RESEARCH ARTICLE

4-Hydroxynonenal Promotes Growth and Angiogenesis of Breast Cancer Cells through HIF-1α Stabilization

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Abstract

4-Hydroxynonenal (4-HNE) is a stable end product of lipid peroxidation, which has been shown to play an important role in cell signal transduction, while increasing cell growth and differentiation. 4-HNE could inhibit phosphatase and tensin homolog (PTEN) activity in hepatocytes and increased levels have been found in human invasive breast cancer. Here we report that 4-HNE increased the cell growth of breast cancer cells as revealed by colony formation assay. Moreover, vascular endothelial growth factor (VEGF) expression was elevated, while protein levels of hypoxia inducible factor 1 alpha (HIF-1α) were up-regulated. Sirtuin-3 (SIRT3), a major mitochondria NAD+-dependent deacetylase, is reported to destabilize HIF-1α. Here, 4-HNE could inhibit the deacetylase activity of SIRT3 by thiol-specific modification. We further demonstrated that the regulation by 4-HNE of levels of HIF-1α and VEGF depends on SIRT3. Consistent with this, 4-HNE could not increase the cell growth in SIRT3 knockdown breast cancer cells. Additionally, 4-HNE promoted angiogenesis and invasion of breast cancer cells in a SIRT3-dependent manner. In conclusion, we propose that 4-HNE promotes growth, invasion and angiogenesis of breast cancer cells through the SIRT3-HIF-1α-VEGF axis.

Keywords: 4-Hydroxynonenal - breast cancer - growth - invasion - HIF-1α - VEGF - SIRT3 - angiogenesis

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Introduction

4-Hydroxynonenal (4-HNE) is the primary α/β-unsaturated-unsaturated hydroxyalkenal that is produced by lipid peroxidation in cells (Patrick et al., 2005; Christov et al., 2013). 4-HNE can bind to cysteine, histidine, and lysine residues of proteins via Michael addition reaction (Bennaars-Eiden et al., 2002; Macpherson et al., 2007). Thus, 4-HNE can increase oxidative post translational modification (PTM) and then alter protein function and cell signaling (Jin and Zangar, 2009; Ryan et al., 2014). 4-HNE could inhibit PTEN (Phosphatase and tensin homolog) activity in the hepatocytes (Shearn et al., 2011; Ayala et al., 2014). Modification of AKT2 (v-akt murine thymoma viral oncogene homolog 2) by 4-hydroxynonenal inhibits insulin-dependent AKT signaling (Shearn et al., 2011). 4-HNE modification induces phosphorylation of DAXX at Ser668 and Ser671 to facilitate its cytoplasmic export (Sharma et al., 2008). Additionally, 4-HNE causes induction, phosphorylation, and nuclear accumulation of p53 (Cao et al., 2014). 4-HNE was reported to increase cell growth and differentiation (Dalleau et al., 2013). Increased levels of 4-HNE was also found in human invasive breast cancer (Jin and Zangar, 2009).

Hypoxia inducible factor 1 (HIF-1), consisting of HIF-1α and HIF-1β, is essential mediator of the cellular oxygen-signaling pathway (Li et al., 2013). HIF-1β expresses constitutively, whereas HIF-1α proteins are rapidly degraded and undetectable under normoxic conditions (Stroka et al., 2001; Park et al., 2003). HIF-1α-activated genes include VEGF, which promotes angiogenesis (Ahn et al., 2014). The stability of HIF-1α was regulated by its post-translational modifications such as hydroxylation, ubiquitination, acetylation, sumoylation and phosphorylation (Ke and Costa, 2006; Ulrich, 2007; van Hagen et al., 2010). Sirtuin-3 (SIRT3), a major mitochondria NAD+-dependent deacetylase, was reported to destabilize HIF-1α (Finley et al., 2011; Chen et al., 2014). Under the normoxic conditions, HIF-1α is degraded via the ubiquitin-proteasome pathway by binding of the von Hippel-Lindau tumor suppressor protein (pVHL) to the oxygen-dependent degradation domain (ODD) (Maxwell et al., 1999). HIF-1α protein is rapidly degraded and difficult to detect from cell lysates under normoxic conditions (Groulx and Lee, 2002) however, various factors can activate HIF-1α even under normoxic conditions and HIF-1α is detectable from isolated nuclei under normoxic conditions (Finley et al., 2011). This provides the possibility to study HIF-1α protein under normoxic conditions.

