Dihydroartemisinin Inhibits Angiogenesis in Breast Cancer via Suppressing NF-κB/MMP-2/-9 Pathway

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Research Article

Keywords: dihydroartemisinin, angiogenesis, breast cancer, NF-κB, MMP-2/-9

Posted Date: February 21st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1310186/v1

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Abstract

Purpose

Dihydroartemisinin (DHA), a derivative of artemisinin that is well-known as an antimalarial drug, has been reported to have anti-tumor and anti-angiogenesis effects. However, whether and how it inhibits angiogenesis in breast cancer is poorly understood. In this study, we detected the anti-angiogenesis effect of DHA on breast cancer.

Methods

Firstly, we detect the anti-angiogenesis effect of DHA on breast cancer in a chick chorioallantois membrane model. Then, we collected the conditioned medium of MDA-MB-231 cells used in aortic ring angiogenesis assay, and HUVECs migration and tube formation assay. Finally, we used gelatin zymography, cellular immunofluorescence assay and western blot analysis to study signaling modulators regulated by DHA in MDA-MB-231 cells.

Results

The results showed that angiogenesis induced by MDA-MB-231 cells was attenuated by DHA. Vessel sprout and tube-formation of vascular endothelial cells were also depressed when cultured with CM from MDA-MB-231 pretreated with DHA. What’s more, the expression, and activities of matrix metalloproteinase 2 and 9 (MMP-2/-9) in MDA-MB-231 cells were downregulated by DHA. Further studies showed that DHA downregulated the expression of p-PI3K, p-AKT, p-ERK, and p-NF-κB proteins in tumor cells.

Conclusion

DHA was highly efficacious in inhibiting angiogenesis induced by breast cancer cells. The downregulating of MMP-2/9 through inhibiting phosphorylation of PI3K, AKT, and ERK in tumor cells may be the key factors in the inhibitory effect of DHA on angiogenesis.

1. Introduction

Tumor angiogenesis is necessary for solid tumor development[1]. New blood vessels develop continuously during tumor growth, providing oxygen and nutrition for tumor cells and acting as a medium for tumor invasion and metastasis. To be specific, with the degradation of the vascular matrix, vascular endothelial cells proliferate, migrate, and form new blood vessels that weave a vasculature network around the tumor, and the whole process can be driven by tumor cells secreting angiogenic factors[2–4]. As with tumors, tumor angiogenesis loses growth control. what’s more, the newly formed tumor vessels
are immature, irregular, twisty, with thin sinusoidal walls, and without continuous endothelial cells[5]. These aberrant morphologies vessel with a high permeability allowing tumor cells to enter the bloodstream more easily and metastasize to other tissues[4]. In breast cancer, higher microvascular density predicts a higher risk of metastasis and a poorer clinical prognosis[6]. Therefore, inhibition of tumor angiogenesis is a meaningful approach to inhibit breast cancer metastasis and achieve long-term effective control worth exploring.

Dihydroartemisinin (DHA) is the first-generation derivative of Artemisinin, an antimalarial drug derived by Tu Youyou from Artemisia annua Linn., which was recorded and used as a traditional medicine in ancient China thousands of years ago [7]. DHA was generated from artemisinin by reducing the C-10 carbonyl group with a higher bioavailability and solubility (Figure 1A). As a first-line antimalarial drug, it has cured many millions of malaria patients since its discovery[8]. What's more, the anticancer effects of DHA have been reported in several studies. Mounting evidence shows it acts against cancer cells both in vitro and in vivo. The profound effects of DHA on cancer cells most are verified through xenograft tumor models and revealed effects such as inhibition of cell proliferation and migration, inducing apoptosis, ferroptosis and oxidative stress[9]. Noteworthily, DHA has been reported to have an anti-angiogenic effect in model of chicken chorioallantois membrane (CAM) and human pancreatic cancer xenografts transplant to BALB/c nude mice[10, 11]. Since tumor cells play an important role in the induction of angiogenesis. We focus on whether DHA alters paracrine secretion of cancer cells that inhibits angiogenesis in breast cancer.

