Melittin Inhibits the Expression of Key Genes Involved in Tumor Microenvironment Formation by Suppressing HIF-1α Signaling in Breast Cancer Cells

Zabih Mir Hassani
Kharazmi University
Mohammad Nabiuni (devbiokharazmi@gmail.com)
Kharazmi University https://orcid.org/0000-0002-0349-6676
Kazem Parivar
Kharazmi University
Somayeh Abdirad
Kharazmi University
Latifeh Karimzadeh
Tehran University: University of Tehran

Research Article

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Abstract

Purpose: HIF-1α has critical roles in formation of Tumor microenvironment by regulating genes involved in angiogenesis and anaerobic respiration. TME fuels tumors growth and metastasis and presents therapy with several challenges. Therefore, we aimed to investigate if Melittin disrupts HIF-1α signaling pathway in breast adenocarcinoma cell line MDA-MB-231.

Methods: breast adenocarcinoma cell line MDA-MB-231 was cultured in presence of different doses of Melittin and MTT assay was carried out to measure Melittin's cytotoxic. Cells were exposed to 5% C_2 to mimic hypoxic conditions and Melittin. Western blot was used to measure HIF-1α protein levels. Gene expression analysis was performed using real-time PCR to measure relative mRNA abundance of genes involved in tumor microenvironment formation.

Findings: Our results revealed that Melittin effectively inhibits HIF-1α at transcriptional and translation/post-translational level. HIF-1α protein and mRNA level was significantly decreased in Melittin-treated groups. It is found that inhibition of HIF-1α by Melittin is through downregulation of NFκB gene expression. Furthermore, gene expression analysis showed a downregulation in VEGFA and LDHA expression due to inhibition of HIF-1α protein by Melittin. In addition, cell toxicity assay showed that Melittin inhibits the growth of MDA-MB-231 cell line through activation of extrinsic and intrinsic apoptotic pathways by upregulating TNF and BAX expression.

Conclusions: Melittin suppresses the expression of genes responsible for formation of TME physiological hallmarks by suppressing HIF-1α signaling pathway. Our results suggest that Melittin can modulate tumor microenvironment by inhibition of VEGFA and LDHA.

Introduction

Hypoxia is the imbalance between oxygen consumption and supply[1]. Hypoxic regions within tumor is a common characteristic to a vast variety of solid tumors[2]. Hypoxia-inducible factor 1(HIF-1) is the main regulator of O_2 homeostasis in mammalian cells[3]. HIF1 activity is dependent on stabilization of its alpha subunit(HIF-1α) which is under tight control of O_2 availability in the cytosol. Even though, HIF-1α gene transcription is mainly regulated in an O_2-independent manner by NF-kB and STAT3. NF-kB interacts with HIF-1α promoter at -197/-188 bp through its p50 and p65 subunits[4]. Moreover, it has been shown that HIF-1α expression is regulated by JAK/Stat signaling through a binding motif at -363/-355 bp of the HIF-1 α promoter[5]. Under normoxic conditions, HIF-1α is constantly synthesized and degraded. When oxygenation is sufficient, von Hippel–Lindau (VHL) binds HIF-1α and through interaction with Elongin C, recruits an E3 ubiquitin-protein ligase complex that ubiquitinates HIF-1 α and targets it for degradation by the 26S proteasome. In order to bind HIF-1α, VHL needs to be hydroxylated on proline residue 402 or 564 or both by prolyl hydroxylase PHD2. VHL uses O_2 and α-ketoglutarate as substrates[6]. During hypoxia, this degradation mechanism is disturbed, resulting in accumulation of HIF-1α in cytoplasm. Upon

stabilization of HIF-1α, it dimerizes with HIF-1β, HIF1 transcription factor forms in the nucleus and triggers the expression of more than 1500 genes[7].

HIF-1 is a major driver of tumor microenvironment(TME) formation. Elevated expression of glycolytic genes[8]–[11], and HIF1 induction of pyruvate dehydrogenase kinase 1(PDK1)[12] and lactate dehydrogenase A(LDHA)[10], drives tumor cell's metabolism through anaerobic respiration, resulting in declined pH of solid tumor microenvironment[13], [14]. HIF1 upregulates carbonic anhydrase IX (CAIX)[15] which also has a role in decreased TME acidity[16], [17]. On the other hand, HIF1 upregulates proangiogenic genes Ang-1 and − 2, Tie-2 and VEGF[18], [19]. High permeability of newly formed vasculature and lack of lymphatics result in elevated interstitial fluid pressure (IFP) of tumors, another hallmark of TME[20]. This environment favors tumor cells with resistance to current chemotherapy and radiotherapy strategies[21].

