The Protective Effects of Melittin on *Propionibacterium acnes*–Induced Inflammatory Responses *In Vitro* and *In Vivo*

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Melittin is the main component in the venom of the honey bee (*Apis mellifera*). It has multiple effects including antibacterial, antiviral, and anti-inflammatory activities in various cell types. However, the anti-inflammatory mechanisms of melittin have not been elucidated in *Propionibactierium acnes* (*P. acnes*)–induced keratinocyte or inflammatory skin disease animal models. In this study, we examined the effects of melittin on the production of inflammatory cytokines in heat-killed *P. acnes*–induced HaCaT cells. Heat-killed *P. acnes*–treated keratinocytes increased the expression of pro-inflammatory cytokines and Toll-like receptor 2. However, melittin treatment significantly suppressed the expression of these cytokines through regulation of the NF-κB and MAPK signaling pathways. Subsequently, the living *P. acnes* (1×10^7 CFU) were intradermally injected into the ear of mice. Living *P. acnes*–injected ears showed cutaneous erythema, swelling, and granulomatous response at 24 hours after injection. However, melittin-treated ears showed markedly reduced swelling and granulomatous responses compared with ears injected with only living *P. acnes*. These results demonstrate the feasibility of applying melittin for the prevention of inflammatory skin diseases induced by *P. acnes*.

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INTRODUCTION

Acne is an inflammatory disease of the sebaceous glands, and is a common skin disease that induces inflammation on the skin surface of the face, neck, chest, and back. Acne develops mostly in young people because of several factors: hormonal imbalance, bacterial infection, stress, food, and cosmetic application (Marples, 1974). In particular, *Propionibacterium acnes* (*P. acnes*) is a Gram-positive anaerobic bacterium residing in pilosebaceous follicles as a member of resident bacterial flora in the skin (Leyden, 2001). Once it overgrows and colonizes sebaceous hair follicles, *P. acnes* is pertinent to the development of inflammatory acne vulgaris, the most common skin disease afflicting up to 80% of individuals throughout their lives (Bojar and Holland, 2004). *P. acnes* acts as an immunostimulator and produces a variety of enzymes as well as biologically active molecules that are involved in the development of inflammatory skin diseases. The main components of the pilosebaceous unit of the skin, such as keratinocytes, can be activated by *P. acnes* and lead to the production of pro-inflammatory cytokines (Leeming *et al.*, 1985; Vowels *et al.*, 1995). It has been reported that a secreted peptidoglycan of *P. acnes* can stimulate the production of pro-inflammatory cytokines or chemokines, such as interleukin (IL)-1, IL-8, and TNF-α, by monocytic cell lines; thereby triggering granulomatous reactions of inflammatory skin disease (Chen *et al.*, 2002; Jain and Basal, 2003). Furthermore, *P. acnes* stimulates the production of pro-inflammatory cytokines via Toll-like receptor (TLR)2 and TLR4 (Webster, 2002; Heymann, 2006). During inflammatory reactions, TLR activation results in the activation of the MAPK and the transcription factor NF-κB signaling pathways. These pathways then modulate inflammatory gene expression, which is crucial in shaping the innate immune response within the inflammatory skin disease (Grange *et al.*, 2009).

Antibiotics are typical therapeutic agents for *P. acnes*–induced inflammatory skin diseases; they are administered to inhibit inflammation or kill the bacteria. For example, triclosan, benzoyl peroxide, azelaic acid, retinoid, tetracycline, erythromycin, macrolide, and clindamycin are among such antibiotics. However, these antibiotics have been known to induce side effects. Therefore, many researchers have tried to develop therapeutic agents for acne that have no side effects, but high antibacterial activity (Iwasaki and Medzhitov, 2004).

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Abbreviations: LPS, lipopolysaccharide; PA, living P. acnes–injected group; P. acnes, Propionibactierium acnes; Sup, culture supernatant of P. acnes

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Melittin is a cationic, hemolytic peptide that is the main toxic component in the venom of honey bee (*Apis mellifera*). It has multiple effects, including antibacterial, antiviral, and anti-inflammatory activities, in various cell types (Raghuraman and Chattopadhyay, 2007). Recent studies have shown that melittin can induce cell cycle arrest, growth inhibition, and apoptosis in various tumor cells (Chu *et al.*, 2007; Zhang *et al.*, 2007). We previously demonstrated that melittin efficiently suppresses the expression of specific genes in the animal model of liver cirrhosis and atherosclerosis (Kim *et al.*, 2011; Park *et al.*, 2012). These studies are informative, but they are not enough to demonstrate that melittin can prevent the development of inflammatory molecular mechanisms of skin diseases in *in vitro* and *in vivo* models. Therefore, in this study, we investigated the potential therapeutic effects of melittin as an alternative agent for inflammatory skin diseases. We examined the effects of melittin on the production of inflammatory cytokines in heat-killed *P. acnes*–induced HaCaT cells *in vitro*. Furthermore, the molecular pathogenesis of anti-inflammatory effects of melittin was investigated in living *P. acnes*–induced inflammatory skin disease animal models.

RESULTS

Heat-killed *P. acnes* is capable of inducing inflammatory responses in HaCaT cells

We first examined whether heat-killed *P. acnes* was capable of inducing secreted inflammatory cytokines. HaCaT cells were treated with various stimulations to determine the inflammatory effects of these stimulants. TNF-α, IL-1β, IL-8, and IFN-γ secretions were increased by lipopolysaccharide (LPS), culture supernatant of *P. acnes* (Sup), and heat-killed *P. acnes* in the HaCaT cell culture medium. However, the expression levels were lower in LPS- and Sup-treated cells than those in heat-killed *P. acnes* (1.0×10^7 CFU ml^{-1})–treated cells (Figure 1a). In particular, 1.0×10^7 CFU ml^{-1} of heat-killed *P. acnes* significantly increased TNF-α and mature IL-1β (17 kDa) expression levels compared with other stimulations (Figure 1b). We investigated the effects of LPS, Sup, and heat-killed *P. acnes* ($1.0 \times 10^{5\text{-}7}$ CFU ml^{-1}) on cell