In this study, we used an in vivo CAM model to detect the angiogenesis induced by MDA-MB-231 cells pretreated by DHA. Further evidence for anti-angiogenesis effects of DHA was provided by using the MDA-MB-231 CM as a culture medium in endothelial cells migration and tube formation assay. As for the mechanism, we examined the expression of MMP-2/-9 in and out of cells after being treated by DHA. Finally, we examined the expression of proteins associated with MMP-2/-9 and the signal pathway of DHA anti-angiogenesis.

2. Materials And Methods

2.1 Chemicals and Cell Culture Materials

MDA-MB-231 cells, a triple negative breast cancer cell line, were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and stored in our laboratory and resuscitated once every half year[12]. Human umbilical vein endothelial cells (HUVECs) were kindly provided by Shanghai Whelab Bioscience Limited. Both cells were cultured in DMEM medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 2 mmol/l L-glutamine, 100 U/ml penicillin, 0.1 µg/ml streptomycin and 10% fetal bovine serum (FBS, Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.) at 37°C in a humidified atmosphere containing 5% CO2. The culture medium was replaced once every two days. DHA was purchased from Dalian Meilun Biotechnology Co., Ltd. (Liaoning, China).

2.2 CCK8 assay
Cells were seeded in a 96-well plate at a concentration of $4 \times 10^3$ cells/well. While incubated at 37°C and humidified atmosphere containing 5% CO2 for 12h, culture medium with different concentrations of DHA (0, 6.25, 12.5, 25, 50, 100, 200, 400μM) was replaced in each well. After 24 or 48 h of incubation, 200μL PBS with 10% CCK-8 solution (Beyotime, Shanghai, China) was replaced in each well, followed by incubating at 37°C for 2 hours. The cell viability was measured at an absorbance of 490 nm using a microplate reader (Thermo Fisher Scientific, US). Cell viability = OD 490 nm of the treatment/OD 490 nm of the control.

2.3 Chick chorioallantois membrane (CAM) assay

To assess the effect of DHA on the tumor cells induced vasculature, we performed a CAM assay according to the previously described method [13]. Briefly, embryonic eggs were incubated in a humidified (65-70%) incubator at 37.6°C. The normally developing eggs were randomly divided into four groups of five. Group without tumor cells was named as control group, and group with tumor cells pretreated by DHA (50 and 100μM) or not were name as DHA (50μM) group, DHA (100μM) group and MDA-MB-231 group separately as follows. After 7-days incubation, a small window was cut on the top of each egg to expose the CAM on which was put an absorbable gelatin sponge loaded with basal medium or indicated MDA-MB-231 cells as above. Then the window was sealed with laboratory film and incubated for an additional 2 days. CAM photos were taken under a stereoscopic microscope equipped with a digital camera. Excellent as the CAM model for assessing tumor angiogenesis, the scientific statistics and assessment of vascular patterning in it are challenging[14]. In view of this, vessels around the seed point are divided into three grades according to their branches, with the last capillary being grade III. Total vascular area and the number of vessels of each class in visual field are measured using ImageJ Software.

It is interesting to note that there are blood vessels drilling into the gelatin sponge in some group. To find it out, the sponge was fixed with paraformaldehyde and carefully isolate. 5μm thick paraffin-embedded sections were used to stain with hematoxylin and eosin (H&E). Sections after staining are observed and photographed under a microscope and the number of vessels in 5 random field were counted.