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Increased levels of 4-HNE was also found in human invasive breast cancer. Here, we investigated the role of 4-HNE in the growth, invasion and angiogenesis of breast cancer.

Materials and Methods

Cell lines and Cell culture

The human breast cell lines, MDA-MB-231 and MCF-7 were obtained from American Type Culture Collection (ATCC). 293T cells and human umbilical vein endothelial cells (HUVECs) were obtained from the Institute of Cell and Biochemistry Research of Chinese Academy of Science. HUVECs were cultured in Kaighn's Modified Ham's F-12K medium supplemented with endothelial cell growth supplement (BD Biosciences) and 10% fetal bovine serum in 5% CO_2 atmosphere at 37°C. The other cell lines were maintained in DMEM medium with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37 °C.

Reagents

4-Hydroxynonenal was purchased from Merck Millipore. Antibodies used from this study were purchased from: β-actin (Merck); VEGF (Santa Cruz Technology); HIF-1α (BD Biosciences); LSD1 (Cell Signaling Technology); SIRT3 (Abcam).

RNA isolation and Real-time PCR

RNA was extracted from cancer cells using the TRIzol reagent (Life Technologies). The cDNA synthesis was performed using a SuperScript First-Standard Synthesis System for RT-PCR (Life Technologies) according to the manufacturer. VEGF mRNA expression levels were measured using a real-time PCR assay with SYBR Green (TOYOBO) and ABI Prism 7500 real-time PCR system (Applied Bio-systems). The primers of real-time PCR for human VEGF: Forward, 5'-CTTTTCTGCTGTCTTGGGTGC-3'; Reverse, 5'-ATCGCATCAGGGGCACACAG-3'. The primers of real-time PCR for human β-actin: Forward, 5'-ATCATGTTTGAGACCTTCAACA-3'; Reverse, 5'-CATCTCTTGCTCGAAGTCCA-3'. β-actin was used as the normalization control for normalization. Real-time PCR was performed with 2 min incubation at 95 °C and 40 amplification cycles (95°C, 10 s; 55°C, 15 s; 72°C, 35 s).

Plasmid construction and production of lentiviral vectors

Lentiviral vectors PLKO.1 containing shRNA sequences against SIRT3 were purchased from Sigma-Aldrich. The target sequences: shSRIT-1: 5'-TCTTGCTGCATGTGGTTGATT-3' (CDS); shSIRT3-2: 5'-GTGGGTGCTTCAAGTGTTGTT-3' (5UTR). The sequence of non-target pLKO.1 shRNA (shNS): 5'-CTTGTGGAAAGGACGAAACACCG-3'. The control and SIRT3 expressing lentiviral vectors were from Genesent (coding region of NM_001017524.2). Lentiviral vectors were transfected into HEK 293T cells with the packaging plasmids. After 48 h, the transfected HEK 293T cell supernatants with target-containing virus were collected. Then, the lentiviral particles were mixed with 8 μg/ml polybrene (Sigma-Aldrich) in human breast cancer cells. Cells were then selected with 2 mg/ml puromycin for 48 h.

Colony formation assays

The Cells were seeded at a density of 300 cells/dish (MDA-MB-231) and of 200 cells/dish (MCF-7) into the 6-well plate. 24 hours later, cells were incubated (37°C, 5% CO_2) in DMEM (10% FBS) with ethanol vehicle control or 1 μM 4-Hydroxynonenal. Two weeks later, colonies were fixed with 10% buffered formalin and stained with 2% crystal violet. Colony formation rate was determined as colony formation rate=colonies/input cells×100%.

