Casein-Derived Tripeptide, Val-Pro-Pro (VPP), Modulates Monocyte Adhesion to Vascular Endothelium

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Aim: A food-derived bioactive tripeptide, Val-Pro-Pro (VPP), has been shown to possess angiotensin I-converting enzyme (ACE) inhibitory activity and foods containing such peptides exhibit an antihypertensive effect in clinical settings.

Methods: The present study focused on the effect of VPP on monocyte adhesion to endothelium under flow conditions using phorbol 12-myristate 13-acetate (PMA)-stimulated monocytic THP-1 cells.

Results: Pre-incubation of THP-1 cells with VPP (1 mM, 24 hours) significantly decreased the PMA-induced adhesion of THP-1 cells (p<0.05) to human umbilical vein endothelial cells (HUVECs). PMA-induced up-regulation of β1 and β2 integrin activation in THP-1 cells was downregulated by VPP, which significantly suppressed only the PMA-induced phosphorylation of JNK (p<0.05) in THP-1 cells. In addition, preincubation of THP-1 with SP600125, a specific inhibitor of JNK, resulted in significant reduction of the PMA-induced adhesion of THP-1. Interestingly, another tripeptide with comparable ACE inhibitory activity, Leu-Gly-Pro (LGP), failed to reduce the PMA-induced adhesion of THP-1, suggesting a distinct anti-inflammatory effect of VPP on THP-1 adhesion.

Conclusion: These observations suggest that VPP moderates monocyte adhesion to inflamed endothelia via attenuation of the JNK pathway in monocytes, which might contribute to the primary prevention of atherosclerosis.


Key words: Food-derived tripeptide, Monocyte, Adhesion, PMA, Atherosclerosis

Introduction

The risk of cardiovascular events increases proportional to lifestyle-related diseases, such as diabetes mellitus, hyperlipidemia or hypertension. For primary prevention of these diseases, lifestyle modifications, such as weight reduction, reduction in salt intake, increased physical activity, or healthy eating patterns is recommended. With respect to the dietary approach, a recent clinical trial demonstrated that a healthy diet with reduced saturated fat and partial substitution of carbohydrate with protein can lower blood pressure, improve lipid levels, and reduce the estimated cardiovascular risk¹. The mechanisms by which protein could exert its beneficial effects include an increased intake of biologically active amino acids or peptides². In particular, the beneficial effects of bioactive peptides derived from various food protein sources, such as milk, animal and fish meat, maize, wheat, soybean and egg, have been addressed³. These peptides are considered to promote various physiological functions, including immunomodulatory, antihypertensive, antioxidant, antithrombotic, and antimicrobial actions. Among these, several peptides have been shown to have an angiotensin I-converting enzyme (ACE) inhibitory action⁴,⁵. ACE inhibitors not only reduce high blood pressure but also decrease the incidence of cardiovascular diseases⁶, and
anti-atherosclerotic effects of ACE inhibitors have been reported in several animal studies, independently of their blood pressure-lowering properties

Two naturally existing bioactive tripeptides, iso-leucyl-prolyl-proline (IPP) and valyl-prolyl-proline (VPP), which are produced from milk caseins during lactic acid bacteria fermentation or milk casein hydrolysate, have ACE inhibitory activities, and several clinical studies have shown their blood pressure-lowering effect. Furthermore, a weekly intake of casein hydrolysate containing VPP and IPP improves vascular endothelial dysfunction without change in systemic blood pressure in subjects with mild hypertension. These data suggest that VPP and IPP have direct beneficial effects on the vasculature.

In the development of atherosclerosis, monocyte-endothelial interaction preceding plaque formation is one of the crucial steps. In the present study, we attempted to elucidate the anti-atherogenic effects of VPP, focusing on in vitro monocyte-endothelial interaction and the inflammatory status. We demonstrated that VPP moderated PMA-stimulated monocyte-endothelial interaction, and elucidated the possible involvement of the inhibition of JNK phosphorylation in the effect.

Materials and Methods

Materials

VPP, Val-Pro-Pro, and IPP, Ile-Pro-Pro, were provided by Calpis Co., Ltd. (Tokyo, Japan).