Crucial involvement of HIF1 in several steps of carcinogenesis and tumor invasion and metastasis has made it a great target for cancer therapy. Though resistance to current therapeutic reagents has been a sustaining challenge. Melittin(MEL) is a short polypeptide of 26 aminoacids and constitutes about 50% of total dried weight of honey bee venom[22]. Several studies have reported anti-inflammatory, anti-arthritic, and anti-viral effects of MEL in various cell types. It also induces apoptosis, cell cycle arrest and growth inhibition in several cancer cell types[23]. it has been shown that MEL can inhibit angiogenesis through downregulation of VEGF in cervical cancer. MEL specifically suppressed EGF-induced VEGF secretion and new blood vessel formation by inhibiting HIF1 signaling pathway[24].

Nevertheless, there are no previous reports on how MEL can affect HIF-1α in breast cancer. in this study, we have investigated the effect of MEL on breast cancer cell line MDA-MB-231, and its inhibitory effect on HIF-1α mRNA expression and protein level. Also, the expression of genes involved in the formation of TME and downstream to HIF1 signaling has been investigated.

Materials And Methods

Cell and materials

Human triple negative breast adenocarcinoma cell line MDA-MB-231 was obtained from Pasteur Institute of Iran Cell Bank (Tehran, Iran) and cultured in RPMI 1640 medium(GIBCO) supplemented with 1% penicillin-rapamycin antibiotic mixture and 10% fetal bovine serum(FBS). Cultures were incubated at 37°C in presence of 5% CO_2. To mimic hypoxic conditions, cells were cultured at 37°C in presence of 5% CO_2 and 5% O_2 as described by Bakmiwewa et al[25].All reagents used were purchased from Sigma (St. Louis, US) except otherwise mentioned.

Cell toxicity assay

Cell toxicity effects of MEL on MDA-MB-231 cell lines was evaluated by MTT assay. Cells were seeded in a 96-well plate at $2*10^4$ cell/well in RPMI 1640 and incubated for 24 hours to attach. Then media was

removed, cell were washed with phosphate buffer saline (PBS) once, and new RPMI 1640 containing MEL at 1, 2 and 4 µg/ml concentrations were added to corresponding wells. Cells were incubated for 24 hours. Then 100 µl MTT reagent was added to each well and incubated for 4 hours. Finally, formazan deposits were resolved by adding Dimethyl sulfoxide (DMSO) to each well. Absorbance was measured at 570nm using Epoch™ Microplate Spectrophotometer (Vermont, US).

Western Blot Analysis

To measure relative protein levels of HIF-1α, western blotting analysis was used. Total protein was extracted using NP40 buffer. Briefly, cells were resuspended in 50 µl NP40 lysis buffer (10mM Tris, 10mM NaCl, 5 mM EDTA, 1 mM DTT, 0.5% Nonidet P-40, 0.1mM phenylmethylsulfonyl fluoride, 2 µM aprotinin, and 2 µM leupeptin) and vortexed and then incubated on ice for 30 minutes followed by centrifugation at 13000rpm at 4°C. Supernatant was collected and stored at -20°C. Total protein was quantified using Bradford's method. 30µg of total protein was electrophoresed and transferred onto a nitrocellulose membrane. β-actin was used as housekeeping protein. Primary antibody against HIF-1α was obtained from Abcam (Cambridge, UK). Primary antibody for β-actin and HRP-labelled secondary antibody was purchased from Biorad (California, US). Detection of specific target proteins was carried out using Biorad Clarity Western ECL Substrate (California, US), as instructed by manufacturer.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using Invitrogen TRIzol reagent (California, US) as described by supplier. First strand cDNA was synthesized using 1µg of total RNA using Geneall's Hyperscript™ first strand synthesis kit (Seoul, Korea). Quantitative realtime PCR was performed in triplicates using Ampliqon's RealQ Plus 2x Master Mix Green (Odense, Denamark). β-2 microglobulin (B2M) was used as reference gene. Primer sequences used for realtime PCR are presented in Table 1.