Cell invasion assay

A total of 1×10^5 MDA-MB-231 cells were suspended in 100 μl DMEM with 10% FBS and seeded with ethanol vehicle control or 4-Hydroxynonenal (1 μM) onto the upper compartment of Matrigel-coated (BD Biosciences) Transwell chambers (24-well, pore size 8 μm; Millipore). The cells were incubated for 24 hours at 37°C in a 5% CO_2 chamber. The cells that did not invade through the pores were removed by a cotton swab. Cells on the lower surface of the membrane were stained with hematoxylin-eosin (H&E) and counted.

ELISA analysis

The supernatants from above experiments were assessed for VEGF using a commercially quantitative ELISA kit (R and D Systems). Human breast cancer cells (5×10^4 cell per well) were plated in 48-well plates overnight at 37°C. At 90% confluence, the medium was replaced with serum-free medium. Cells were treated with ethanol vehicle control or 1 μM 4-Hydroxynonenal and the conditioned medium was collected after 48 hours. VEGF concentrations were determined by measuring absorbance at 420 nm, and VEGF values were normalized to the total protein concentration in each well.

Tube formation assay of HUVECs

An *in vitro* tube formation assay was performed to evaluate the properties of MDA-MB-231 cells treated with or without 4-Hydroxynonenal (1 μM) on HMVECs tube formation. The upper chamber was prepared by plating a monolayer of HUVECs onto a very thick layer of Matrigel (BD Biosciences) in Transwell filters (0.4 μm pore size, Costar) followed by incubation at 37°C for 24 hours. The HUVECs were then co-cultured with the MDA-MB-231 human breast cancer cells with ethanol vehicle control or

4-Hydroxynonenal (1 μM) at 37°C for 4 days. Then tube-like structures that formed in the gel were photographed.

Western blot analysis

For the whole-cell lysates: cells were suspended in lysis buffer (150 mM NaCl, 100 mM Tris, pH7.4, 5 mM EDTA and 1% Triton X-100) containing a mixture of protease and phosphatase inhibitors (Roche). Then cell lysates were centrifuged at 16000×g for 15 min at 4°C. For isolation of nuclei: nuclei were isolated from

cultured cells using the NE-PER nuclear and cytoplasmic extraction kit (Pierce) according to manufacturer instructions. The protein concentration was determined by protein-dye (Bio-Rad). Equal amounts of protein (50 μg) were separated by SDS-PAGE (10%) and transferred onto PVDF membrane (Millipore). After blotted with primary antibodies overnight, peroxidase conjugated secondary antibodies were incubated with the membrane. Bound antibodies were detected by Chemiluminescent horseradish peroxidase (HRP) (Pierce) and photographed by ImageQuant LAS 4000 luminescent image system (GE Healthcare).

Statistical analysis

The unpaired Student's t-test was used for the data analysis. All data were shown as mean±standard error (SE). A statistical difference of $p<0.05$ was considered significant.

Results

4-Hydroxynonenal promotes the growth and invasion of breast cancer cells

Increased levels of 4-hydroxynonenal (4-HNE) has been reported in breast cancer (Sharma et al., 2012). Herein, we detected the effects of 4-HNE on the growth of breast cancer cells by performing the colony formation assay. As shown in Figure 1A, 4-HNE (1μM) increased the cell growth in both MDA-MB-231 and MCF-7 breast cancer cell lines. To further investigate the function of 4-HNE in the invasion of breast cancer cells, we performed matrigel invasion assays. The results revealed that 4-HNE remarkably promoted the invasion of MDA-MB-231 breast cancer cells (Figure 1B). Therefore, these findings indicate that 4-HNE act as an endogenous tumor promoter in breast cancer cells.

4-Hydroxynonenal up-regulates the expression of VEGF

Previous studies (Vatsyayan et al., 2012) demonstrated that 4-HNE could increase the VEGF expression and secretion. This might be the cause of 4-HNE to promote cell growth and invasion of breast cancer cells. To confirm this, MDA-MB-231 and MCF-7 cells were treated with 4-HNE for 6 or 12 hours (1μM). Western Blot analysis showed that the expression of VEGF protein was up-regulated by 4-HNE in the whole-cell lysates of breast cancer cells (Figure 2A). In order to further investigate the function of 4-HNE in the regulation of VEGF, quantitative RT-PCR was performed to detect the levels of VEGF affected by 4-HNE. As shown in Figure 2B, we found that 4-HNE significantly increased the levels of VEGF mRNA. These results imply that 4-HNE increases the expression of VEGF due to activation of transcription. Meanwhile, increased VEGF secretion was also found under the treatment with 4-HNE in breast cancer cells (Figure 2C).