The reagents and antibodies used in the present study were obtained from the companies indicated as follows: SP600125 (Calbiochem, San Diego, CA); phorbol 12-myristate 13-acetate (PMA) (Wako Pure Chemicals, Tokyo, Japan); recombinant human interleukin (IL)-1β (Genzyme, Cambridge, MA); LGP, Leu-Gly-Pro (Bachem AG, Bubendorf, Switzerland); recombinant human monocyte chemoattractant protein (MCP)-1 (R&D Systems, Minneapolis, MN); enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Biosciences, Buckinghamshire, UK); Alexa Flour 488-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA); RPMI 1640 medium, Dulbecco's phosphate-buffered saline (PBS), and rabbit anti-actin polyclonal antibodies (Sigma-Aldrich, St. Louis, MO); α 4 integrin antibodies (CD49d; Upstate Biotechnology, Charlottesville, VA); β1 integrin antibodies (clone B44), ACE antibodies (clone 9B9; Chemicon International, Temecula, CA); protein kinase C (PKC)α and -δ antibodies, phospho-specific ERK1/2 antibodies, ERK1/2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific JNK antibodies, and JNK antibodies (Cell Signaling Technology, Beverly, MA).

Cell Culture

THP-1, a human monocytic cell line, and hybridomas producing an anti-β2 integrin antibody, KIM127, were obtained from the American Type Culture Collection (Manassas, VA), and cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine in a humidified atmosphere of 5% CO2 at 37°C. Human umbilical vein endothelial cells (HUVECs) were purchased from Sanko Junyaku (Tokyo, Japan), cultured in 0.1% gelatin-coated tissue culture dishes, and then plated on 22-mm fibronectincoated glass cover slips after 2 or 3 passages for use in a flow chamber apparatus. To examine cell viability, THP-1 cells were stained with a 0.25% trypan blue solution after incubation with VPP.

Monocyte Adhesion Assay

The protocols of the adhesion assay under flow conditions have been described in detail previously. Briefly, HUVEC monolayers on coverslips were stimulated with 10 U/mL IL-1β for 4 h and then positioned in a flow chamber mounted on an inverted microscope (TX-100; Nikon, Tokyo, Japan). Perfusion medium (PBS containing 0.2% human serum albumin), followed by THP-1 cells (1 × 10⁶/mL) resuspended with the perfusion medium were drawn through the chamber with a syringe pump (PHD2000; Harvard Apparatus Inc., Holliston, MA) for 10 min at a controlled flow rate to generate a shear stress of 1.0 dyne/cm². Prior to the experiment, THP-1 cells were preincubated in the presence or absence of appropriate concentrations of each peptide for 24 hours or SP600125 for one hour, followed by PMA treatment (250 nM; 30 min). The entire period of perfusion was recorded on videotape and then transferred to a personal computer for image analysis to determine the number of rolling and adherent cells on HUVEC monolayers in 10 randomly selected 20x microscope fields.

Flow Cytometry

THP-1 cells (1 × 10⁶/mL) were washed with RPMI 1640 medium containing 5% FCS, and then incubated with each primary antibody (1:300 dilution; non-diluted medium of the hybridoma producing KIM127 was used as the anti-β2 integrin antibody), followed by incubation with Alexa Flour
488-conjugated goat anti-mouse antibody (1:250 dilution). Fluorescence intensity was analyzed using a FACS calibur (BD Biosciences, San Jose, CA).

Western Blotting
Total cell lysates and membrane fraction of THP-1 cells (1 × 10⁶ condition) were prepared as described previously¹⁸. Lysates from each condition were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blotting analysis was carried out with the antibodies described above as primary antibodies, followed by incubation with a secondary IgG conjugated to HRP. Immunoreactive proteins were detected using an ECL kit.

Isolation and Culture of Human Peripheral Blood Mononuclear Cells (PBMNCs)
PBMNCs were isolated from heparinized peripheral venous blood from healthy donors by centrifugation over HISTOPAQUE (Sigma-Aldrich, St. Louis, MO). Isolated PBMNCs were cultured in RPMI 1640 medium containing 10% AB human serum (The Interstate Companies, Bloomington, MN), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine at 37°C and 5% CO₂.