Table 1
Sequence of primers used for gene expression analysis

Gene	Sequence(5' to 3')	Product size
HIF-1α	CCAGCAGACTCAAATACAAGAACC	138 bps
	TGTATGTGGGTAGGAGATGGAGAT	
NFκB	CACAAGGCAGCAAATAGACGAG	133 bps
	CATTTTGTTGAGAGTTAGCAGTGAG	
VEGFA	CTTGTTCAGAGCGGAGAAAGC	125 bps
	ACATCTGCAAGTACGTTCGTT	
LDHA	GTTTGTTCACCTCATAAGCACTCTC	104 bps
	CTCTCTGAAGACTCTCCACCC	
BAX	GGAAACTGGTGCTCAAGGC	161 bps
	GGTCATCAGTCGCTTCAGTG	
BCL2	GGATAACGGAGGCTGGGATG	165 bps
	CAGGGATGTTGACTTCTCTTGTG	
TNF	TCCACCCATGTGCTCCTCAC	97 bps
	TCTGGCAGGGGCTCTTGATG	
β-2M	AGATGAGTATGCCTGCCGTG	104 bps
	CGGCATCTTCAAACCTCCA	

Statistical Analysis

Data are drawn from triplicate experiments. Analysis was performed using R package dplyr on R Studio version 1.3 platform. Realtime PCR data were analyzed using $2^{-\Delta\Delta Ct}$ method. One-way ANOVA has been used to detect statistically significant differences. P values less than 0.05 was considered as significant.

Results

Melitin inhibits the proliferation of breast cancer cell line MDA-MB-231 through induction of apoptosis by upregulating BAX and TNF expression

In order to understand how MEL can modulate the growth of MDA-MB-231 cell line, we first evaluated its cytotoxic effects using MTT assay. The viability of MDA-MB-231 cells did not change at 1 and 2 µg/ml of

MEL, but a significant change in viability was observed at 4 mg/ml (Fig. 1A). Melittin's IC_{50} for MDA-MB-231 cell line was measured 3.8 µg/ml. To find the mechanism of growth inhibition by Melittin, expression of BAX, BCL2 and TNF was measured using real-time PCR. Melittin treatment at dose of 4 µg/ml induced a 4.4-fold increase in expression level of BAX($p < 0.001$), where no significant change in the expression level of BCL2 was observed (Fig. 1B). On the other hand, TNF expression increased with different doses of MEL constantly (Fig. 1C). Our findings suggest that growth inhibitory effect of MEL is primarily exerted through induction of TNF expression and subsequent activation of extrinsic apoptotic cascade, while it triggers the intrinsic cascade by upregulating the expression of pro-apoptotic gene BAX, too. Melittin is not toxic to MDA-MB-231 cells at concentrations 1 and 2 µg/ml and thus further experiments were performed using these concentrations.

Melittin inhibits HIF-1α protein level by downregulating its mRNA expression through inhibition of NF-kB expression

To induce HIF-1α protein, cells were cultured in 5%O_2 for 6 hours. Low oxygen concentration resulted in significant elevated levels of HIF-1α protein (Fig. 2A). In order to investigate effect of MEL on HIF-1α, cells were treated with different concentrations of MEL and incubated at 5%O_2 for 6 hours. Melittin reverted the elevated levels of HIF-1α protein in a dose-dependent manner presenting approximately a 10-fold decrease in levels of hypoxia-induced HIF-1α protein at 2 µg/ml (Fig. 2B). Real-time PCR experiment showed that Melittin inhibits the expression of HIF-1α mRNA compared to untreated and hypoxic groups (FC = 0.1, $p < 0.001$ and FC = 0.2, $p < 0.001$ at 1µg/ml and 2 µg/ml respectively; Fig. 2C). This suggests that during hypoxia, HIF-1α is induced at transcriptional and translational/post-translational level. This upregulation was reverted significantly when cells were treated with different doses of MEL compared to untreated and hypoxia-treated group (Fig. 2C), indicating that Melittin inhibits HIF-1α protein levels by downregulating its mRNA transcription. Further analysis of NF-kB gene expression, direct regulator HIF-1α transcription, showed decreased expression levels when cells were treated with 2 µg/ml of Melittin (Fig. 2D). These findings show that Melittin inhibits HIF-1α through downregulation of its transcription by NF-kB.