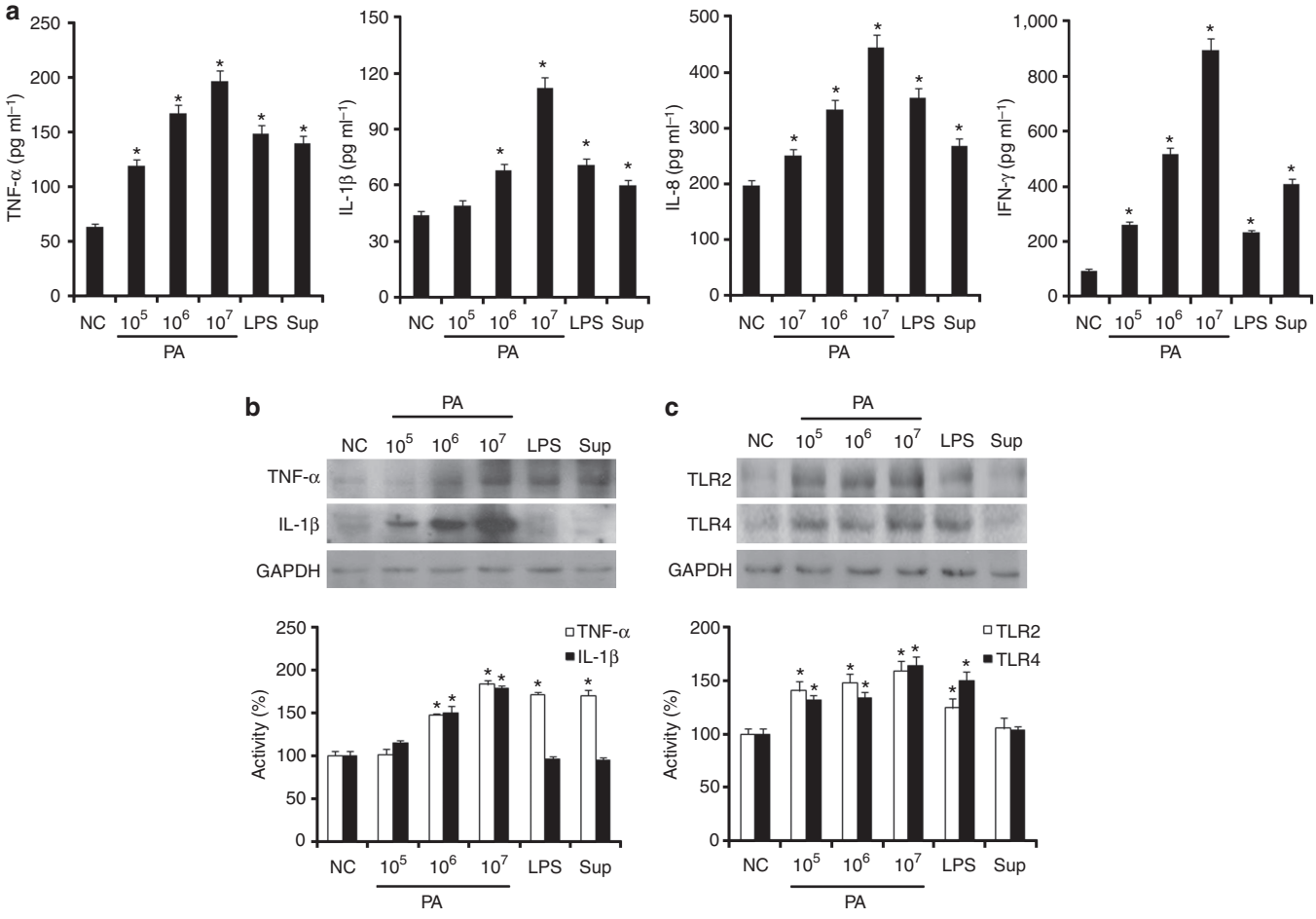

Figure 1. Various stimulants induced pro-inflammatory cytokines in keratinocytes. HaCaT cells were treated with heat-killed *Propionibactierium acnes* ($1.0 \times 10^{5\text{-}7}$ CFU ml^{-1}), LPS (100 ng ml^{-1}), or Sup (50 μl ml^{-1}). (**a**) ELISA results with culture medium show that TNF-α, IL-1β, IL-8, and IFN-γ were increased by heat-killed *P. acnes*, LPS, and Sup treatment. (**b**) Western blot analysis demonstrates that TNF-α and IL-1β (17 kDa) were increased by heat-killed *P. acnes*, LPS, and Sup treatment. (**c**) Western blot analysis demonstrates that TLR2 and 4 were increased by heat-killed *P. acnes*, LPS, and Sup treatment. Results are expressed as mean ± SE of three independent determinations. *$P<0.05$ compared with the normal control (NC). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PA, living *P. acnes*–injected group; Sup, culture supernatant of *P. acnes*; TLR, Toll-like receptor.

cytotoxicity by measurement of LDH release. The LDH assay signifies membrane integrity and direct measurement of cell death. Our results show that LPS, Sup, and heat-killed *P. acnes* ($1.0 \times 10^{5\text{-}7}$ CFU ml^{-1}) did not influence LDH release in HaCaT cell culture medium (Supplementary Figure S1 online). Recent papers have reported that *P. acnes* may induce inflammation through activation of TLRs, especially TLR2 and 4, which are expressed in keratinocytes (Lyte *et al.*, 2009). Figure 1c shows increased expression of TLR2 and 4 by heat-killed *P. acnes*. In addition, the expression levels of TLR2 were higher in heat-killed *P. acnes*–treated cells compared with those in LPS- or Sup-treated cells. These results suggest that TLR2 responded to *P. acnes* and that heat-killed *P. acnes* can initiate inflammation in keratinocytes.