Statistical Analysis
Results are presented as the mean ± S.D.
Data were analyzed using one-way analysis of variance (ANOVA) with Tukey’s post hoc analysis. *p < 0.05 was considered significant.

Results
Effects of Valyl-Prolyl-Proline (VPP) on Adhesion of THP-1 Cells to HUVECs
We examined the effect of VPP on monocyte-endothelial interactions under flow conditions (shear stress of 1.0 dyn/cm²). In a dose–response study (Fig. 1A), PMA increased THP-1 cell adhesion to activated HUVECs (pre-PMA, 3.8 ± 1.5/highpower field [HPF]; post-PMA, 15.7 ± 3.8/HPF; n = 10, p < 0.001). When THP-1 cells were preincubated in the presence of VPP (1 mM) for 24 h, the amount of adhesion to activated HUVECs was significantly decreased (VPP post-PMA, 10.8 ± 3.1/HPF; n = 10, p < 0.05 vs post-PMA without VPP). VPP did not affect THP-1 cell adhesion in the absence of PMA stimulation (Fig. 1A).

In a time–response study with 1 mM VPP, the inhibitory effect was observed at 24 h (Fig. 1B). Preliminary experiments with trypan blue staining revealed that THP-1 cells were not damaged by VPP treatment up to a concentration of 1 mM (data not shown). When the effect of VPP was compared with an ACE inhibitory peptide, Leu-Gly-Pro (LGP), which has less in vitro ACE inhibitory activity (IC₅₀ = 117 µM) than that of VPP (IC₅₀ = 9 µM), the amount of adhesion of THP-1 to activated HUVECs was not significantly altered (Fig. 1C).

Fig. 1. (A) and (B) Effects of Val-Pro-Pro (VPP) on adhesion of THP-1 cells to HUVECs. THP-1 cells (10⁴/mL) were incubated in the presence or absence of VPP, followed by stimulation with PMA (250 nM) for 30 min, and then perfused over HUVEC monolayers activated with 10 U/mL IL-1β for 4 hours at a shear stress of 1.0 dyn/cm². Rolling and adherent THP-1 cells on HUVEC monolayers were counted under 10 microscope fields for 10 minutes as described in Materials and Methods. (A) Dose–response effect of VPP after 24 h treatment. (B) Time–response effect of 1 mM of VPP. Data are representative of 3 separate experiments. *p < 0.05 vs PMA (+), VPP (−). (C) Effect of a tripeptide that has lower ACE inhibitory activity than VPP on adhesion of THP-1 cells to HUVECs. THP-1 cells (10⁴/mL) were incubated in the presence (1 mM) or absence (−) of VPP or Leu-Gly-Pro (LGP) for 24 hours, followed by stimulation with PMA (250 nM) for 30 min, and then their adhesion to HUVECs was examined under the flow conditions described above. Data are representative of 3 separate experiments. **p < 0.01 vs PMA (+), VPP (−), LGP (−).
Effects of VPP on PMA-Induced Integrin Expression in THP-1 Cells

To investigate the effect of VPP on integrin activation in THP-1 cells, flow cytometric analysis was performed with monoclonal antibodies B44 and KIM127 to detect activation-dependent epitopes of β1- and β2-integrins, respectively. After stimulation with PMA, both B44-positive β1-integrin (Fig. 2A) and KIM127-positive β2-integrin (Fig. 2B) were increased in THP-1 cells, which were decreased by VPP treatment. Furthermore, we found at MCP-1-induced β1-integrin activation was decreased by the treatment of 1 mM VPP for 24 h in THP-1 cells (Fig. 2C) and monocytes (Fig. 2D). In contrast, VPP did not alter MCP-1-induced α4-integrin expression in THP-1 cells (Fig. 2E).