Melittin controls the expression of key players of tumor microenvironment formation by modulating HIF-1α signaling

HIF-1α plays a crucial role in formation of tumor microenvironment by regulating expression of genes involved in different steps of development of TME. Thus we further analyzed the expression of genes that are down-stream to HIF-1α and are involved in angiogenesis and anaerobic respiration. HIF-1α mediated anti-angiogenic effect of MEL was investigated by analyzing the expression of VEGF-A, the leading character of the angiogenesis story. Declined O_2 concentration induced a 2.2-fold increase in expression of VEGFA gene compared to untreated group (Fig. 3A, $p < 0.05$) where MEL treatment at concentrations of 1 and 2 µg/ml reverted the change and induced significant downregulation of VEGFA (FC = 0.1, $p < 0.05$

and FC = 0.07, $p < 0.05$, respectively). This observation indicates that MEL can inhibit angiogenesis by inhibition of VEGFA expression through downregulation of HIF-1α protein level (Fig. 3A). For evaluation of effect of declined HIF-1α protein levels by MEL on anaerobic respiration, gene expression of lactate dehydrogenase A(LDHA), the key driver of anaerobic respiration switch was measured. Elevated levels of HIF-1α induced by low O2 concentration, upregulated expression level of LDHA mRNA slightly, compared to untreated group (Fig. 3B). Subsequently, MEL treatment reduced the expression level of LDHA significantly in comparison to hypoxia-treated group (FC = 0.4 and FC = 0.3 at 1µg/ml and 2 µg/ml respectively, $p < 0.05$; Fig. 3B). This suggests that MEL can inhibit anaerobic respiration through downregulation of LDHA. These data suggest inhibitory effects of Melittin against physiological characteristics of TME through regulation of angiogenesis and anaerobic respiration.

Discussion

Emerging evidences have refined the definition of cancer as complex heterotypic multicellular interactions between transformed tumor cells and the neighboring universe, termed as tumor microenvironment, which favors the development and growth of a malignancy. TME contains physiochemical and cellular microenvironment and changes in its composition have profound effects on cancer progression[26]. In this study, we have put a key regulator of TME under investigation. As results of uncontrolled proliferation of O_2-consuming tumor cells and inefficiency of newly formed vasculature, chronic and cycling(intermittent) hypoxia is a prominent feature of solid tumors microenvironment[27]. Mammalian cells respond to any fluctuation in oxygen concentration by HIF1[3]. HIF1 is a transcription factor and controls the expression of more than 1500 genes at transcriptional level. Many of these genes are involved in tumor cell proliferation, immune evasion, angiogenesis, EMT, invasion and metastasis[7]. HIF1 is a dimer of HIF-1β and O2-regulated HIF-1α. In normoxia, HIF-1α is immediately targeted for proteasomal degradation by VHL that needs O_2 as a cofactor, while during hypoxia it accumulates in cytoplasm, enters the nucleus where it dimerizes with HIF-1β and HIF1 complex is formed[7]. This makes HIF-1α an ideal target for cancer therapy.

In current study we have investigated the pharmacological effects of Melittin from honey bee venom on HIF-1α function in breast cancer cell line MDA-MB-231. Previous studies have demonstrated anti-proliferative effects of Melittin on cervix and non-small cell lung cancer cell lines[24], [28]. We found that Melittin inhibits the growth of breast cancer cell line MDA-MB-231 (Fig. 1A). An IC_{50} of 3.8 µg/ml was measured. Melittin exerts its inhibitory effect on proliferation of MDA-MB-231 cell line primarily through upregulation of TNF and subsequent activation of extrinsic apoptosis pathway (Fig. 1C). Furthermore, anti-proliferative effect of Melittin may be attributed to upregulation of pro-apoptotic gene BAX (Fig. 1B). Shu et al. reported a similar mechanism for growth inhibition by Melittin in renal tubule epithelial cells in mouse model of acute kidney injuries; Upregulation of TNF corresponding to elevated levels of TNF in mouse serum indicative of TNF signaling pathway activation and upregulation of BAX/BCL2 expression ratio[29]. Besides, it has been reported that Melittin inhibits cell growth and induces apoptosis by inhibiting the MAPK, Akt, and NF-kB pathways in cancer cells[24]. In another study, it has been suggested

that anti-proliferative effects of Melittin include activation of caspases and matrix metalloproteinases[28].