4-Hydroxynonenal promotes the growth and invasion of breast cancer cells

Sirtuin-3 and Sirtuin-1 (SIRT3 and SIRT1) were reported to be covalently modified by 4-HNE (Caito et al., 2010; Fritz et al., 2011). This modification by 4-HNE can inhibit SIRT3 deacetylase activity (Fritz et al., 2011). Moreover, SIRT3 was reported to destabilize HIF-1α protein and inhibit its activity (Finley et al., 2011). Thus we suppose that the regulation of cell growth, invasion and VEGF expression by 4-HNE to the breast cancer cells may be the results of 4-HNE induced SIRT3 inhibition and stabilization of HIF-1α protein. HIF-1α protein is rapidly degraded and difficult to detect from cell lysates under normoxic conditions (Ke and Costa, 2006), however,

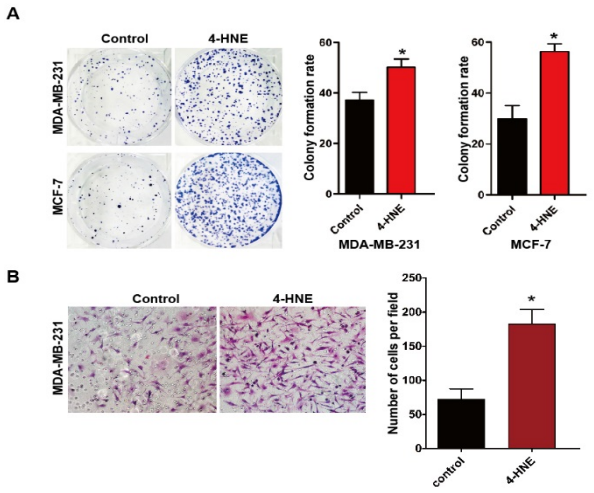

Figure 1. 4-Hydroxynonenal Increases the Growth and Invasion of Breast Cancer Cells. MDA-MB-231 and MCF-7 cells were treated with ethanol vehicle control or 4-HNE (1μM). **A)** Representative photographs of colony formation in breast cancer cells. Quantification of the colony formation rate in breast cancer cells; *$p<0.002$ compared with the control group. **B)** Invade cells in invasion assay were stained and counted in control and 4-HNE groups; *$p<0.001$ compared with the control group

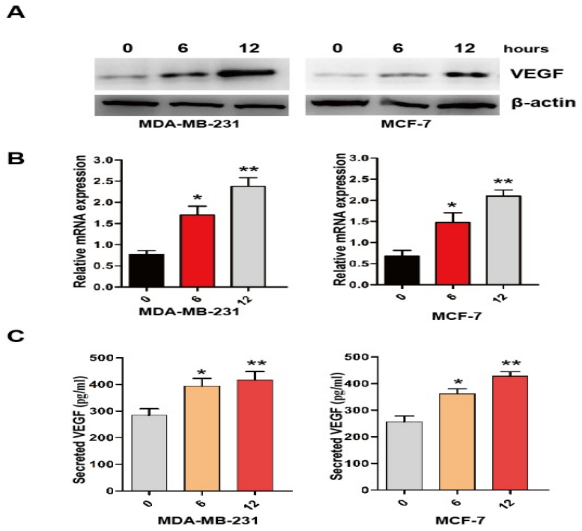

Figure 2. 4-Hydroxynonenal Increases the Expression and Secretion of VEGF. MDA-MB-231 and MCF-7 cells were treated with 4-HNE for 0-12 hours (1μM). (A) The levels of VEGF and β-actin protein were examined by Western Blot with whole-cell lysates. (B) The levels of VEGF mRNA were examined by real-time PCR. *$p<0.02$, **$p<0.004$ compared with the control group (C) VEGF levels in the culture medium determined by ELISA