Effects of VPP on the Expression of PKCs or the Phosphorylation of MAPKs in THP-1 Cells

To investigate the intracellular mechanisms
underlying the inhibitory effect of VPP, we first examined the expression of PKC in THP-1 cells. PMA is a potent PKC activator, and PKC is considered as one of the key molecules controlling the adhesion of monocytes to endothelial cells. While PMA activated PKCα and δ, VPP treatment (1 mM; 24 h) did not inhibit PKC activation induced by PMA (Fig. 3A, B). We next examined the effects of VPP on the phosphorylation of MAP kinases in THP-1 cells. It has been reported that MAP kinase activation is involved in the inflammatory status in human monocyteic cells. As shown in Fig. 4, PMA induced the phosphorylation of ERK1/2, p38 and JNK in THP-1 cells. When THP-1 cells were preincubated in the presence of VPP for 24 hours, phosphorylation of JNK was significantly reduced (Fig. 4C). In order to confirm the involvement of JNK in the adhesion mechanism of THP-1 cells to endothelium, we examined the effect of SP600125, a highly specific inhibitor of JNK, on the adhesion of THP-1 cells to endothelium. Treatment with SP600125 inhibited PMA-induced THP-1 cell adhesion (Fig. 5A) and β1-integrin activation on THP-1 (Fig. 5B), similar to that observed in VPP treatment, suggesting that JNK phosphorylation is correlated with the adhesion of THP-1 to endothelium. Furthermore, we examined whether LGP peptide, which showed less inhibitory activity in the adhesion of THP-1 cells to endothelium than VPP, has a modulating effect on PMA-induced JNK phosphorylation in THP-1 cells. When THP-1 cells were preincubated in the presence of LGP for 24 hours, JNK phosphorylation was slightly reduced, but not significantly (Fig. 5C). LGP failed to inhibit PMA-induced β1-integrin activation in THP-1 cells (Fig. 5D).

We also examined the effect of an MEK inhibitor, PD98059, on the adhesion of THP-1 cells to endothelium in order to confirm the involvement of ERK in the mechanism. The treatment of THP-1 cells with PD98059 suppressed PMA-induced adhesion to HUVECs (Fig. 5E) and B44-positive β1-integrin activation (Fig. 5F).

### Effects of Isoleucyl-Prolyl-Proline (IPP) on Adhesion of THP-1 Cells to HUVECs, JNK Phosphorylation, and Integrin Activation in THP-1 Cells

Another ACE inhibitory tripeptide, IPP, which has similar ACE inhibitory activity (in vitro IC₅₀ is 5 μM) with VPP, had an inhibitory effect on THP-1 cell adhesion (Fig. 6A), JNK phosphorylation (Fig. 6B), and integrin activation (Fig. 6C).

### Expression Level of ACE in THP-1 Cells After PMA Treatment

Since ACE expression on monocytes may contribute to vascular inflammation, such as monocyte-endothelial adhesion through local renin angiotensin system activation, we examined the expression levels of ACE in THP-1 cells by flow cytometry and Western blotting. THP-1 cells (10⁶/mL) were incubated in the presence or absence of VPP (1 mM) for 24 h, followed by stimulation with PMA for 30 min. PMA did not increase ACE expression in THP-1 cells, and VPP did not affect ACE expression (data not shown).

### Discussion

We demonstrated that a bioactive tripeptide, VPP, significantly reduced PMA-stimulated monocyteic cell THP-1 adhesion to activated HUVECs under flow conditions (Fig. 1), and inhibition of JNK phosphorylation might be involved in the underlying mechanism of the phenomenon. The present study is, to our knowledge, the first to describe food derived bioactive peptide inhibitory effects on human monocyte-endothelial interaction under flow.

As previously described, ACE inhibitory action of VPP is moderate, when compared to clinically approved inhibitors. A previous study showed similar
inhibitory effects of ACE inhibitory drugs, imidaprilat and captopril, on MCP-1 or PMA-induced monocyte adhesion to endothelium\(^1\). In that study, the anti-adhesive effect of imidaprilat was observed at 50 nM as early as 4 h after incubation with THP-1, while the anti-adhesive effect of VPP was observed by treatment for 24 h at 1 mM in the present study. In addition, no inhibitory effect of VPP on MCP-1-induced \(\alpha_4\)-integrin expression in THP-1 cells was observed (Fig. 2E), which differs from the data in the previous study; therefore, the anti-adhesive potential of VPP is relatively mild compared to that of imidaprilat. Involvement of ACE in the progression of atherosclerosis has been suggested\(^2\), and ACE expression on monocyte-derived cells may activate the local renin angiotensin system in the vascular wall and subsequently progress atherosclerotic plaque\(^3\). Though PMA treatment increases ACE expression and its activity in HUVECs\(^4\), imidaprilat treatment did not affect ACE expression in THP-1 cells following MCP-1 or PMA treatment\(^2\). Similarly, VPP minimally reduced ACE expression after PMA treatment, which may be attributed to the cleavage of cell-surface ACE upon activation, as reported previously\(^5\).