Nevertheless, no previous reports regarding modulation of breast cancer TME by Melittin are available. HIF-1α is a crucial modulator of TME physiology. Declined pH and high interstitial fluid pressure in TME due to anaerobic respiration and leaky vasculature provide a barrier to current therapy strategies. HIF-1α regulates genes responsible for anaerobic respiration and angiogenesis. In current study we examined effects of Melittin treatment on HIF-1α and its downstream genes required for the formation of TME. Melittin treatment suppressed hypoxia-induced HIF-1α protein and mRNA levels (Fig. 2B and 2C). Gene expression analysis of NF-kB revealed that Melittin inhibits NF-kB expression (Fig. 2D). NF-kB is a direct regulator of HIF-1α expression[30]. Our finding suggests that Melittin inhibits HIF-1α primarily at transcriptional level through inhibition of NF-kB expression. This is in contrast to a previous study by shin et al., that reported that Melittin inhibits HIF-1α at translational level but not its mRNA, by Inhibition of ERK and mTOR/p70S6K Pathway in Human Cervical Carcinoma Cells. Mitogen-activated protein (MAP) kinases and phosphatidylinositol 3-kinases (PI3K)/Akt pathways are involved in translation of HIF-1α[24]. STAT3, another regulator of HIF-1α transcription, enhances HIF-1α expression through PKM2/HIF-1α positive feedback loop[31]. Several studies have reported that Melittin suppresses STAT3 signaling pathway. Kim and colleagues demonstrated that Melittin inhibits STAT3 activation and translocation of NFκB into the nucleus in human fibroblast-like synoviocytes[32]. In another study, it was reported that Melittin inhibits phosphorylation of JAK2 and STAT3 in SKOV3 and PA-1 ovarian cancer cell[33]. Inhibition of HIF-1α protein level by Melittin resulted in downregulation of VEGFA and LDHA (Fig. 3A and 3B). Several studies have reported VEGFA downregulation by Melittin in human cervical carcinoma cells[24], hepatocellular carcinoma cell lines[34], bladder cancer cells[35], lung cancer cells[36] and ehrlich carcinoma mouse model[37]. Inhibition of ERK and mTOR/p70S6K, VEGFR-2 and the COX-2-mediated MAPK signaling pathways has been implicated in these studies. Putting these together, Melittin can disrupt the formation of TME by suppressing HIF-1α protein in hypoxic conditions (Fig. 4).

Conclusion

In conclusion, we report for the first time that Melittin inhibits the growth of breast cancer cells by upregulation of extrinsic and intrinsic apoptotic pathway genes. Moreover, we demonstrate that Melittin can disrupt tumor microenvironment formation by suppressing the expression of genes responsible for anaerobic respiration and angiogenesis through inhibition of HIF-1α at transcriptional and translational/post-translational level in breast cancer cells. This is of importance as TME has been established as a barrier to cancer therapy during recent years. Our study provides further evidence on anti-cancer effects of Melittin.

Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

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Availability of data

Not applicable.

