Melittin, a Major Component of Bee Venom, Sensitizes Human Hepatocellular Carcinoma Cells to Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL)-induced Apoptosis by Activating CaMKII-TAK1-JNK/p38 and Inhibiting IκBα Kinase-NFκB

Received for publication, September 16, 2008, and in revised form, December 11, 2008 Published, JBC Papers in Press, December 12, 2008, DOI 10.1074/jbc.M807191200

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Promoting apoptosis is a strategy for cancer drug discovery. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in a wide range of malignant cells. However, several cancers, including human hepatocellular carcinoma (HCC), exhibit a major resistance to TRAIL-induced cell death. Melittin, a water-soluble 26-amino acid peptide derived from bee venom of Apis mellifera, can exert toxic or inhibitory effects on many types of tumor cells. Here we report that melittin can induce apoptosis of HCC cells by activating Ca2+/calmodulin-dependent protein kinase, transforming growth factor-β-activated kinase 1 (TAK1), and JNK/p38 MAPK. We show that melittin-induced apoptosis can be inhibited by calcium chelator, by inhibitors for Ca2+/calmodulin-dependent protein kinase, JNK and p38, and by dominant negative TAK1. In the presence of melittin, TRAIL-induced apoptosis is significantly increased in TRAIL-resistant HCC cells, which may be attributed to melittin-induced TAK1-JNK/p38 activation and melittin-mediated inhibition of IκBα kinase-NFκB. Our data suggest that melittin can synergize with TRAIL in the induction of HCC cell apoptosis by activating the TAK1-JNK/p38 pathway but inhibiting the IκBα kinase-NFκB pathway. Therefore, the combination of melittin with TRAIL may be a promising therapeutic approach in the treatment of TRAIL-resistant human cancer.

At present about 20 different ligands that belong to the tumor necrosis factor (TNF) superfamily have been identified, among which TNFα, lymphotoxin α, Fas ligand, apo3L, and TNF-related apoptosis-inducing ligand (TRAIL) have been characterized as major mediators of apoptosis (1, 2). TRAIL, in its soluble form, is emerging as an attractive anticancer agent because of its cancer cell specificity and potent antitumor activity (1, 2).

TRAIL signals by interacting with its receptors (1). Thus far, five receptors (Fas ligand) have been identified, namely the two agonistic receptors, TRAIL-R1 and TRAIL-R2, and the three antagonistic receptors TRAIL-R3, TRAIL-R4, and osteoprotegerin (3–6). Binding of TRAIL to the extracellular domain of agonistic receptors results in the trimerization of the receptors and clustering of the intracellular death domains, which lead to the recruitment of the adaptor molecule Fas-associated protein with death domain (FADD) (1). Subsequently, FADD recruits and activates initiator caspase-8 and caspase-10, leading to cellular disassembly (1). Meanwhile, TRAIL-initiated apoptotic signaling requires an amplification loop by mitochondrial pathway engagement through impairment of the mitochondrial membrane permeability regulated by Bcl-2 family members, which sequentially leads to cytochrome c or Smac/DIABLO (second mitochondrial activator of caspases/direct IAP-binding protein with low isoelectric point (pI)) release, apoptosis formation, and the final DNA fragmentation (7).

Similar to TNF-induced activation of the nuclear factor κB (NFκB) transcription factor and the MAPK pathway (1, 2), TRAIL can also initiate the activation of signaling pathways

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1 This work was supported by National Key Project of Scientific and Technical Supporting Programs Grant 2006BAI04A06 provided by the Ministry of Science and Technology of China, 100 Talents Programme Grant 97BR044-2 provided by the Shanghai Municipal Health Bureau, National Natural Science Foundation of Grant 30572122, National Outstanding Doctor Academic Degree Thesis Foundation of China Grant 200775, and National 115 Key Project of Liver Cancer Grant 2008ZX10002-023. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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4 The abbreviations used are: TNF, tumor necrosis factor; CaMKII, Ca2+/calmodulin-dependent protein kinase; HCC, hepatocellular carcinoma; IKK, IκB kinase; FADD, Fas-associated protein with death domain; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; M KK, MAPK kinase; NFκB, nuclear factor κB; ROS, reactive oxygen species; Smac/Diablo, second mitochondrial activator of caspases/direct IAP-binding protein with low isoelectric point (pI); release, apoptosis formation, and the final DNA fragmentation (7).
that involve the adaptor molecules TNF receptor-associated factor-2, receptor-interacting protein, and transforming growth factor-β-activated kinase 1 (TAK1), finally leading to the activation of the MAPK pathway (including ERK1/2, c-Jun NH₂-terminal kinase JNK1/2, and p38) and the IκBα kinase (IKK)-NFκB pathway (8–12).