Effects of melittin on pro-inflammatory cytokines and TLR expressions in heat-killed *P. acnes*–treated HaCaT cells

We determined the cytotoxicity of melittin at different doses and times by the CCK-8 assay. HaCaT cells were treated with different concentrations of melittin (0.1, 0.5, and 1 μg ml^{-1}) and at different time points (8, 12, and 24 hours). After treatment with melittin, the viability of HaCaT cells was decreased at 12 and 24 hours. However, an 8-hour treatment with melittin did not affect the cell viability of HaCaT cells. Thus, the effects of melittin on HaCaT cells were minimal at 8 hours. On the basis of these results, an optimal treatment time of 8 hours for melittin was used for subsequent experiments in HaCaT cells (Supplementary Figures S2 and S3 online). Subsequently, we examined the effects of melittin on the production of inflammatory cytokines and chemokines in heat-killed *P. acnes*–induced HaCaT cells. Cells were treated with different concentrations of melittin in the presence of heat-killed *P. acnes*. Melittin treatment significantly suppressed the secretion of TNF-α, IL-1β, IL-8, and IFN-γ in 1.0×10^{7} CFU ml^{-1} of heat-killed *P. acnes*–treated cells. Thus, these observations suggest that melittin effectively inhibits the secretion of TNF-α, IL-1β, IL-8, and IFN-γ in HaCaT cells (Figure 2a). Also, we assessed the effects of melittin on heat-killed *P. acnes*–induced pro-inflammatory cytokine expression in HaCaT cells. Cells expressed TNF-α and IL-1β (17 kDa) after exposure to heat-killed *P. acnes*. On the other hand, melittin treatment markedly suppressed the expression of TNF-α and IL-1β (17 kDa) in a concentration-dependent manner (Figure 2b). In addition, melittin-treated HaCaT cells displayed a significantly decreased expression of TLR2 and 4, especially with the administration of 1 μg ml^{-1} of melittin (Figure 2c). Several papers have reported that the activity of TLR2 results in IL-8 secretion of keratinocytes (Kim *et al.*, 2002). Thus, this study examined the expression of TLR2 and IL-8 in HaCaT cells by immunofluorescence labeling (Figure 2d). The

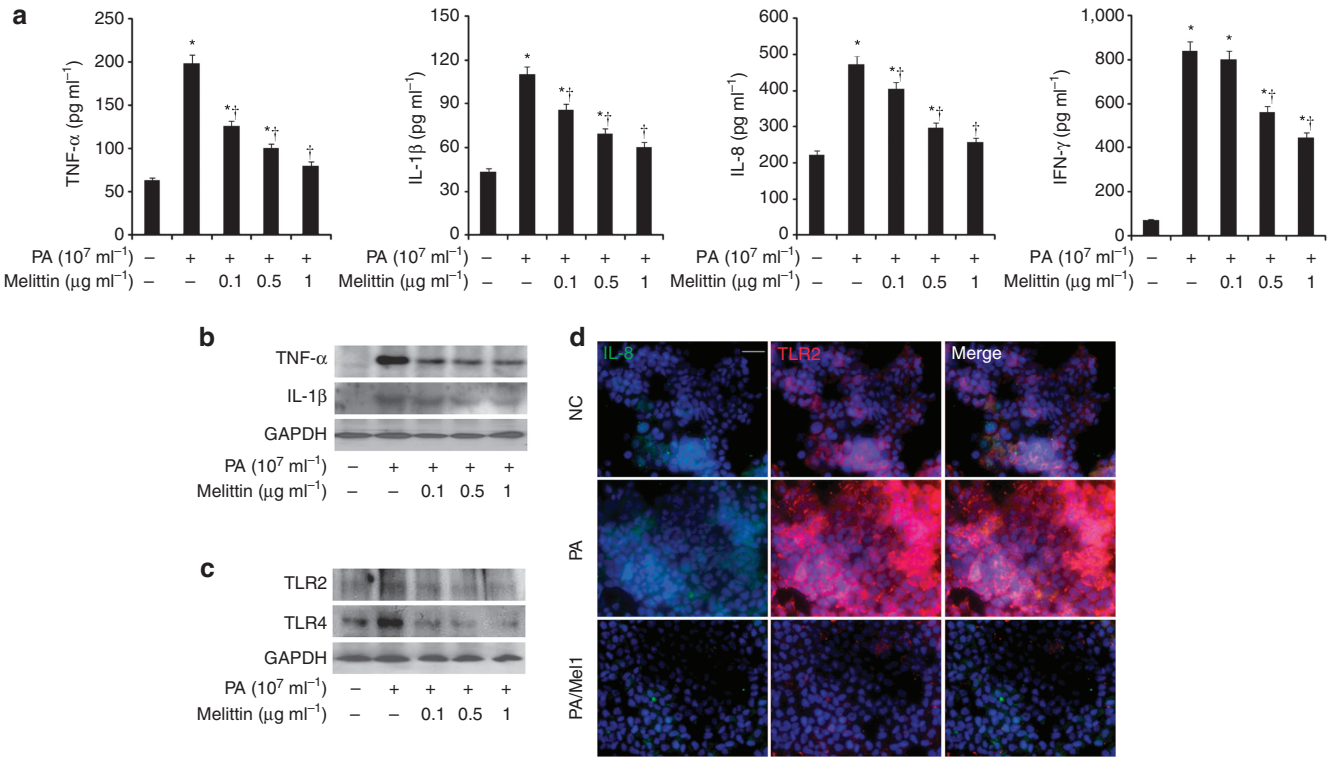

Figure 2. Melittin effectively inhibits pro-inflammatory cytokines and TLRs in HaCaT cells. (**a**) ELISA results demonstrate that melittin suppressed the secretion of TNF-α, IL-1β, IL-8, and IFN-γ in culture medium with HaCaT cells. (**b**, **c**) Western blot analysis shows that melittin inhibited the expression of TNF-α and IL-1β (17 kDa), and the regulation of TLR2 and 4. (**d**) Melittin treatment reduced IL-8 and TLR2 in heat-killed *P. acnes*–treated HaCaT cells. Immune complexes were detected by anti-mouse FITC (green), anti-rabbit Texas red (red), and nuclei were stained with Hoechst 33342 (blue). Magnification ×400, bar = 100 μm. Results are expressed as mean ± SE of three independent determinations. **P*<0.05 compared with the normal control (NC). †*P*<0.05 compared with the only heat-killed *P. acnes*–treated cells. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PA, living *P. acnes*–injected group; TLR, Toll-like receptor.

distribution of TLR2 (FITC, green) and IL-8 (Texas red, red) in normal HaCaT cells displayed little TLR2 and IL-8 expression in the cytoplasm and plasma membrane. In contrast, heat-killed *P. acnes*–treated cells showed a strong labeling intensity of TLR2 and IL-8 in the cytoplasm of HaCaT cells. Staining with TLR2 revealed that colonies of heat-killed *P. acnes* were present on the surface area of the cytoplasm. In particular, the expression of TLR2 was clearly increased in heat-killed *P. acnes*–treated cells. However, treatment with 1 μg ml^{-1} of melittin exhibited decreased expression of TLR2. These data suggest that heat-killed *P. acnes* induced activation of TLR2 and resulted in IL-8 expression, and that melittin reduced the expression of TLR2 and IL-8. These results demonstrate that melittin seems to be capable of reducing pro-inflammatory cytokine secretion or expression and related TLR2 expression in heat-killed *P. acnes*–induced keratinocytes.