Kojima et al.\(^6\) also showed that MCP-1 or PMA increased PKC translocation in THP-1 cells, and imidaprilat treatment inhibited PKC translocation via chelating intracellular Zinc; however, VPP treatment followed by PMA stimulation to THP-1 cells did not affect PKC\(\alpha\) and \(\delta\) translocation (Fig. 3). Thus, VPP may not affect intracellular Zinc in THP-1 cells.

On the other hand, the inhibitory effect of VPP and other tripeptides on the adhesion of THP-1 cells was correlated with their inhibitory potential for ACE (in vitro IC\(_{50}\) of VPP, LGP and captopril are 9 \(\mu\)M, 117 \(\mu\)M and 0.023 \(\mu\)M, respectively), suggesting a potential important site within ACE or an other molecule having a similar binding site that is involved in the adhesion of THP-1 cells to HUVECs. In fact, when we also examined the effects of another ACE inhibitory tripeptide, IPP (IC\(_{50}\)=5 \(\mu\)M), it showed similar anti-adhesive properties to VPP (Fig. 6).

Circulating leukocytes maintain their integrins in largely low-affinity conformational (nonadhesive) states\(^5\). Activation of leukocyte integrins, transition from a bent low-affinity to an extended high-affinity conformation by agonists such as chemokines\(^7\) increases the activity of integrin-mediated adhesion to the endothelium. In the present study, transition from a low- to high-affinity conformation of THP-1 integrins was stimulated by PMA and detected using anti-\(\beta_1\) and \(\beta_2\) integrin monoclonal antibody recognizing the activation-dependent epitope of each integrin\(^8\). The results show that PMA stimulation enhanced the activation of THP-1 integrins, which was suppressed by pretreatment of THP-1 cells with VPP (Fig. 2A, B). These findings suggest that VPP inhibits the agonist-induced transition from low- to high-affinity conformation of monocyte \(\beta_1\) and \(\beta_2\) integrins, which could subsequently inhibit the interaction between

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**Fig. 4.** Effects of VPP on MAPK phosphorylation in THP-1 cells. THP-1 cells (10\(^6\)/mL) were treated as described in the legend of Fig. 1. Western blotting (10 mg protein/lane) detected the expression of total and phosphorylated forms of MAPK in lysates of THP-1 cells (10\(^6\)/mL) for each condition. Blots are representative of 3 separate experiments. Band densities were quantified using LAS1000 (Fuji Film) and are expressed in the bar graph. *\(p<0.05\) vs PMA(\(+\)), VPP(\(-\)).
integrins and ligands of the endothelium. A previous study reported that HUTS21-positive β1-integrin activation was inhibited by the pretreatment of THP-1 cells with calphostin C, a potent inhibitor of protein kinase C. As mentioned above, VPP treatment followed by PMA stimulation of THP-1 cells did not affect PKC translocation, but inhibited JNK phosphorylation, suggesting the involvement of JNK in the activation of integrins.

Involvement of MAP kinase in monocyte cell adhesion or integrin activation has been described previously, in which MCP-1-induced activation of ERK in THP-1 cells resulted in integrin activation. We observed that a MEK inhibitor, PD98059, decreased PMA-induced adhesion of THP-1 cells to HUVECs and B44-positive β1-integrin in THP-1 cells (Fig. 5E, F), suggesting that ERK activation as well as JNK activation is involved in the integrin activation and adhesion of THP-1 cells. If PMA-induced JNK activation leads to integrin activation in THP-1 cells, VPP might inhibit JNK-dependent signals, resulting in reduced adhesion. In fact, we demonstrated that treatment of THP-1 cells with a JNK inhibitor, SP600125, followed by PMA stimulation resulted in reduced adhesion of THP-1 to HUVECs (Fig. 5A) and suppression of B44-positive β1-integrin on THP-1 (Fig. 5B), suggesting a critical role of JNK activation in the adhesion of monocytes to endothelial.
Food-derived bioactive peptides have been shown to possess properties, such as antihypertensive, antioxidant, antithrombotic, or antihyperlipidemic, that may reduce the risk of cardiovascular disease. ACE inhibitory peptides derived from some food protein sources are currently the best-known class of bioactive peptides. These nutritional peptides are expected to exert their effectiveness in both the prevention and treatment of hypertension. Numerous studies in spontaneously hypertensive rats (SHR) as well as in hypertensive human volunteers have been conducted to demonstrate the potent antihypertensive effects of these peptides. These in vivo studies have revealed that several ACE inhibitory peptides, including two casein-derived tripeptides, IPP and VPP, significantly reduce high blood pressure.