2.4 Aortic ring angiogenesis assay of angiogenesis

MDA-MB-231 cells ($5 \times 10^5$ cells/culture flask) were exposed to different concentrations of DHA (0, 50, and 100μM) for 24 h then the medium was replaced with fresh medium containing 1% FBS after being rinsed with PBS (pH 7.4) to remove unreacted drug. Pretreated MDA-MB-231 cells were cultured for another 24 h, and the supernatant was collected and referred to MDA-MB-231-derived conditioned medium (MDA-MB-231 CM)

Aortic rings are prepared from the thoracic aorta of 2-month-old rats. The aorta is cleaned of blood and fibroadipose tissue under a dissecting microscope and then cross-sectioned into 1 mm long rings using a scalpel blade. Aortic ring cultures are set in a 96-well culture plate with clotting media of Matrigel with serum-free medium mixed at 1:1. Then the growth medium (DMEM with 20% FBS) was added to the
wells. After 3 days of culture in a humidified CO\textsubscript{2} incubator at 37 °C, rings were randomly divided into four groups of five, and the growth media was replaced with indicated MDA-MB-231 CM or serum-free medium in each group [15]. After another 7−days in incubator, the images were taken under a microscope and the average microvessel sprouting number was counted from each group discharging the highest and lowest sample.

2.5 Transwell Assay

HUEVC cells were cultured in low-serum media supplemented with 1% FBS for 24 h, and then were suspended in the upper chamber of an 8μm aperture transwell in a density of 2×10\textsuperscript{5} cell/well with MDA-MB-231 CM or basal medium 200μL. The lower chamber was filled with 10% FBS medium (700μl) and incubated at 37°C for 12 hours. Subsequently, the migrated cells on the lower surface were stained with crystal violet. The number of migratory cells of each well was counted at five random fields. Results were from triplicate experiments.

2.6 Tube formation assay

Growth factor-reduced Matrigel (Corning, New York, US) was coated on a pre-cooled 96-well culture plate, and incubated for 30 min at 37°C. HUVEC cells were suspended in MDA-MB-231 CM pretreated with DHA or not and then seeded into each well at a density of 1×10\textsuperscript{5} cells/well. After 4 h in the incubator, the tubular structure was visualized at random fields and photographs were taken using Nikon's Eclipse TS 100 microscope. Tubulogenic was assessed by counting the number of closed intercellular compartments (closed rings or pro-angiogenic structures) according to reported methods using ImageJ Software. Results were from triplicate experiments, and representative views were shown.

2.7 Gelatin zymography for the detection of MMPs

Gelatin zymography was used to assess the enzymatic activities of MMPs as described previously[16]. Briefly, MDA-MB-231 CM was collected as mentioned above, and 30 μL CM in each group was resuspended in nonreducing laemmli sample buffer and resolved by 8% SDS-PAGE containing 1 mg/mL of gelatin (Yuanyebio, Shanghai, China). Following electrophoresis, gels were washed with 2.5% Triton X-100 to remove SDS and incubated in substrate buffer (50 mM, pH 8 Tris buffer containing 5 mM CaCl\textsubscript{2}) for 18 h at 37°C. Gels were then stained with 0.5% Coomassie brilliant blue R-250 (Service, Wuhan, China), followed by de-staining for 2 h. The gelatinolytic activity was visualized as negative staining bands using a digital camera.

2.8 Cellular immunofluorescence assay

MDA-MB-231 cells were set in a 6-well plat with a coverslip in each well. After being treated with different concentrations of DHA for 24 h, the cells were fixed by using 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton-X-100 for 20 min, and blocked with standard goat serum albumin for 1 h at room temperature. The cells were then incubated with primary anti-NF-κB monoclonal anti-body at 4°C
overnight. The secondary anti-rabbit Cyanine 3 (Cy3) anti-body (Cell Signaling Technology, USA) was incubated on another day at room temperature for 1 h. Subsequently the 4’,6-diamidino-2-phenylindole (DAPI) was stained for 5 min. The coverslips were taken out and set on the slide, captured, and analyzed by a confocal laser scanning microscopy system using a 400 × magnification (LSM710, Carl Zeiss, Germany).