Effects of melittin on the NF-κB and MAPK signaling pathways in heat-killed *P. acnes*–treated HaCaT cells

To determine the involvement of the NF-κB signaling pathways in the anti-inflammatory properties of melittin, the activation of these proteins was examined by western blots (Figure 3a). Following the administration of heat-killed *P. acnes*, increased expressions of cytosolic phosphorylated IKK, IκB, and nuclear NF-κB were found in the

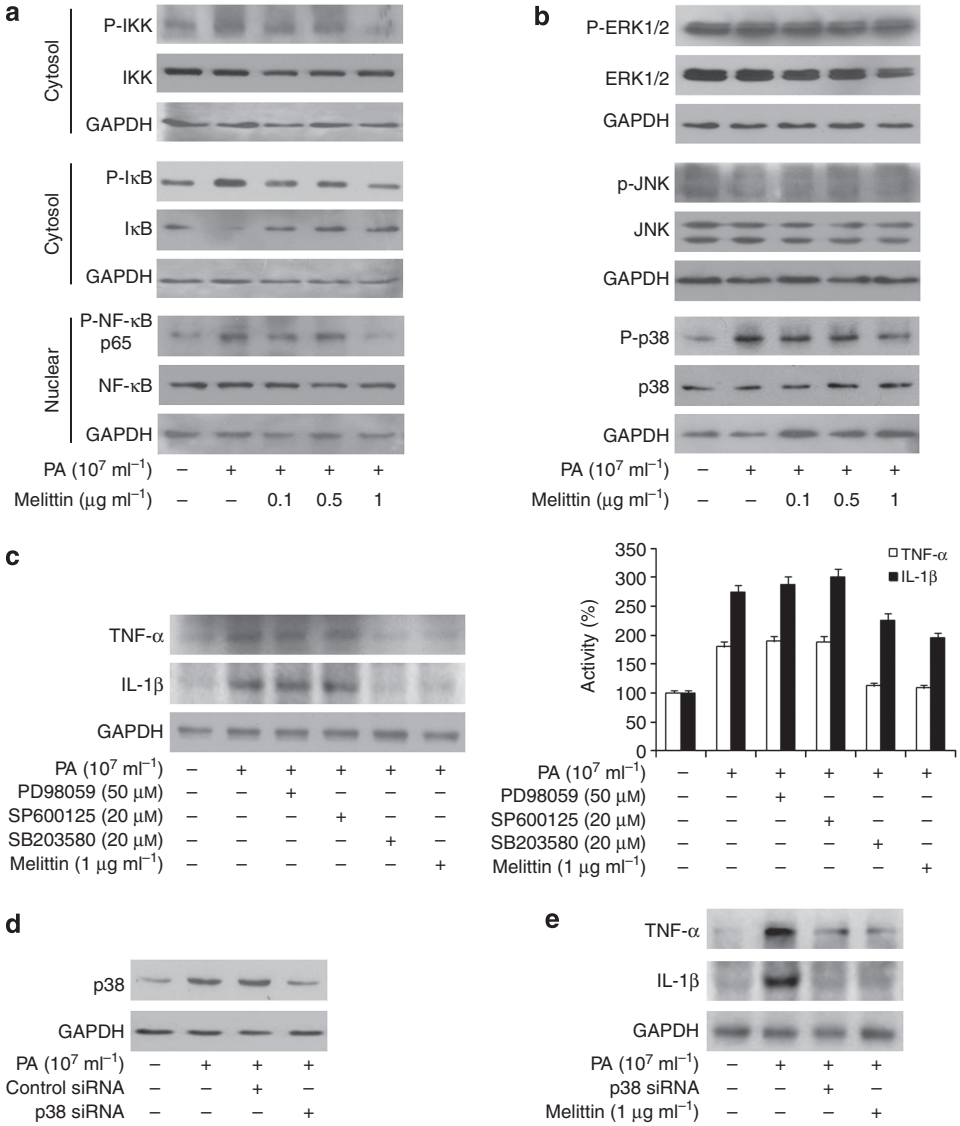

Figure 3. Melittin effectively inhibits the NF-κB and MAPK signaling pathway in *P. acnes*–treated HaCaT cells. (**a**) Western blot analysis shows that phosphorylation of IKK, IκB, and NF-κB is suppressed by melittin treatment. (**b**) Melittin treatment almost completely blocked the phosphorylation of p38 after heat-killed *P. acnes* treatment of HaCaT cells. (**c**) Expression levels of TNF-α and IL-1β suppressed by melittin and SB203580 treatment in HaCaT cells. (**d**) HaCaT cells were transfected with control or specific p38 siRNA for 48 hours and then treated with heat-killed *P. acnes* for 8 hours. The expression of p38 was suppressed by p38 siRNA treatment in heat-killed *P. acnes*–treated HaCaT cells. (**e**) Expression levels of TNF-α and IL-1β suppressed by p38 siRNA and melittin-treated HaCaT cells. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PA, living *P. acnes*–injected group.