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Figures

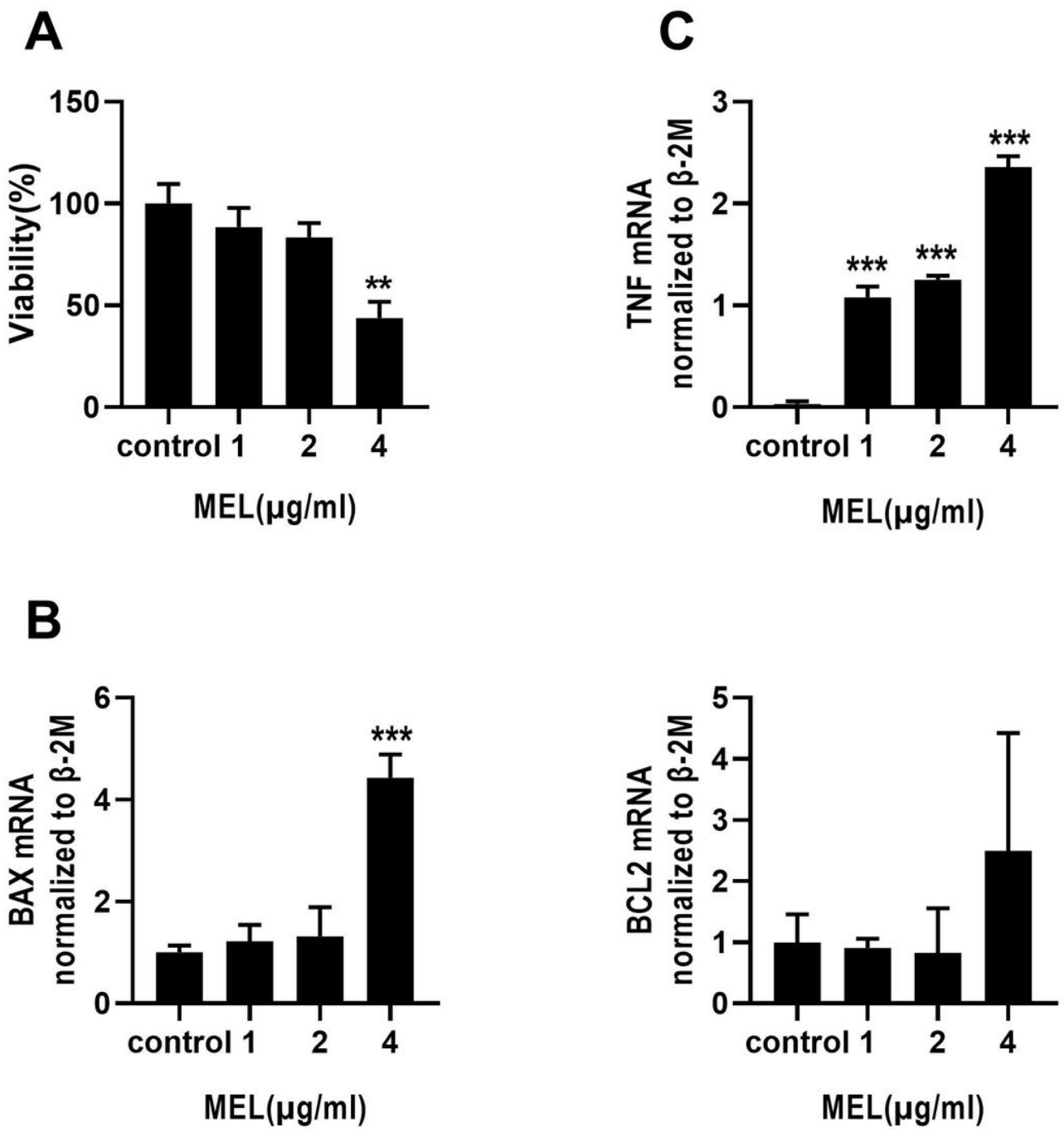

Figure 1

Melitin inhibits the proliferation of breast cancer cell line MDA-MB-231 through induction of apoptosis by upregulating BAX and TNFα expression. (A)Cells were treated with deiiferent doses of MEL for 24 hours. viability was measured using MTT assay. significant change in viability was observed at 4 µg/ml. (B) Gene expression analysis of BAX and BCL2 showed that MEL treatment upregulated the expression of BAX, while no significant change was observed for BCL2. (C) TNF expression was upregulated with different doses of MEL, suggestive of induction of extrinsic apoptotic pathway (**, $p<0.01$; ***, $p<0.001$).