2.9 Western blot analysis

Cells were seeded in a 6-well plate and treated with DHA at indicated concentration for 24 h. Whole cell lysates were prepared using RIPA lysis buffer (INVITROGEN, LLC) following the manufacturer’s instructions. While proteins were boiling properly about 40 μg of total protein was resolved in 8-12% SDS-PAGE. After transferring resolved protein onto PVDF membrane (Millipore), 10% BSA were used to block the membrane. The membranes were then incubated with primary antibodies in 4°C overnight, washed, incubated with appropriate HRP-conjugated secondary antibodies for 2 h at room temperature. Finally, the membranes were visualized using the chemiluminescence detection substrate (Beyotime, Wuhan China) on Image Lab (Bio Chem. USA). The protein bands were quantified using ImageJ software and normalized with respective β-actin.

2.10 Statistical Analysis

GraphPad Prism (Version 4.00, 1992-2003 GraphPad Software Inc.; San Diego, CA, USA) was used for statistical analysis using one-way ANOVA plus Tukey post hoc test to determine the significant changes. P < 0.05 or P < 0.01 were considered significant.

3. Results

3.1 DHA decreases the viability of MDA-MB-231 cells in a dose-dependent manner.

We first detected the toxicity of DHA to MDA-MB-231 cells by CCK-8 assay. MDA-MB-231 cells were exposed with DHA at different concentrations for 24 h or 48 h, and then cell viability was measured using CCK-8 assays. As shown in Figure 1B, when the concentration of DHA is below 100 μM, there was no significant decrease in cell viability compared to the control group (P > 0.05). When DHA was added up to 200 μM, cell viability was significantly reduced. The inhibition-effect of DHA was more notable at the dose of 400 μM, which implies that DHA decreases the viability of MDA-MB-231 cells in a dose-dependent manner. However, when the cells were treated for 48 h (Figure 1C), even low dose (6.25 μM) DHA showed certain cytotoxic effects on cells. For the consider to reduce the interference of drug toxicity in angiogenesis inhibition experiments, we choose the concentration of 50 μM and 100 μM for 24 h for the following experiments.

3.2 DHA inhibits angiogenesis induced by MDA-MB-231 cells in vivo

To investigate the inhibitory effect of DHA on angiogenesis induced by MDA-MB-231 cells, we used a chorioallantois membrane assays. MDA-MB-231 cells were pretreated by DHA for 24 h, and then seeded
upon chick chorioallantois membrane through gelatin sponge. 48h later, CAM photos were taken under a stereoscopic microscope. The area and number of vessels were analyzed as that described in method section. As shown in Figure 2A, B. The coverage area was increased in presence of MDA-MB-231 cells, indicating that MDA-MB-231 cells could promote angiogenesis on CAM. But the area of neovascularization significantly decreased in DHA groups compared with the MDA-MB-231 cell group. Meanwhile, the number of blood vessels at different levels decreased with increased DHA concentration (Figure 2A, C). To further explore the vessel formation in gelatin sponge, we made the H&E staining of the gelatin sponge (Figure 2D) and showed that angiogenesis in the sponge trabecular was formed only in MDA-MB-231 cell group. Overall, MDA-MB-231 cells induced angiogenesis in CAM but which could be inhibited or even eliminate by pretreated with DHA, and implying DHA inhibits angiogenesis induced by MDA-MB-231 cells in vivo.

3.3 DHA pretreated MDA-MB-231 CM suppresses vessel sprout formation ex vivo

Soluble cytokines in the tumor microenvironment secreted by tumor cells recruit vascular cells to form vessel sprouts[17]. To reveal how DHA participates in this procedure, we collected CM from indicated MDA-MB-231 cells to culture aortic ring and observe the formation of micro vessel sprouting. The result showed that the micro vessel sprouting number in the group cultured the CM of MDA-MB-231 cells were much more than the control group. But DHA abrogated the promoting effect of MDA-MB-231 cells on micro vessel sprouting (Figure 3, p<0.01). What's more, the number of micro vessel sprouts decreased in a dose-dependent manner. These results suggest that MDA-MB-231 cells secret soluble cytokines in the medium to promote angiogenesis, which can be inhibited by DHA.