heat-killed *P. acnes*–treated cells. However, addition of melittin significantly reduced IKK and IκB phosphorylation in the heat-killed *P. acnes*–treated cells. In the nuclear fraction, phosphorylated NF-κB was also decreased at 1 μg ml^{-1} of melittin. These results indicate that treatment with melittin abrogated the effect of *P. acnes* on altering the expression levels of genes, which are relevant to skin inflammation through NF-κB signaling. Activation of MAPK is significant in the regulation of inflammation to control the activation of NF-κB and IKKs (Kim *et al.*, 2006). In the present study, we investigated whether melittin modulates MAPK signals in heat-killed *P. acnes*–treated HaCaT cells. Figure 3b shows that phosphorylated p38 was markedly increased after treatment with heat-killed *P. acnes*. However, phosphorylated p38 was decreased after treatment with melittin. More specifically, treatment with 1 μg ml^{-1} of melittin almost completely blocked the phosphorylation of p38 after treatment with heat-killed *P. acnes* in HaCaT cells. However, ERK1/2 and JNK phosphorylated forms were not changed after treatment with heat-killed *P. acnes* or melittin. Subsequently, we confirmed the p38 MAPK-dependent TNF-α and IL-1β upregulation in HaCaT cells that were pretreated with MAPK inhibitors or p38 siRNA. Pretreatment with PD98059 and SP600125 increased the expression levels of TNF-α and IL-1β (17 kDa) in heat-killed *P. acnes*–treated HaCaT cells. However, SB203580 or 1 μg ml^{-1} of melittin specifically inhibited the expression of TNF-α and IL-1β (17 kDa), respectively, in heat-killed *P. acnes*–treated cells (Figure 3c and Supplementary Figure S4 online). After transfection with p38 siRNA for 48 hours, the expression of p38 showed a decline in heat-killed *P. acnes*–treated HaCaT cells. However, transfection with control siRNA did not have any effect on p38 accumulation (Figure 3d). Next, we examined the effects of melittin on TNF-α and IL-1β (17 kDa) expression in heat-killed *P. acnes*–treated HaCaT cells. Figure 3e shows that pretreatment with p38 siRNA inhibited the expression of TNF-α and IL-1β. Furthermore, melittin suppressed the expression of TNF-α and IL-1β in heat-killed *P. acnes*–treated HaCaT cells. These results support the explanation that melittin inhibits TNF-α and IL-1β expression by suppression of p38 MAPK phosphorylation in heat-killed *P. acnes*–treated HaCaT cells.

Effects of melittin on *P. acnes*–treated inflammatory animal model

To investigate the effects of living *P. acnes* on the development of inflammatory skin diseases, ear tissues were harvested to observe their histological changes (Supplementary Figure S5 online). Living *P. acnes*–injected ears showed cutaneous erythema, swelling, and granulomatous response at 24 hours after injection. Supplementary Figure S5b online shows that the left ear was significantly thickened compared with the PBS-injected right ear. These results demonstrate that living *P. acnes* is suitable to induce inflammation in the skin *in vivo*. Subsequently, we attempted to determine the protective effects of melittin against *P. acnes*–induced inflammatory skin tissues. To do this, different concentrations of melittin mixed with vaseline were applied to the right ears of mice. Histological observation revealed that *P. acnes* induced a considerable increase in the number of infiltrated inflammatory cells (Figure 4a–c). However, melittin-treated ears showed markedly reduced swelling and granulomatous response compared with ears injected with only living *P. acnes*. In particular, 100 μg of melittin resulted in a 1.3-fold reduction of ear thickness compared with ears injected with only living *P. acnes* (Figure 4f).

Effects of melittin on the gene expression of TNF-α and IL-1β in the inflammatory animal model

Bacterial infection stimulates the production of pro-inflammatory cytokines, including TNF-α and IL-1β (Kim, 2005). As shown in Figure 5a and b, the expression levels of TNF-α and IL-1β were barely detected in normal skin tissue from the NC group. However, more significant upregulation of these expression levels was observed in the PA and Vas groups, whereas treatment with melittin led to the evident down-regulation of TNF-α and IL-1β expression. Concomitant with western blot and RT–PCR, the results show that the expression levels of TNF-α and IL-1β were significantly increased in the PA and Vas groups. Also, TNF-α, IL-1β, IL-8, and IFN-γ mRNA levels were increased in the PA and Vas groups. However, these expression levels were decreased in the PA/Mel100 group (Figure 5c-e). These results show that melittin resulted in suppression of pro-inflammatory cytokines in the *P. acnes*–treated inflammatory animal model.

Effects of melittin on the DNA-binding activity of transcription factors and TLR2 expression in the inflammatory animal model

We evaluated the underlying mechanisms of melittin in skin inflammation *in vivo*. The expression levels of cytosolic phospho-IKK, phospho-IκB, and nuclear NF-κB were determined. After *P. acnes* administration, there was an increase in cytosolic phospho-IKK, phospho-IκB, and nuclear NF-κB expression in the PA and PA/Vas groups. However, melittin treatment reduced the phosphorylation of IKK and IκB in the PA/Mel100 group. Also, treatment with melittin reduced the amount of the nuclear NF-κB protein (Figure 6a). Subsequently, to examine whether melittin effectively blocks the DNA-binding activity of NF-κB and AP-1, gel mobility shift assays were performed. As shown in Figure 6b and c, the binding activity of NF-κB and AP-1 was increased in the PA and Vas groups. In contrast, this enhancement of binding activity was markedly withdrawn after treatment with melittin. These results show that melittin effectively blocked the DNA-binding activity of NF-κB and AP-1 at the transcriptional level. Afterwards, we examined the effects of melittin on the expression of TLR2 and CD14 in inflammatory skin. Merged images show that the PA and Vas groups predominantly expressed CD14 in the dermis and TLR2 in the epithelium, both of which are localized at different parts of the tissue. These results suggest that an injection of *P. acnes* induces the infiltration of macrophages and activation of TLR2 in the inflammatory skin tissue. However, melittin treatment resulted in noticeable inhibition of CD14 and TLR2 expression (Figure 6d). These results suggest that melittin inhibits the expression of CD14 and IL-8. Moreover, melittin modulates DNA-binding activity of NF-κB and AP-1 in inflammatory skin.

