active IKKβ, FLAG-IKKβ was transiently overexpressed in human embryonic kidney (HEK) 293 cells and collected by immunoprecipitation using anti-FLAG antibody. It has been shown that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-deoxy-$\Delta^{12,14}$-PGJ_2) directly inhibits IKKβ activity.[17,18] Consistent with this, we found that 15-deoxy-$\Delta^{12,14}$-PGJ_2 strongly inhibited IKKβ activity (Fig. 4B and Supplementary Fig. 5B). However, under the same experimental conditions, peperomin E and 2,6-didehydropeperomin B had no effect on IKKβ activity (Fig. 4B and Supplementary Fig. 5B). These data indicate that peperomin E and 2,6-didehydropeperomin B inhibit IKK complex activation in a manner distinct from 15-deoxy-$\Delta^{12,14}$-PGJ_2.[17,18]

Studies of SAR revealed that the α-methylene γ-lactone moiety is important for peperomin E and 2,6-didehydropeperomin B to exhibit the inhibitory activity. It is known that the α-methylene γ-lactone moiety can bind covalently to reactive thiol residues, such as cysteine residues, of proteins via the Michael addition reaction. IKKα and IKKβ have a cysteine residue (Cys-179) within their activation loop.[17,19] 15-Deoxy-$\Delta^{12,14}$-PGJ_2 as well as 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid methyl ester (CDDO-Me) or 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) contains an α,β-unsaturated carbonyl moiety that can form reversible adducts with reactive thiol groups. It has been shown that 15-deoxy-$\Delta^{12,14}$-PGJ_2, CDDO-Me, and CDDO-Im inhibit IKKβ activity through direct modification of Cys-179.[17,18,20,21] Similar to these compounds, the sesquiterpene lactone parthenolide contains an α-methylene γ-lactone group and inhibits IKKβ by covalently binding to Cys-179.[22] The Cys-179 residue was also shown to be essential for the inactivation of IKKβ in the case of arsenite and a gold compound, auranofin.[19,23] However, in contrast to these previous studies, under the experimental conditions in which 15-deoxy-$\Delta^{12,14}$-PGJ_2 directly inhibited IKKβ activity, peperomin E and 2,6-didehydropeperomin B were unable to inhibit the IKKβ activity. Therefore, it seems likely that peperomin E and 2,6-didehydropeperomin B do not primarily block IKKβ activity via the modification of Cys-179, but may target other protein(s) required for IKK activation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.06.029.

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