Melittin is the principal toxic component in the venom of the European honey bee *Apis mellifera* and is a cationic, hemolytic peptide (13). It is a small linear peptide composed of 26 amino acid residues in which the amino-terminal region is predominantly hydrophobic, whereas the carboxyl-terminal region is hydrophilic. It has been reported that melittin has multiple effects, including antibacteria, antivirus, and anti-inflammatory, in various cell types (13). We and others have shown that melittin can induce cell cycle arrest, growth inhibition, and apoptosis in various tumor cells (14–18). However, the mechanisms of the anti-cancer effects of melittin have not been fully elucidated.

HCC is one of the most common cancers in the world. Unfortunately, human hepatoma-derived cell types exhibit a major resistance to TRAIL-induced cell death (19–26). In this study, we tested the effects of melittin in the induction of apoptosis of HCC cells and explored the mechanisms involved in melittin-induced apoptosis of TRAIL-resistant HepG2 cells. We show that melittin can initiate an apoptotic machinery that depends on calcium influx and activation of Ca²⁺/calmodulin-dependent protein kinase (CaMKII)-TAK1-JNK/p38 signaling pathway. Moreover, we find that melittin can sensitize HCC cells to TRAIL-induced apoptosis by activating CaMKII-TAK1-JNK/p38 but inhibiting IKK-NFκB pathways.

**EXPERIMENTAL PROCEDURES**

**Cells, Antibodies, and Reagents**—The HeLa, Jurkat, Hep3B, and HepG2 cells were obtained from the ATCC (Manassas, VA) and cultured under standard conditions. The human HCC cell lines SMMC-7721 and BEL-7402 were obtained from TCC Cell Bank (Shanghai, China) and maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 25 units/ml penicillin, and 25 μg/ml streptomycin. The antibodies against Bcl-XI, caspase-3, caspase-8, caspase-9, inhibitor of apoptosis protein 1 (c-IAP1), c-Jun, poly(ADP-ribose) polymerase (PARP), and Smac/DIABLO; the antibodies against CaMKII, IκBα, IKKβ, JNK1/2, MKK3, MKK4, p38, p65 subunit of NFκB (p65/RelA), TAK1, and voltage-dependent anion channel; and the antibodies against phosphorylated CaMKII (Thr-286), IκBα (Ser-18/22), JNK1/2 (Thr-183/Tyr-185), MKK3/6 (Ser-189/207), MKK4 (Ser-257/Thr-261), p38 (Thr-180/Tyr-182), p65/RelA (Ser-32), JNK1/2 (Thr-183/Tyr-185), and TAK1 (Thr-184/187) were from Cell Signaling Technology (Beverly, MA). Purified recombinant IκBα was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Recombinant MKK4 was obtained from Merck. Melittin and the antibody against β-actin were from Sigma. The caspase inhibitors Z-DEVD-FMK (for caspase-3) and Z-LEHD-FMK (for caspase-9), the kinase inhibitors SB203580 (for p38), KN62 (for CaMKII), and SP600125 (for JNK1/2), the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′,N,N′,N,N′-tetraacetic acid (BAPTA), and the reactive oxygen species (ROS) inhibitor N-acetyl-l-cysteine (NAC) were from Calbiochem. The quantitative ELISA kits for phosphorylated IκBα (Ser-32), JNK1/2 (Thr-180/Tyr-182), and p38 (Thr-180/Tyr-182) were from Calbiochem.