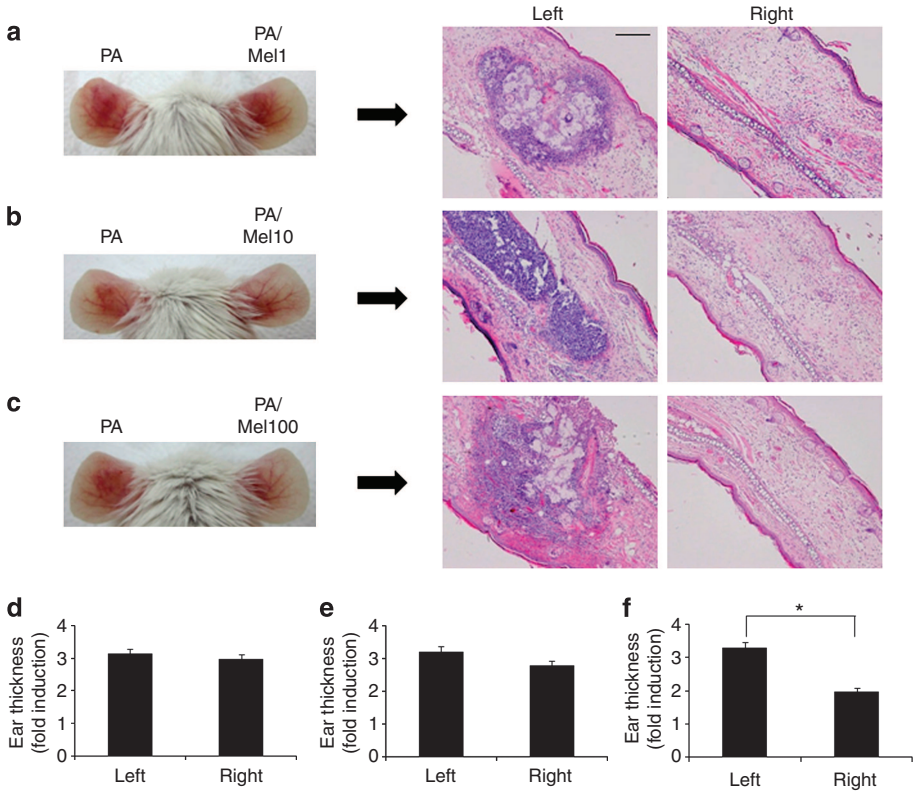

Figure 4. Melittin reduced ear thickness in *P. acnes*–injected inflammatory skin. Living *P. acnes*, 1.0×10^7 CFU per 20 μl in PBS, was intradermally injected into the ears of ICR mice. After (**a**, **d**) 1, (**b**, **e**) 10, and (**c**, **f**) 100 μg of different concentrations of melittin mixed with 0.05 g of vaseline were applied to the surface of the right ear. Representative hematoxylin and eosin staining images from each study group (five mice per group). PA, living *P. acnes*–injected group; PA/Mel 1, 10, 100, living *P. acnes*–injected group with application of 1, 10, and 100 μg of melittin mixed with vaseline. Magnification ×100, bar = 100 μm. Results are expressed as mean ± SE of three independent determinations. *$P<0.05$ compared with the right ear.

DISCUSSION

A direct effect of *P. acnes* on keratinocytes during immune responses of skin lesions has been implicated in the initiation of the inflammatory processes. *P. acnes* has a critical role in the development of inflammatory skin diseases (Vowels *et al.*, 1995; Thiboutot, 1997). Several papers have reported that infection of *P. acnes* involves an interaction of TLR2 and 4 with keratinocytes (Webster, 2002; Heymann, 2006). Activation of TLR2 and 4 induces the release of inflammatory cytokines and chemokines, such as TNF-α, IL-1β, IFN-γ, and IL-8 in skin diseases (Chen *et al.*, 2002; Pivarcsi *et al.*, 2003). TNF-α is a multifunctional cytokine involved in the regulation of immunity and inflammation (Jain and Basal, 2003). Recent studies have indicated that TNF-α, IL-1β, and IL-8 accelerate skin inflammation in mice. Also, these cytokines or chemokines were shown to modulate inflammatory responses in keratinocytes and monocytes (Kang *et al.*, 2005; Shibata *et al.*, 2009). In particular, IL-8 is a CXC chemokine with mitogenic activity in keratinocytes. It is a chemoattractant involved in the recruitment of neutrophils, the predominant cell type in inflammation-related skin lesions (Layton *et al.*, 1998; Koreck *et al.*, 2003). During inflammatory reactions in the skin, IFN-γ has an essential role in host defense against various bacteria by activating phagocytes and inflammatory reactions (Kawa *et al.*, 2010). Recent papers have reported that LPS, Sup, and *P. acnes* directly stimulate the production of TNF-α, IL-8, and IFN-γ via TLR expression (Vowels *et al.*, 1995; Basal *et al.*, 2004). Our results show that LPS, Sup, and *P. acnes* induced secretion of TNF-α, IL-8, and IFN-γ in HaCaT cells. Moreover, melittin treatments effectively inhibited the expression of these cytokines. These results demonstrate that LPS, Sup, and *P. acnes* are capable of inducing inflammatory responses in HaCaT cells. In addition, melittin treatments effectively inhibited the expression of TNF-α, IL-1β, IFN-γ, and IL-8, and modulated TLR activation.

The major pathway used by most TLRs leads to the activation of the NF-κB and MAPK signaling pathways. They then modulate inflammatory gene expression, which is crucial for the innate immune response of inflammation (Hari *et al.*, 2010). NF-κB comprises a family of inducible transcription factors that serve as important regulators of the host immune and inflammatory responses (Yaron *et al.*, 1998; Spencer *et al.*, 1999). Other important signaling pathways of MAPK have been implicated in multiple cellular events, such as proliferation, survival, differentiation, and inflammation (Duesbery *et al.*, 1998; Grange *et al.*, 2009). Activation of MAPK leads to increased production of pro-inflammatory mediators, such as TNF-α and IL-1β (Kim *et al.*, 2006; Moon *et al.*, 2007). In this study, melittin suppressed heat-killed *P. acnes*–inducible IκB phosphorylation and nuclear NF-κB p65 activation in HaCaT