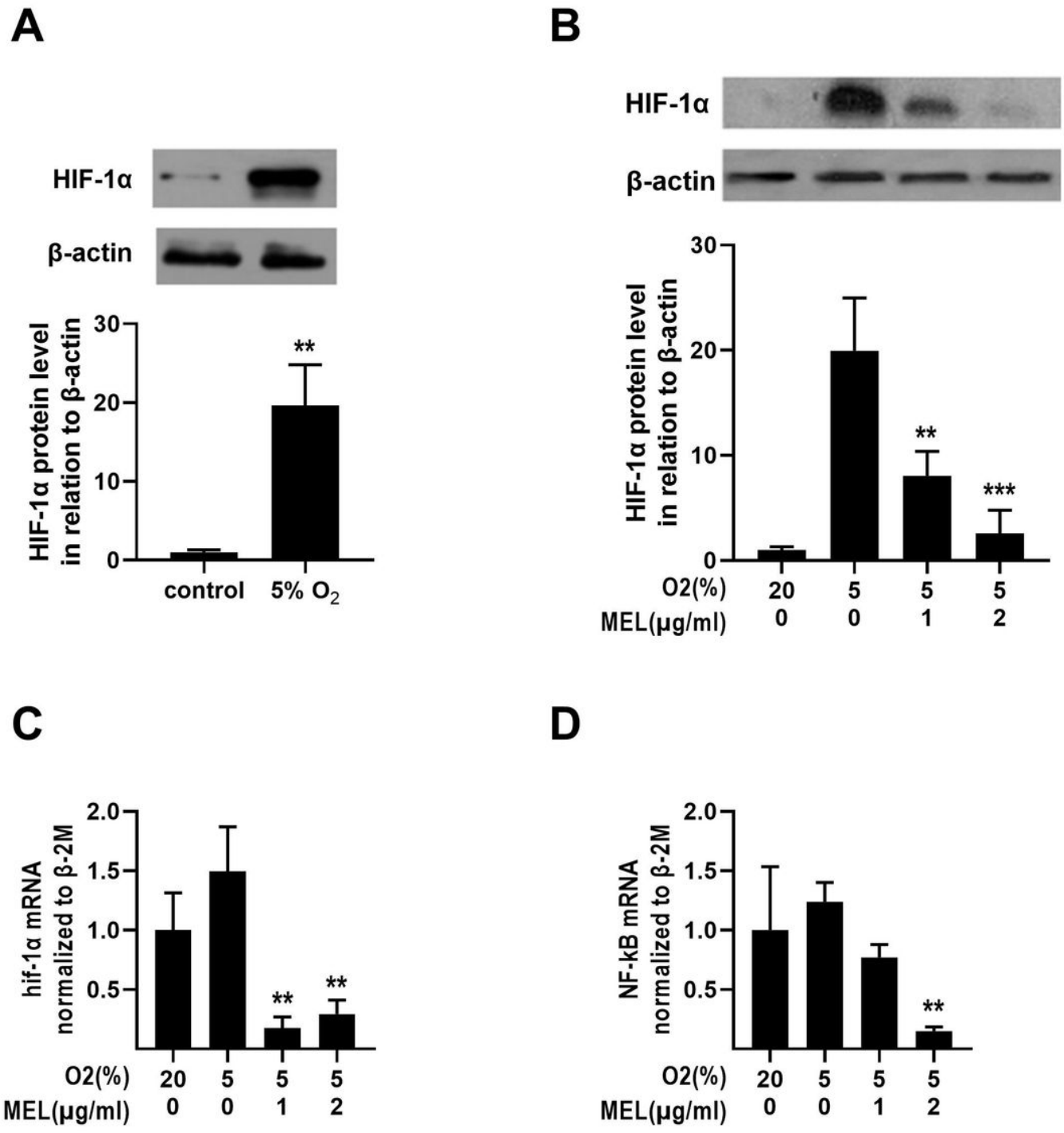

Figure 2

Melittin inhibits HIF-1α protein level by downregulating its mRNA expression through inhibition of NF-kB expression. (A) Cells were incubated at 5% O2 for 6 hours and HIF-1α protein level was measured using western blotting. HIF-1α protein level increased approximately 20 times. (B) cells were treated with different doses of MEL and incubated at 5% O2 for 6 hours. MEL decreased HIF-1α protein level significantly in comparison to hyopia-treated group at 2 μg/ml. (C) mRNA levels of hif-1α gene did not change in hypoxic group compared to untreated control. Significant decrease was observed when treated

with different doses of MEL. (D) further analysis of NF-kB showed no significant change in its expression in hypoxia-treated group in comparison to untreated control. NF-kB expression was downregulated at presence of 2 µg/ml MEL, sugeestive of HIF-1α downregulation as a result of declined expression of NF-kB (**, p<0.01; ***, p<0.001).