**Plasmids, Vector Construction, and Transfection**—For construction of HA-tagged full-length TAK1 (GenBank™ accession number D76446), dominant negative TAK1 (mutation of Lys-63 into Trp, TAK1K63W), full-length p65/RelA (GenBank™ accession number M62399), and full-length IKKβ (GenBank™ accession number AF026524) expression vectors, the pcDNA3.1 vector (Invitrogen) was used and performed as described previously (27). All the expression vectors used in this study were confirmed by sequencing and then prepared using Endofree plasmid maxi kit (Qiagen, Hilden, Germany). For the transfection of expression vectors in mammalian cells, the jetPEI reagents were used (Polyplus-Transfection Co., Illkirch, France). The pGL3.5×κB-luciferase reporter plasmid was kindly provided by Seamus J. Martin (Trinity College, Dublin, Ireland) (28). The pRL-TK-Renilla-luciferase plasmid was obtained from Promega (Madison, WI).

**Apoptosis Assay**—After treatments with melittin or TRAIL, cells were labeled with annexin V and propidium iodide (PI) provided by Molecular Probes (Eugene, OR). Mitochondrial membrane potential was measured by labeling cells with 1 μM rhodamine 123 (Rho123, Molecular Probes) at 37 °C for 15 min. The production of ROS was analyzed by labeling cells with 10 μM dihydrorhodamine 123 (DHR123, Molecular Probes) for 15 min. Samples were examined by fluorescence-activated cell sorter (FACS) analysis, and the results were analyzed using CellQuest software (BD Biosciences) as described (29).

**Mitochondrial Isolation**—For the isolation of mitochondria after melittin treatments, the mitochondrial isolation kit for cultured cells (Pierce) was used as instructed.

**RNA Quantification**—Quantitative real time reverse transcription-PCR analysis was performed by LightCycler (Roche Applied Science) and SYBR reverse transcription-PCR kit (Takara, Dalian, China). Data were normalized by the level of β-actin. The experiments were performed as described previously (30).

**Western Blot Assay**—Western blot assay was performed as described by us previously (29). Bands were revealed using Supersignal West Femto maximum sensitivity substrate (Pierce).

**Caspase-3 Activity Assay**—For the examination of caspase-3 activation, whole cell lysates were subjected to ELISAs of cleaved caspase-3 by using sandwich ELISA kit (Cell Signaling Technology) as instructed.

**Assay of Luciferase Reporter Gene Expression**—Cells were transfected with the pGL3.5κB-luciferase reporter plasmid, RL-TK-Renilla-luciferase plasmid, and treated as indicated. Total amounts of plasmid DNA were equalized via empty control vector. Luciferase activities were measured with the dual luciferase reporter assay system (Promega) as described previously (30). Data are normalized for transfection efficiency by dividing firefly luciferase activity with that of Renilla luciferase.

**In Vitro Kinase Assay**—For the analysis of kinases activity, cells were lysed in lysis buffer containing 20 mM Tris, pH 7.5, 300 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 2 mM...
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Melittin induces apoptosis of HepG2 cells. A and B, HepG2 cells were treated with melittin for 12 h (A) or as indicated, stained with annexin V and PI, and analyzed by FACS. The apoptotic cells (the annexin V-positive cells) were indicated as the percentage of gated cells. Results in B were expressed as mean ± S.D. of triplicate samples. C, HCC cells Hep3B (B), BEL-7420 (B), and SMMC-7721 (SMMC) were treated with melittin as indicated. Annexin V+ cells were evaluated by FACS. Results were expressed as mean ± S.D. of triplicate samples. D, HepG2 cells were treated for 12 h, stained with Rho123, and analyzed by FACS. Results are presented as percentage of Rho123low cells. E, HepG2 cells were treated for 12 h, stained with DHR123, and analyzed by FACS. Results are presented as percentage of DHR123high cells.

RESULTS

Melittin Induces Apoptosis of HCC Cells—To determine the effects of melittin on HCC cells, we treated HepG2 cells with melittin at the concentration of 5 or 10 μg/ml and examined the apoptosis by annexin V/PI double staining. We found that melittin could induce apoptosis of HepG2 cells (Fig. 1A and B). Similar effects were observed in SMMC-7721, BEL-7402, and Hep3B HCC cell lines (Fig. 1C).

Moreover, we found that the mitochondrial membrane potential of HepG2 cells was decreased by melittin, as evidenced by the increase in Rho123low cells (Fig. 1D). The production of ROS (DHR123high cells) was also significantly increased after melittin treatments (Fig. 1E).