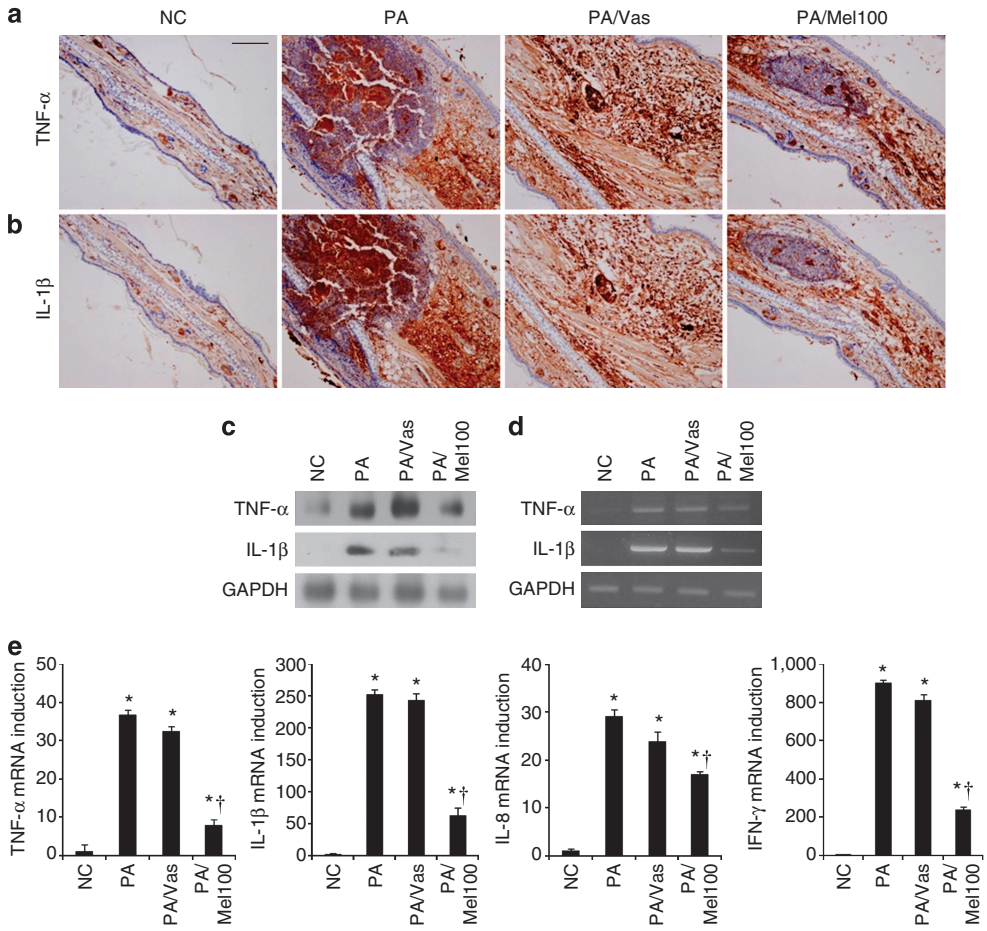

Figure 5. Melittin effectively inhibits the pro-inflammatory cytokine in *P. acnes*–injected inflammatory skin. (**a**, **b**) Immunohistochemical results demonstrate that melittin suppresses the expression of TNF-α and IL-1β. (**c**, **d**) Western blot and RT–PCR analyses show that melittin treatment inhibited the expression of TNF-α and IL-1β. (**e**) Real time–PCR analyses show that melittin treatment inhibited the mRNA levels of TNF-α, IL-1β, IL-8, and IFN-γ. Representative images from each study group (five mice per group). *$P<0.05$ compared to the NC. †$P<0.05$ compared to the PA. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NC, normal control; PA, living *P. acnes*–injected group; PA/Vas, living *P. acnes*–injected group with application of vaseline; PA/Mel100, living *P. acnes*–injected group with application of 100 μg of melittin mixed with vaseline. Magnification ×100, bar = 100 μm.

cells. Moreover, the heat-killed *P. acnes*–induced p38 MAPK signal was specifically inhibited by melittin. These results demonstrate that melittin mediates anti-inflammatory effect via NF-κB signaling and that activation of the p38 pathway is important in the activation of TNF-α and IL-1β during inflammatory reactions.

On the basis of the *in vitro* results, we investigated the effects of melittin on the *P. acnes*–treated animal model. Recent studies have reported that an injection of living *P. acnes* leads to the development of inflammatory skin diseases in animal models (Nakatsuji *et al.*, 2008, 2009). However, the precise mechanism of the anti-inflammatory effects of melittin has not been elucidated in inflammatory skin diseases. In this study, intradermal injection of living *P. acnes* into the mouse ear induced an increase in ear thickness and in granulomatous response. Subsequently, we examined the effects of melittin on the living *P. acnes*–injected inflammatory animal model. The major findings of *in vivo* results are that melittin attenuates the manifestation of inflammatory skin pathologies and decreases the expression of pro-inflammatory cytokines. These inflammatory cytokines are regulated by transcription factors, such as NF-κB and AP-1, in acne lesions (Iwasaki and Medzhitov, 2004; Trinchieri and Sher, 2007; Shibata *et al.*, 2009). The results of this study show that the binding activity of NF-κB and AP-1 was increased in living *P. acnes*–induced inflammatory skin disease. In contrast, melittin markedly withdrew the responses induced by living *P. acnes* by suppression of inflammatory cytokines through modulation of NF-κB and AP-1 transcription factors. In effect, melittin inhibits degeneration of skin inflammation.

In conclusion, we demonstrated the protective effects of melittin on the *P. acnes*–induced *in vitro* and *in vivo* inflammatory models. Administration of melittin significantly decreased the expression of various inflammatory cytokines in heat-killed *P. acnes*–treated keratinocytes. In particular, melittin suppressed the expression of TNF-α and IL-1β through regulation of the NF-κB and MAPK signaling pathways in keratinocytes. In addition, melittin exerted anti-inflammatory effects against the living *P. acnes*–treated animal model. These protective effects were mainly because of the suppression of NF-κB and AP-1, which regulate the production of inflammatory cytokines. These results demonstrate the feasibility of