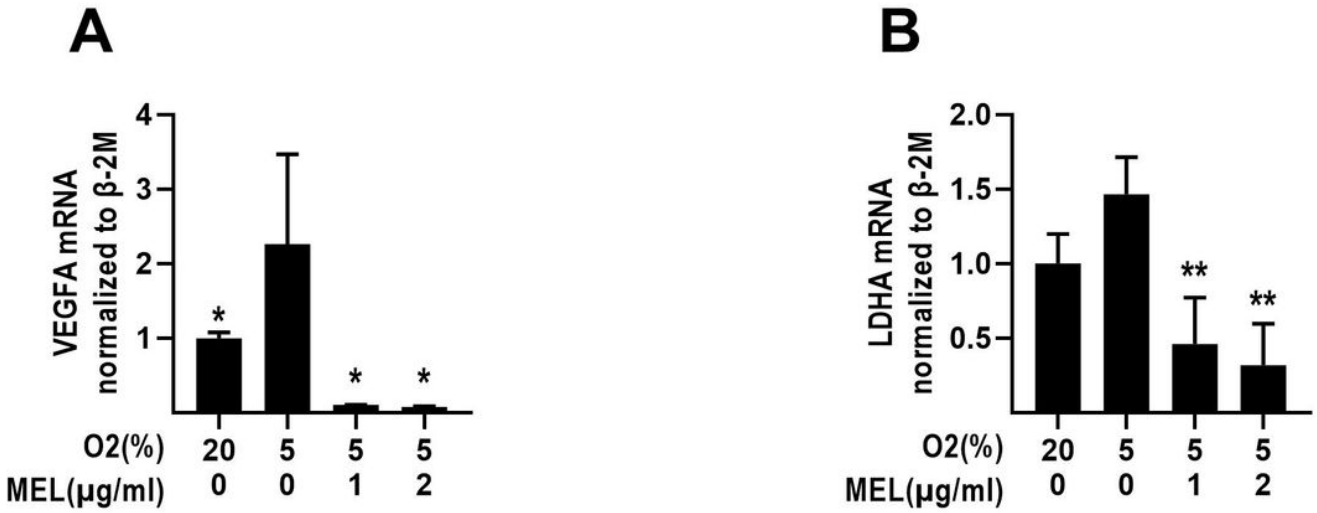

Figure 3

Melittin controls the expression of key players of tumor microenvironment formation by modulating HIF-1α signaling. Cells were incubated at 5% O2 for 6 hours and gene expression analysis was performed using realtime PCR. (A) hypoxic treatment induced expression of VEGFA compared to untreated control. MEL inhibited expression of hypoxia-induced VEGFA expression significantly. (B) low concentration of O2 upregulated LDHA slightly, though it was not significant. MEL treatment supressed LDHA expression significantly. These observations implicate inhibitory effect of MEL on TME physiological hallmarks (*, p<0.05; **, p<0.01).

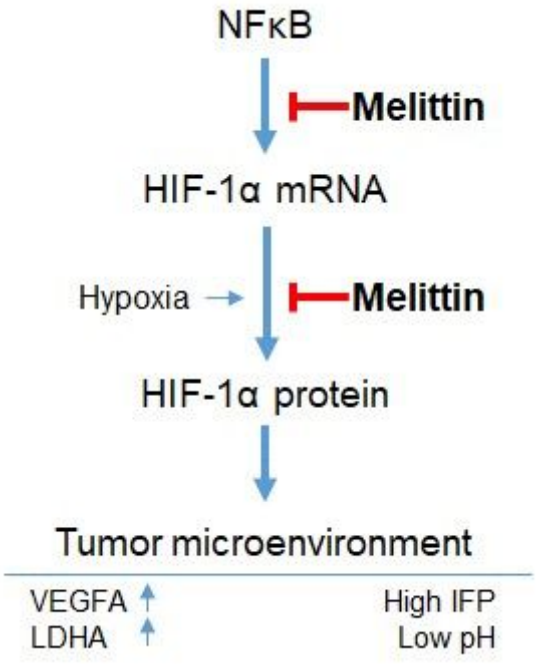

Figure 4

Melittin and TME. HIF-1α drives the formation of TME by regulating the expression of key genes involved in the process. Melittin disrupts the cascade by inhibiting HIF-1α at transcriptional and translational/post-translational level.