Melittin Activates Caspase-3 and -9 and PARP and Promotes the Release of Cytochrome c and Smac/DIABLO from Mitochondria—To elucidate the mechanisms involved in melittin-induced apoptosis of HCC cells, we first examined the activation of caspases by melittin. We found that melittin could induce the cleavage of caspase-3 and -9 and PARP but not caspase-8, which could be blocked by the calcium chelator BAPTA and ROS inhibitor NAC, indicating that melittin-induced activation of caspase-3 and -9 and PARP was initiated by the calcium influx and the production of ROS (Fig. 2A). Moreover, we found that the pretreatment of cells with caspase-3 inhibitor Z-DEVD-FMK, caspase-9 inhibitor Z-LEHD-FMK, BAPTA, or NAC could inhibit the melittin-induced apoptosis (Fig. 2B).

The data (Fig. 1D) suggested that melittin treatments may have disrupted the membrane permeability of mitochondria. To verify this mechanism, we isolated mitochondrial and cytosol fractions of HepG2 cells. We found that melittin treatments could induce the release of cytochrome c and Smac/DIABLO from mitochondria to cytosol (Fig. 2C), indicating that melittin-induced apoptosis may also involve the mitochondrial pathway of apoptosis.
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FIGURE 2. Melittin-induced apoptosis is associated with caspase activation, cleavage of PARP, and disruption of mitochondrial permeability. HepG2 cells were treated with 10 μg/ml melittin or vehicle control (DMSO) for 12 h with or without pretreatments with BAPTA (5 μM), NAC (100 μM), Z-DEVD-FMK (50 μM), or Z-LEHD-FMK (25 μM) for 30 min. A, cell lysates (30 μg/lane) were examined for phosphorylated (p) or total levels of signaling molecules using indicated antibodies. B, HepG2 cells were stained for annexin V and PI. Apoptosis was evaluated by determining the percentage of annexin V-positive cells, and the results are presented as mean ± S.D. of triplicate samples. *, p < 0.05; **, p < 0.01; ***, p < 0.001. C, HepG2 cells were extracted for cytosol and mitochondrial protein and examined for the release of cytochrome c and Smac/DIABLO by Western blot. The efficiency of the isolation was evaluated by examining the caveolin-1 (cytosol marker) and voltage-dependent anion channel (mitochondrial marker).

Then we tested the roles of calcium influx and ROS production on mitochondrial release of cytochrome c and Smac/DIABLO. We found that both BAPTA and NAC could inhibit the release of cytochrome c and Smac/DIABLO into the cytosol (Fig. 2C), indicating that the mitochondrial disruption by melittin may be subsequent to calcium influx and ROS production.

Melittin Activates CaMKII-TAK1-JNK/p38 Pathway—Previously it has been demonstrated that melittin can activate the calcium channel and induce the influx of calcium from extracellular medium (13–15, 32, 33). However, the signaling pathways activated by melittin have not been fully understood.

CaMKII is a ubiquitous mediator of Ca2+-linked signaling that phosphorylates a wide range of substrates to coordinate and regulate Ca2+-mediated alterations in cellular function (34). We find that melittin treatments could activate CaMKII, which could be blocked by the calcium chelator BAPTA (Fig. 3A) in HepG2 cells, indicating that melittin could activate CaMKII by increasing cytosol Ca2+-concentration.

TAK1 is a MAPK kinase (MEKK) that has been shown to activate MKK3, -4, and -6 and subsequent JNK1/2 and p38 after melittin treatments, which could also be blocked by CaMKII inhibitor KN62, indicating that melittin could activate CaMKII by increasing cytosol Ca2+-concentration.
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JNK1/2, and p38 was inhibited (Fig. 3C). The activation of JNK/p38 by melittin and the involvement of TAK1 in melittin-induced JNK/p38 activation were also verified by ELISAs of the levels of phosphorylated JNK/p38 (Fig. 3, D and E). These data together suggest that melittin could activate a signaling pathway sequentially involving CaMKII-TAK1-MKK-JNK/p38.