In light of monocyte-endothelial cell interaction, previous studies demonstrated that thoracic aorta from SHR bind monocytic cells with greater activity than aorta from normotensive Wistar-Kyoto rats. Although the inhibitory effect of ACE inhibitors on in vivo monocyte adhesion to endothelium also has not been reported, the VPP peptide, having an antihypertensive effect on SHR, might affect monocyte-endothelial cell interaction.

Among ACE inhibitory peptides, IPP, VPP, and VY are shown to be absorbed in part through the intestine, enter the systemic circulation without significant degradation, and then exert bioactive properties at the tissue level. Previously, casein hydrolysate containing VPP and IPP improved vascular endothelial dysfunction in subjects with mild hypertension independent of the change in systemic blood pressure. Sardine muscle-derived ACE inhibitory dipeptide, VY, showed in vitro an antiproliferative effect on human vascular smooth muscle cells independent of their ACE inhibitory properties. These data seem to indicate a possible role of these peptides in atherogenic vasculatures; however, recent intravital observation of mechanically injured femoral artery in mice suggested the dominant contribution of oxidative stress in leukocytes but not in the vasculature and a crucial role of monocytes but not vascular wall cells in vascular injury. To clarify the remaining regulatory pathways by VPP controlling monocyte adhesion, further studies are necessary in an in vivo setting as well as in vitro.

In conclusion, we demonstrated that a food-derived ACE inhibitory tripeptide, VPP, attenuated PMA-stimulated adhesion of THP-1 to activated HUVECs. The mechanism of action may be attributed in part to the inhibition of JNK phosphorylation in THP-1. These data indicate that VPP could have a potentially antithrombotic, or antihyperlipidemic, that may reduce the risk of cardiovascular disease. ACE inhibitory peptides derived from some food protein sources are currently the best-known class of bioactive peptides. These nutritional peptides are expected to exert their effectiveness in both the prevention and treatment of hypertension. Numerous studies in spontaneously hypertensive rats (SHR) as well as in hypertensive human volunteers have been conducted to demonstrate the potent antihypertensive effects of these peptides. These in vivo studies have revealed that several ACE inhibitory peptides, including two casein-derived tripeptides, IPP and VPP, significantly reduce high blood pressure.

Fig. 6. (A) Effects of Ile-Pro-Pro (IPP) on adhesion of THP-1 cells to HUVECs. THP-1 cells (10^5/mL) were incubated in the presence of IPP (1 mM) for 24 h, followed by stimulation with PMA (250 nM) for 30 min, and then their adhesion to HUVECs was examined under the flow conditions described in Fig. 1. Data are representative of 3 separate experiments. *p<0.05 vs PMA (+), IPP (−). (B) Effects of IPP, LGP, and VPP on JNK phosphorylation in THP-1 cells. THP-1 cells (10^5/mL) were treated as described in the legend of Fig. 1. Western blotting (10 mg protein/lane) detected the expression of total and phosphorylated forms of JNK in lysates of THP-1 cells (10^5/mL) for each condition. Blots are representative of 5 separate experiments. Band densities were quantified using LAS1000 (Fuji Film) and are expressed in the bar graph. *p<0.05, **p<0.01 vs PMA (+), Peptide (−). (C) Activation of β1-integrin was reduced after IPP treatment. THP-1 cells (10^4/mL) were incubated in the presence or absence of IPP (1 mM) for 24 h, followed by stimulation with PMA for 30 min. β1-integrin (B44) expression was detected for each condition by flow cytometric analysis as described in Materials and Methods. Data are representative of 3 separate experiments.

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