3.4 DHA treated MDA-MB-231 CM suppresses the migration and tube-formation of HUVECs

Migration of vascular endothelial cells is an important step in angiogenesis. Here we investigated the migration and tube-formation ability of HUVECs by Transwell assay and tube-formation assay. Transwell assay showed that the CM collected from the control MDA-MB-231 cells stimulated migration of HUVECs. However, HUVECs incubated with CM from MDA-MB-231 pretreated with DHA migrated to the lower compartment was reduced (Figure 4). The result of tube formation assay indicated that HUVECs formed tube-like structures in matrigel and the number of tubes of HUVECs incubated with the CM from MDA-MB-231 pretreated by DHA (50 and 100µM) was significantly reduced compared to the MDA-MB-231 CM group (Figure 4). In conclusion, DHA inhibited migration and tube formation of HUVECs induced by MDA-MB-231 cells.

3.5 DHA downregulated the protein expression and enzymatic activity of MMP-2/MMP-9 in MDA-MB-231 Cells

MMPs are highly expressed in tumor cells and secreted into the microenvironment, which are involved in a variety of tumor biological behaviors including angiogenesis [18]. Our previous results suggest that DHA may inhibit angiogenesis by influencing paracrine secretion in tumor cells. So, we took it a step to explode the mechanism. In our study, the activity of MMPs secreted in the MDA-MB-231 CM was detected
using a gelatin zymography assay, and the protein expression in cells was detected by western blot. The results showed that the enzymatic activity of both MMP-2 and MMP-9 were significantly downregulated after tumor cells were treated with DHA (Figure 5), what's more, the expression of protein was reduced as the concentration of DHA increased from 50μM to 100μM. Both MMP-2 and MMP-9 expression and enzymatic activity were downregulated by DHA (Figure5), which means downregulating the secretion and activity of MMP-2/-9 is one of the main ways DHA inhibits angiogenesis induced by MDA-MB-231 cells.

3.6 DHA inhibits PI3K/AKT/NF-κB and ERK/ NF-κB signaling pathways in MDA-MB-231

Previous studies have shown that the expression of MMP-2/-9 could be regulated by NF-κB, and that DHA inhibit the activation of NF-κB [10, 19, 20]. We therefore assumed that DHA could downregulate MMP-2/-9 by inhibiting NF-κB signaling in MDA-MA-231 cells. To test this hypothesis, we first measured Iκbα, phospho-Iκbα, NF-κB/p65 and phosphorylation NF-κB/p65 after DHA treatment in MDA-MB-231 cells by western blot, and as the result showed in Figure 6, DHA significantly inhibited the phosphorylation of Iκbα and NF-κB/p65 compared to the control group. An immunofluorescence assay further confirmed that DHA inhibited the activation of NF-κB/p65 (Figure 6A). Secondly, we detected the expression of PI3K/AKT and ERK that are both associated with NF-κB and showed that DHA also inhibited the phosphorylation of ERK and PI3K/AKT. Finally, we can preliminarily conclude that DHA inhibited NF-κB/p65 activation through ERK and PI3K/AKT signaling pathways and then down-regulated MMP-2/-9 expression.

4. Discussion

In this report, we provide evidence that DHA can decrease breast cancer cell-induced angiogenesis by inhibiting MMP-2/9 releasing of tumor cells.

Folkman et.al [21] first proposed the vascular dependence of tumor growth. Later studies have shown that not only tumor growth, but also its evolution, invasion, and metastasis are closely related to neovascularization. When the tumor volume exceeds 2-3 mm³, simple passive diffusion cannot obtain the oxygen and nutrient supply needed for growth and survival. In a hypoxic environment, Tumor cells tend to release factors that regulating the proliferation, migration, and apoptosis of endothelial cells which are considered as the first step of tumor inducing the angiogenesis toward it from a normal surrounding host. Cancer cells influence angiogenesis by secreting various agents such as growth factors, integrin, interleukins as well as MMPs. Neovascularization promotes the progression and spread of breast cancer in return [22].