Melittin-induced Apoptosis Is Dependent on the Activation of CaMKII-TAK1-JNK-p38 Signaling Pathway—The above results have demonstrated that melittin alone may induce apoptosis of HCC cells by activating caspase-3 and disruption of mitochondria. We then tested the relationship of the CaMKII-TAK1-JNK/p38 signaling pathway with melittin-induced apoptosis. When HepG2 cells were pretreated with kinase inhibitors KN62, SP600125, and SB203580, we found that melittin-induced apoptosis was significantly inhibited (Fig. 3F). Transient transfection of TAK1K63W in HepG2 could also significantly inhibit the apoptosis induced by melittin (Fig. 3G).

Melittin Inhibits TAK1-mediated Activation of IKK-NF-κB Signaling Pathway—To elucidate whether melittin-mediated activation of caspase-3 and CaMKII was dependent on the elevation of cytosol Ca²⁺ concentration, we treated HepG2 cells with ionomycin (calcium ionophore) and examined its effects on melittin-induced apoptosis. We found that ionomycin alone was not sufficient to induce apoptosis of HepG2 cells but was sufficient to activate CaMKII-TAK1-JNK/p38 signaling pathway (data not shown). However, ionomycin alone could increase the kinase activity of IKKβ (Fig. 4A) and the activation of NFκB gene reporter (Fig. 4B), which could be inhibited by CaMKII inhibitor KN62 and the dominant negative TAK1K63W expression (Fig. 4, A–D), indicating that elevation of intracellular Ca²⁺ could activate CaMKII-TAK1-IKK-NFκB pathway.

Previously, it has been reported that melittin can bind IKKα/β and inhibit the expression of inflammatory targets and intermediate mediators in RAW264.7 cells and synovioocytes (38). In HepG2 cells, we found that melittin treatments could not alter the kinase activity of IKKβ, phosphorylation of IKKα, and NFκB gene reporter (Fig. 4, A and B), indicating that melittin alone may not exert inhibitory effects on IKK-NFκB in HCC cells.

Considering that melittin alone could activate CaMKII-TAK1-MKK-JNK-p38 (Fig. 3, A and B) but did not affect IKK-NFκB (Fig. 4, A and B), we tested whether melittin could inhibit TAK1-induced activation of IKK-NFκB. We found that transient transfection of TAK1 and IKKβ could activate the NFκB gene reporter (Fig. 4, E and F). However, melittin could block TAK1- and IKKβ-induced NFκB activation (Fig. 4, E and F), suggesting that melittin inhibited the NFκB activation subsequent to CaMKII-TAK1 activation. However, we found that melittin could not inhibit the NFκB activation induced by p65/RelA expression (Fig. 4G). Therefore, it may be inferred that melittin could simultaneously activate CaMKII-TAK1-MKK-JNK/p38 and inhibit CaMKII-TAK1-IKK-NFκB initiated by calcium influx, which was confirmed by the findings that melittin could inhibit ionomycin-induced activation of NFκB (Fig. 4, A and B).

Melittin Sensitizes HCC Cells to TRAIL-induced Apoptosis—TRAIL-associated strategy in cancer treatments is promising and is being tested in clinical trials (2). However, most of the HCC cells were resistant to TRAIL treatments (19–26). Because melittin could activate CaMKII-TAK1-JNK-p38 but...
inhibit IKK-NFκB in HCC cells, it may be expected that melittin may increase the sensitivity of HCC cells to TRAIL. In HepG2 cells, TRAIL alone was inefficient in the induction of apoptosis (Fig. 5A). The combination of melittin with TRAIL could increase the TRAIL-induced apoptosis of HepG2 cells (Fig. 5A). To further investigate the possible synergistic effects of melittin and TRAIL in the induction of HCC apoptosis, we treated various HCC cells with melittin (5 μg/ml) in combination with different doses of TRAIL (0–200 ng/ml). We found that both in TRAIL-resistant HCC cells (HepG2, Hep3B, SMMC-7721, and BEL-7402, the sensitivity to TRAIL-induced apoptosis that was different) and in TRAIL-sensitive HeLa and Jurkat cells, melittin was capable of promoting TRAIL-induced apoptosis (Fig. 5B and C). Moreover, we found that melittin could augment the effects of TRAIL in activation of caspase-3, cleavage of PARP, and mitochondrial release of cytochrome c and Smac/DIABLO, which confirmed that melittin could promote TRAIL-induced apoptosis (data not shown). Taken together, these results indicated that melittin could sensitize HCC cells (and possibly other tumor cells) to TRAIL-induced apoptosis.