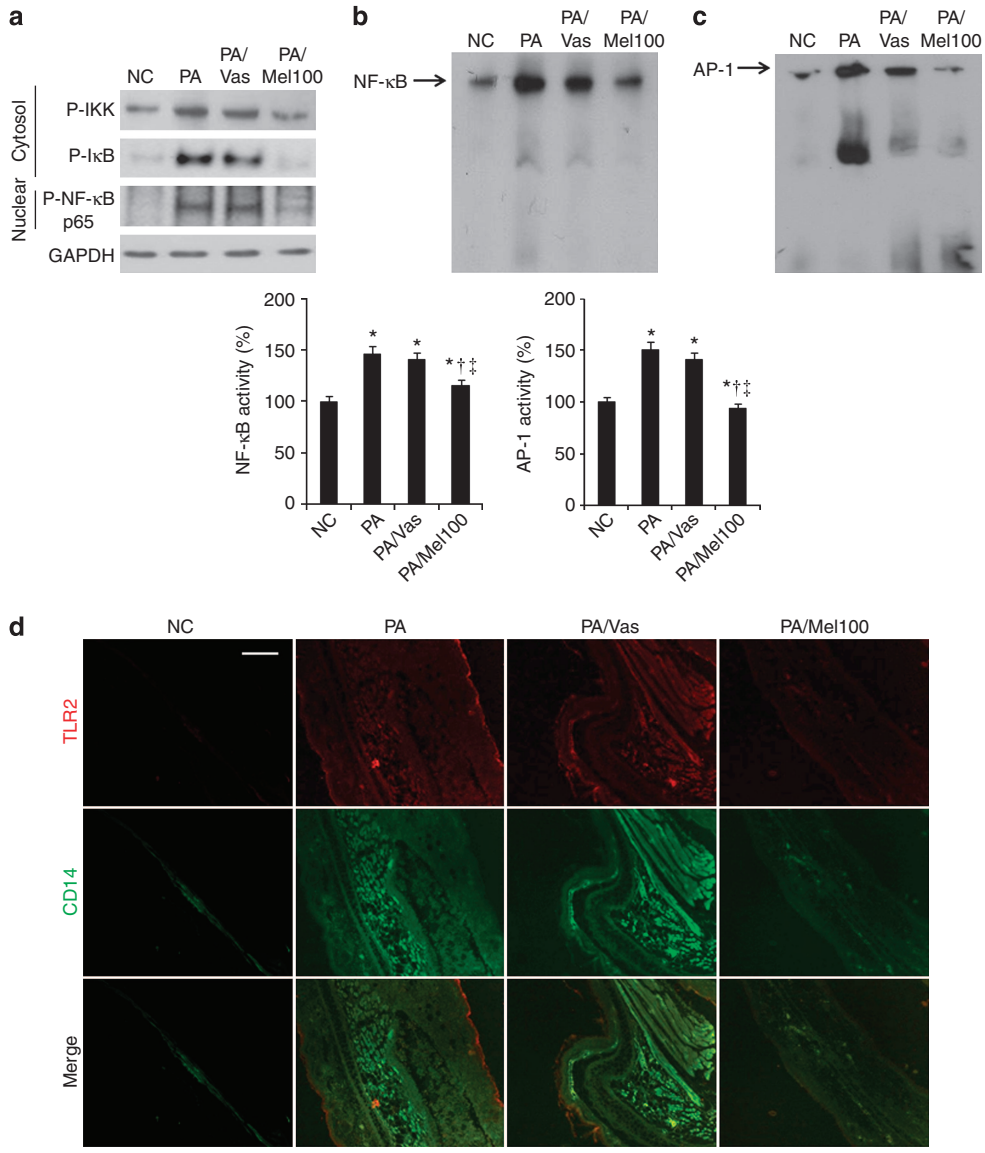

Figure 6. Melittin inhibits the NF-κB signaling and DNA-binding activity. (**a**) Melittin treatment reduced the phosphorylation of IKK, IκB, and NF-κB. Electrophoretic mobility shift assay results show that melittin treatment suppresses the binding activity of NF-κB (**b**) and AP-1 (**c**) in *P. acnes*–treated inflammatory skin. (**d**) Representative immunofluorescence images show that melittin treatment suppresses TLR2 and CD14 in the *P. acnes*–treated inflammatory skin. CD14 and TLR2 immune complexes were detected by anti-mouse FITC (green) and anti-rabbit Texas red (red). Representative images from each study group (five mice per group). Magnification × 100, bar = 100 μm. Results are expressed as mean ± SE of three independent determinations. *$P<0.05$ compared with the NC. †$P<0.05$ compared with the PA. ‡$P<0.05$ compared with the PA/Vas. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NC, normal control; PA, living *P. acnes*–injected group; PA/Vas, living *P. acnes*–injected group with application of vaseline; TLR, Toll-like receptor.

applying melittin for the prevention of progression of inflammatory skin diseases induced by *P. acnes*.

MATERIALS AND METHODS

Cell cultures

HaCaT (5.0×10^5 cells ml^{-1}) cells were seeded in complete medium. After 24 hours, the cells were changed to serum-free medium containing the indicated concentrations of melittin (0.1, 0.5, and 1 μg ml^{-1}). After 30 minutes, the cells were treated with heat-killed *P. acnes* ($1.0 \times 10^{5\text{-}7}$ CFU ml^{-1}, PA), the culture supernatant of *P. acnes* (50 μl ml^{-1}, Sup), and lipopolysaccharide (LPS, 100 ng ml^{-1}) and were cocultured for 8 hours. For further information, see Supplementary Materials and Methods.

ELISA

Concentrations of cytokines and chemokines were measured with ELISA kits (R&D Systems, Minneapolis, MN).

Western blotting

Western blotting was performed as previously described (Kim *et al.*, 2011). For further information, see Supplementary Materials and Methods online.

Animal model

Eight-week-old ICR mice ($n=30$) were randomly subdivided into six groups (five mice per group) and were maintained under various conditions. All surgical and experimental procedures used in the

current study were approved by the institutional review board committee of the Catholic University of Daegu Medical Center. For further information, see Supplementary Materials and Methods online.

RT–PCR

Primer sequences and other details are reported in Supplementary Materials and Methods online.

Histological and immunofluorescent staining

Hematoxylin and eosin, immunohistochemical, and immunofluorescent staining was performed according to the described procedure (Kim *et al.*, 2011). For further information, see Supplementary Materials and Methods online.

Statistical analysis

Data are presented as the means ± SE. The Student's *t*-test was used to assess the significance of independent experiments. The criterion $P<0.05$ was used to determine statistical significance.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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