The CAM is a classical model that be well established and widely used in angiogenesis studies. As well in our experiment, we used gelatin sponges in CAM provide as a carrier of tumor cells to mimic extracellular matrix and tumor microenvironment, and to reflect the dynamic changes between tumor cells and neovascularization[23]. The MDA-MB-231 cells in the sponge can increase angiogenesis in CAM and induce a radially arranged spoked wheel pattern of blood vessels around the sponge. And even more interesting, new blood vessels penetrate into the sponge, which suggest that tumor cells can induce
angiogenesis through paracrine. To explore how DHA plays a role in this process, we select the appropriate concentration (0, 50 and 100µM) of it to pretreat MDA-MB-231 cells. The viability of tumor cells was not significantly decreased after treatment, but the ability to induce neovascularization is obviously weakened, which suggest that DHA may indirectly affect angiogenesis by changing the biological activity of tumor cells.

To further validate this hypothesis, we collected the conditioned medium of MDA-MB-231 cells used in aortic ring angiogenesis assay, and HUVECs migration and tube formation assay. It is consistent with the previous results that the CM from MDA-MB-231 cells induced the migration and tube formation of endothelial cells. However, the promoting effect was significant decreased after tumor cells were pretreated by DHA, which confirmed that DHA can inhibit angiogenesis by affecting the secretion of tumor cells.

Among the angiogenic factors secreted by tumor cells, MMPs family is an important regulator promoting migration and sprouting of endothelial cell while degrading the extracellular matrix (ECM)[24]. It promotes metastasis by promoting epithelial-mesenchymal transformation of tumor cells and facilitating neovascularization. The most closely related to angiogenesis in the MMP family is MMP-9, while some studies indicate a role for MMP-2 as well [25, 26]. Previous studies have shown that DHA regulator the MMP-2/-9 expression to inhibits the development of colon cancer [27]. By the same mechanism, DHA inhibits human fibrosarcoma cells and epithelial ovarian cancer cells proliferation and migration [28, 29]. Consistent with previous studies, our results showed that DHA inhibited the activity and expression of MMP-2/-9 in MDA-MB-231 cells. Since that MMP-2 and MMP-9 are both related to the phosphorylation of ERK1/2 and PI3K by regulating the activation of NF-κB signaling pathways [25, 30, 31]. We further detected the expression of related key proteins in MDA-MB-231 cells after treated by DHA and revealed that DHA inhibits NF-κB(P65) activation by regulating both ERK and PI3K pathways.

Antiangiogenetic drugs have been explored since the discovery of the important role angiogenesis plays in tumor development, and, indeed mature antiangiogenic agents have been applied in clinic [32, 33]. Neutralization antibodies of vascular endothelial growth factor (VEGF) and blockers of VEGF receptors are the most extensively used and efforts have been made to explore inhibitors that target MMP-2 and MMP-9 [24]. However, few antiangiogenic drugs have significantly improved overall survival in cancer patients [34]. Therefore, more effective anti-tumor angiogenesis drugs have yet to be developed. The search for natural products has never stopped, indeed, they often have the advantage of being easy to obtain, having little toxicity and wide range of effects, and continue to surprise people in the treatment of various diseases [35, 36]. DHA, a gift from nature, has great potential as an anti-tumor drug using alone or in combination. Our study highlights the role of DHA in the treatment of breast cancer angiogenesis, and provides experimental basis for DHA as a new antitumor angiogenesis drug.

Declarations

Funding
This work was supported by the National Science Foundation of China (No. 81702920, 82174020).

Conflict of Interest

The authors do not have any conflict of interest.