**Melittin Synergizes with TRAIL in Activation of TAK1-JNK/p38**—TRAIL can induce apoptosis via FADD-caspase-8-caspase-3 pathway and initiate the activation of MAPK and NFκB (1). In the presence of melittin, TRAIL-induced activation of JNK/p38 was augmented (Fig. 6A), as evidenced by ELISAs of the phosphorylated levels of JNK1/2 (p-JNK1/2) and p38 (p-p38). As further evidence, we also examined the activation of JNK/p38 30 min after melittin and TRAIL treatments by Western blot, and we found that melittin did potentiate the TRAIL-induced activation of JNK/p38 (data not shown). More importantly, we found that the melittin-mediated potentiation of TRAIL-induced apoptosis was attenuated by inhibitors specific for JNK1/2 (SP600125) and p38 (SB203580) (Fig. 6B), indicating that melittin-mediated sensitization of HepG2 cells to TRAIL may be exerted via activation of the JNK1/2 and p38 kinases. Similar results were obtained in BEL-7402 and SMMC-7721 HCC cells (data not shown).

TRAIL can activate the JNK/p38 signaling pathways via activation of TAK1-MKK (14–18), whereas melittin may activate JNK1/2 and p38 through the CaMKII-TAK1 signaling pathways (Fig. 3, A–E). Therefore, we examined the effects of melittin on TRAIL-induced activation of TAK1. We found that melittin could increase the phosphorylated levels of TAK1 (p-TAK1)
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and the kinase activity of TAK1 when compared with TRAIL or melittin treatments alone (Fig. 6C).

To investigate the potential roles of TAK1 in melittin-mediated potentiation of TRAIL-induced activation of JNK/p38, we examined the activation status of JNK/p38 after transient transfection of wild type TAK1 and TAK1K63W. We found that TAK1 overexpression could potentiate the activation of JNK/p38 in melittin- and TRAIL-treated cells, but to a lesser extent than that observed in melittin + TRAIL-treated cells (Fig. 6D). However, TAK1K63W overexpression could inhibit the activation of JNK/p38 in melittin-, TRAIL-, and melittin + TRAIL-treated cells. These data (Fig. 6D) together suggest that melittin potentiates TRAIL-induced activation of JNK/p38 via the synergistic activation of TAK1.

Next we examined the roles of TAK1 activation in the synergistic effects of melittin plus TRAIL on apoptosis of HCC cells by overexpressing TAK1 and TAK1K63W. We found that TAK1K63W overexpression inhibited the apoptosis induced by melittin alone and melittin + TRAIL treatments (Fig. 6E), although TAK1 overexpression could partially reverse the melittin-induced promotion of TRAIL-induced apoptosis (Fig. 6E).

Melittin Inhibits TRAIL-induced Activation of IKK-NFκB—NFκB usually plays protective roles in apoptosis of various cells in response to TNF superfamily molecules (1). Previously it has been demonstrated that melittin can inhibit the IKK-IκBα-NFκB signaling pathway in macrophages (38). Furthermore, we have detected the inhibition of TAK1-mediated activation of IKK-IκBα-NFκB by melittin (Fig. 4, D and E). Therefore, we examined the effects of melittin on TRAIL-induced activation of IKK-IκBα-NFκB. We found that TRAIL could increase the kinase activity of IKKβ (Fig. 7A) and the phosphorylated levels of IκBα (Fig. 7B). However, melittin pretreatments could inhibit TRAIL-induced activation of IKKβ and IκBα (Fig. 7, A and B). As further evidence, we found that melittin could inhibit TRAIL-induced activation of the NFκB gene reporter (Fig. 7C).

To elucidate whether melittin inhibits TRAIL-induced activation of IKK-IκBα-NFκB via inhibiting the kinase activity of IKKβ, we overexpressed TAK1, IKKβ, and p65/RelA in HepG2 cells. We found that melittin could inhibit TAK1- and IKKβ-mediated but not p65/RelA-mediated activation of NFκB gene reporter (Fig. 7D), suggesting that melittin-mediated inhibition of TRAIL-induced activation of IKK-IκBα-NFκB may be via inhibition of IKKβ (38).