HIF-1α is detectable from isolated nuclei under normoxic conditions. This provides the possibility to study HIF-1α protein under normoxic conditions (Finley et al., 2011).To test this idea we first investigated whether 4-HNE affects the levels of HIF-1α protein under normoxic conditions. A significant increase of HIF-1α protein was found in breast cancer cells after the treatment with 4-HNE (Figure 3A). We further showed that knockdown of SIRT3 (Figure 3B) resulted in significantly higher levels of HIF-1α and VEGF in MDA-MB-231 cells (Figure 3C). Importantly, 4-HNE could not significantly affect the levels of HIF-1α and VEGF in SIRT3 knockdown cells (Figure 3C). Together, these results support a role of 4-HNE in the regulation of cell growth, invasion and VEGF expression via inhibiting SIRT3 induced destabilization of HIF-1α.

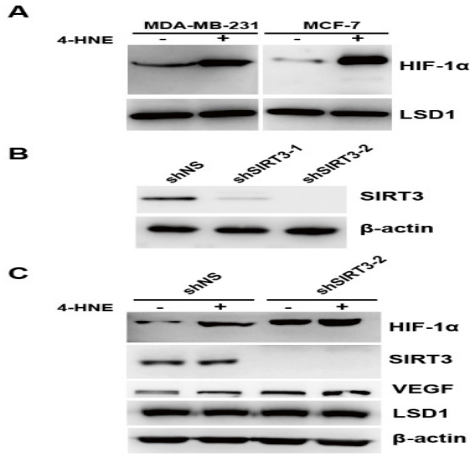

Figure 3. 4-Hydroxynonenal Up-regulates the Levels of HIF-1α. A) MDA-MB-231 and MCF-7 cells were treated with ethanol vehicle control or 4-HNE (1μM) for 12 hours. The levels of HIF-1α and LSD1 protein were examined by Western Blot from the nuclear extracts. **B)** MDA-MB-231 were stably transfected with shRNA control (NS) or with shRNA targeting SIRT3 (shSIRT3-1 and shSIRT3-2). Western Blot showing expression SIRT3 and β-actin in whole-cell lysates. **C)** Control and SIRT3 stable knockdown MDA-MB-231 cells with the treatment of ethanol vehicle control or 4-HNE (1μM) for 12 hours. The expression of HIF-1α and LSD1 protein were examined by Western Blot from the nuclear extracts. The levels of SIRT3, VEGF and β-actin protein were examined by Western Blot in whole-cell lysates

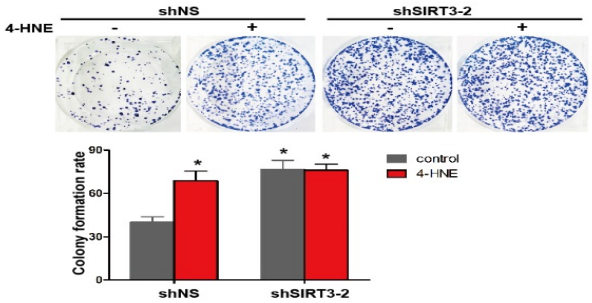

Figure 4. Knockdown of SIRT3 Inhibits Cell Growth Regulation by 4-Hydroxynonenal. Control and SIRT3 stable knockdown MDA-MB-231 cells with the treatment of ethanol vehicle control or 4-HNE (1μM) for 12 hours. Representative photographs of colony formation of MDA-MB-231 cells. Quantification of the colony formation rate in breast cancer cells. *p<0.01 compared with the control group (shNS)

Knockdown of SIRT3 increases the cell growth and inhibits its regulation by 4-Hydroxynonenal

To confirm whether 4-HNE-induced regulation of cell growth depends on SIRT3. We performed the colony formation assay to evaluate the cell growth in mock MDA-MB-231 and SIRT3-knockdown MDA-MB-231 cells under the treatment with 4-HNE. As shown in Figure 4, knockdown of SIRT3 significantly increased the cell growth of breast cancer cells. Differently, 4-HNE could not regulate the cell growth in SIRT3-knockdown MDA-MB-231 cells. This is consistent with the regulation of HIF-1α and VEGF by 4-HNE in SIRT3-knockdown MDA-MB-231 cells (Figure 4). These results further support our idea.