Author Contributions

All authors participated in the design and implementation of the experiment: Qi Rao, He Yu, Ruochan Li, Bin He, Gang Zhao, Fenghua Wu. Qi Rao, He Yu analyzed the data and drafted the manuscript, Gang Zhao and Fenghua Wu conducted experiments, reviewed, and revised manuscripts.

Data availability

Available upon reasonable request.

Ethics approval

No ethical approval is required.

References


Figures
Figure 1

MDA-MB-231 cells were treated by DHA at different concentration 24h or 48h, then the viability was detected using CCK8 assay. (a) Chemical structure of DHA. (b) Cell viability of MDA-MB-231 cells treated with DHA at the indicated concentrations for 24. (c) Cell viability of MDA-MB-231 cells treated with DHA at the indicated concentrations for 48 hours. Values are the means ± S.D. from three independent determinations., *p<0.05, **p<0.01 indicate a significant difference from the control group. DHA: dihydroartemisinin, CCK8: cell counting kit-8.
Figure 2

DHA inhibits angiogenesis induced by MDA-MB-231 cells on CAM. (a) Gelatin sponges were loaded with or without MDA-MB-231 cells that had been pretreated or not with DHA 24 h, seeded on CAM, cultured for 48 h. Photographs were taken under an anatomical microscope. (b) Areas of vessels in different groups. (c) The number of blood vessels at different levels in each group (graded by vessel branch, and level III represents the last branch). (d) H&E staining of gelatin sponges, 40×. Values are the means± S.D. from three independent determinations, n=6. *p<0.05, **p<0.01 indicate a significant difference. DHA: dihydroartemisinin, CAM: chick chorioallantois membrane.
Figure 3

DHA treated MDA-MB-231 CM suppresses the migration and tube-formation of HUVECs. (a, d) MDA-MB-231 CM suppresses vessel sprout formation ex vivo. Aortic ring angiogenesis were inhibited with basal medium or MDA-MB-231 CM from MDA-MB-231 cells pretreated by DHA or not. The pictures show a typical image from three independent experiments (n=6). Micro vessels were counted under the microscope with 200× magnitudes. (b, e) The typical image of migrated HUVECs in transwell, incubated...
with basal medium or MDA-MB-231 CM that from MDA-MB-231 cells pretreated with or without DHA. Photographed by inverted microscope with 200× magnitudes; Migrated cells were counted in 5 random fields. (c, f) Tube formation of HUVECs incubated with basal medium or MDA-MB-231 CM that from MDA-MB-231 cells pretreated with or without DHA. The pictures show a typical image from three independent experiments (n=6). Tube numbers were counted under the microscope with 200× magnitudes. *p<0.05, **p<0.01 indicate a significant difference. DHA: dihydroartemisinin, CM: conditioned medium.

**Figure 4**

DHA inhibited MMP-2/-9 protein expression and activities. (a) Gelatine zymography of MDA-MB-231 CM treated with different concentration of DHA. (b) western blot analyses of the expression of MMP-2/-9 in MDA-MB-231 cells treated with different concentration of DHA. *p<0.05, **p<0.01 indicate a significant difference from the control group. DHA: dihydroartemisinin, CM: conditioned medium, MMP: matrix metalloproteinase.
Figure 5

DHA inhibits PI3K/AKT/NF-κB and ERK/ NF-Kb signalling pathways in MDA-MB-231. (a) Shown are representative images of NF-B (p65) immunostaining in MAD-MB-231 cells treated for 24 hours with DHA (50, 100 M). p65 is in red (Cy3), the nucleus is in blue (DAPI). (b) DHA decreases the level of p-Iκbα and p-NF-κB/p65 in MDA-MB-231 cells. (c, d) DHA inhibited the phosphorylation of ERK and PI3K/AKT in MDA-MB-231 cells. Reported values are the mean ± S.D. of three independent experiments. *p<0.05, **p<0.01 indicate a significant difference from the control group.
Figure 6

Dihydroartemisinin inhibits angiogenesis in breast cancer via suppressing NF-κB/MMP-2/-9 pathway