To investigate the role of melittin-mediated inhibition of TRAIL-induced activation of NFκB in the apoptosis of HCC cells, we examined the effects of melittin in TRAIL-induced apoptosis after overexpressing IKKβ and p65/RelA. We found that melittin-mediated potentiation of TRAIL-induced apoptosis in HepG2 cells could be greatly reversed by overexpressing p65/RelA but not by overexpressing IKKβ (Fig. 7E), indicating that melittin-mediated potentiation of TRAIL-induced apoptosis was partially through the inhibition of IKKβ-mediated activation of NFκB.

NFκB activation usually protects cells from apoptosis by upregulating the expression of anti-apoptotic molecules like Bcl-xl, cIAP, XIAP, and FLIP, etc. (24–26, 39, 40). TRAIL alone could increase the expression of Bcl-xl and c-IAP1 in HepG2 cells, whereas melittin could inhibit the TRAIL-induced up-regulation of these anti-apoptotic molecules (Fig. 7F), indicating that melittin had redirected the TRAIL signaling to inhibition of NFκB pathway.

Melittin Synergizes with TRAIL in the Treatment of Human HCC—To examine the efficiency of combining melittin and TRAIL in treatment of HCC in vivo, we established HepG2 and SMMC-7721 tumor in nude mice and treated the pre-established tumor 7 days after tumor inoculation by intravenous injection of melittin and/or TRAIL daily for seven episodes. We found that the combination of melittin with TRAIL could significantly inhibit the growth of both tumors, which was more remarkable than melittin or TRAIL alone (Fig. 8A).
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**DISCUSSION**

In this study, we have demonstrated that melittin can induce the apoptosis of HCC cells potentially by activating CaMKII-TRAIL induced apoptosis by activating CaMKII-TAK1-JNK/p38 pathway but inhibiting IKK-NFκB pathway. Our study has suggested that the combination of melittin with TRAIL may be promising in the treatment of human HCC.

Melittin has been demonstrated to have multiple functions in various cell types (13). The intracellular targets for melittin have been reported by several groups, such as calcium channel, calmodulin, phospholipase A₂ and phospholipase D, mitochondrial F₁-ATPase, sphingomyelinase, IKKα/β, and Rac1, etc. (13–15, 17, 32, 33, 38, 41, 42). However, the signaling mechanisms, especially the detailed events taking place from the cell membrane to the nuclear activation of transcription factors, responsible for melittin-mediated inhibitory effects in tumor cells have not been elucidated. Upon treatment of cells with melittin, the initial cascade may be the activation of calcium channels and phospholipase A₂, leading to the elevation of intracellular Ca²⁺ concentration and the activation of calcium-sensitive CaMKII. In our study, we show that melittin can rapidly activate CaMKII, which was dependent on calcium elevation, mimicking the effects of the calcium ionophore ionomycin. TAK1 is one of the targets of CaMKII and has been shown previously to be widely involved in the activation of IKKα/β by IL-1R, Toll-like receptors, and TNF receptors (35–37). TAK1 activation can lead to the subsequent activation of MMK-JNK/p38 and IKKα/β-IκBα-NFκB pathways (35, 36). We suggest that melittin can activate TAK1 via calcium-dependent activation of CaMKII, which may provide a linkage between calcium influx and activation of MAPK/NFκB. The inhibition of melittin-induced apoptosis by kinase inhibitors KN62, SP600125, SB203580, and dominant negative TAK1 suggests that melittin-induced apoptosis may be dependent on the activation of the CaMKII-TAK1-MKκ-JNK/p38 pathway.

One controversy in our study is that melittin alone does not affect the IKK-NFκB pathways despite the activation of CaMKII-TAK1 by melittin. We show that ionomycin treatments or overexpression of TAK1 and IKKβ can activate the IKK-NFκB pathways, which can be inhibited by melittin, suggesting that melittin may exert inhibitory effects on IKK-NFκB signaling pathways. The data that melittin cannot inhibit the p65/RelA-induced activation of NFκB suggest that IKK may be the target of melittin in the IKK-NFκB pathways in human HCC cells. Previously melittin has been demonstrated to bind and inhibit the activity of IKKα/β in RAW264.7 macrophages (38). However, a later report suggests that melittin does not affect the NFκB p50-DNA interactions nor the activation of NFκB in human synoviocytes and dermal fibroblasts (43), indicating that the effects of melittin on IKK-NFκB pathways may varied between cell types. Our study suggests inhibitory effects of melittin on IKK-NFκB pathways in human HCC cells, which may contribute to melittin-induced apoptosis of HCC cells.

Resistance of tumor cells to TRAIL treatments is a major barrier of TRAIL application in cancer (1, 2). The combination of TRAIL reagents with other treatments may increase the efficiency of cancer treatments. It has been reported that TRAIL, in combination with chemotherapy, radiotherapy, and other bioactive reagents (such as IFNα), can increase clinical response in...
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various human cancers (1, 2, 19–26). HCC cells are resistant to TRAIL treatments both in vitro and in vivo (19–26). Melittin is a bioactive component of bee venom, although its effects on HCC cells in combination with TRAIL have not been examined. In our study, we demonstrate that melittin can sensitize TRAIL-resistant HepG2 cells to TRAIL treatments by showing melittin can increase the apoptosis of HCC cells in the presence of lower concentrations of TRAIL (as low as 10 ng/ml). The observed synergistic induction of apoptosis by melittin plus TRAIL may not be due to a simple summary effect of individual agents because melittin can significantly increase the apoptosis of TRAIL-resistant HCC cells even when TRAIL alone (10 ng/ml) is unable to induce apoptosis. Additionally, we have detected the synergistic effects of melittin plus TRAIL in other TRAIL-sensitive cell lines. Therefore, melittin may be a novel agent capable of sensitizing HCC cells to TRAIL treatments, which thus awaits clinical trials.

TRAIL signals both apoptosis and survival, which may be differentially regulated by JNK/p38 and NFκB (1). Inhibition of NFκB may thus favor the TRAIL-induced apoptosis. In our study, we showed that melittin can synergize with TRAIL in activation of TAK1-JNK/p38 but inhibit TRAIL-induced activation of IKK-NFκB. Moreover, we provide evidence that melittin can inhibit TRAIL-induced transcription of NFκB-related Bcl-xl and c-IAP1. Therefore, melittin-induced sensitization of HCC cells to TRAIL may be contributed to the dual functions of melittin in the activation of TAK1-JNK/p38 and inhibition of IKK-NFκB. The possible convergence of TRAIL signaling and melittin signaling may be TAK1. Proapoptotic and antiapoptotic roles have been suggested for TAK1 in various cell types under diverse conditions (12, 35, 44–48). In melittin-induced apoptosis, melittin can activate CaMKII-TAK1-JNK/p38 and inhibit TRAIL-induced activation of IKK-NFκB, which together sensitize HCC cells to TRAIL-induced apoptosis.

The differential roles of melittin in activation of TAK1-JNK/p38 but inhibition of IKK-NFκB in response to TRAIL may hold true for other TNF superfamily members because we also find that melittin can potentiate the TNFα-induced activation of TAK1-JNK/p38 but inhibit TNFα-induced activation of IKK-NFκB (data not shown). Additionally, we find that melittin cannot potentiate the drug-induced cell death of BEL-7402 cells that have been selected under the chemotherapeutic drugs adriamycin or 5-fluorouracil to establish multidrug resistance (data not shown). Therefore, the sensitization of HCC cells to apoptosis by melittin may only be applicable to a limited set of apoptotic stimuli that employ apoptotic machinery similarly to TRAIL. Whether melittin can potentiate apoptosis by the other cancer treatments, however, may need further investigation.

In conclusion, we have demonstrated in this study that melittin potentiated the apoptotic effects of TRAIL in human HCC cells by activating the CaMKII-TAK1-JNK/p38 pathway but inhibiting the IKK-NFκB pathway. Our data suggest that melittin may exhibit anti-tumor activity by sensitizing HCC cells to TRAIL-mediated apoptosis, and that the combination of TRAIL with melittin may have therapeutic potential in the treatment of human HCC.

Acknowledgments—We thank Y. Li and M. Jin for expert technical assistance.

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