4-Hydroxynonenal promotes the angiogenesis induced by breast cancer cells in a SIRT3-dependent manner

Increased expression and secretion of VEGF induced by 4-HNE in breast cancer cells may affect the angiogenesis via SIRT3 inhibition. To examine this, we rescued the expression of SIRT3 in SIRT3 stable knockdown

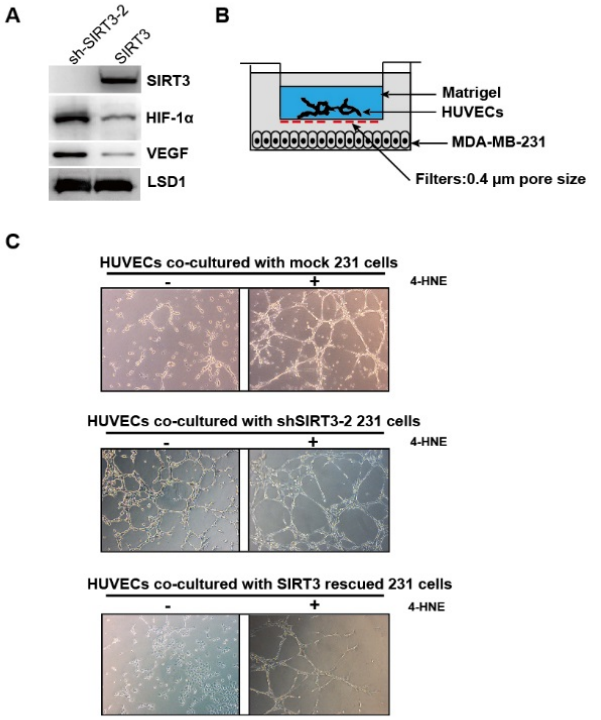

Figure 5. 4-Hydroxynonenal Promotes the Angiogenesis of Breast Cancer Cells in a SIRT3-dependent Manner. A) Rescued the expression of SIRT3 in SIRT3 stable knockdown MDA-MB-231 cells. The expression of HIF-1α and LSD1 protein were examined by Western Blot from the nuclear extracts. The levels of SIRT3 and VEGF protein were examined by Western Blot in whole-cell lysates. **B)** Schematic diagram of the differentiation assay chamber. Mock MDA-MB-231, shSIRT3-2 MDA-MB-231 or SIRT3 rescued MDA-MB-231 cells were grown to confluence in the lower chamber. The upper chamber was prepared by plating a monolayer of HUVECs onto a very thick layer of Matrigel in Transwell filters (0.4μm pore size). HUVECs were then co-cultured with different kinds of MDA-MB-231 cells at 37°C for 4 days. The generation of tubular networks that formed in the gel was photographed.

MDA-MB-231 cells (Figure 5A). ShSIRT3-2 targets a 3'UTR sequence of SIRT3 mRNA, which is absent in SIRT3 cDNA used in rescue (described in Materials and methods). The rescue of SIRT3 expression inhibited the levels of HIF-1α and VEGF in SIRT3 knockdown MDA-MB-231 cells (Figure 5A). Moreover, an in vitro model of angiogenesis was used employing Matrigel and human umbilical vein endothelial cells (HUVECs) co-cultured with mock, SIRT3 knockdown or rescued MDA-MB-231 cells (Figure 5B). As shown in Figure 5C, confluent monolayers of HUVECs co-cultured with mock MDA-MB-231 cells increased the generation of tubular networks under the treatment with 4-HNE. Knockdown of SIRT3 significantly increased generation of tubular networks, whereas 4-HNE had no remarkable effect to the generation of tubular networks in SIRT3 stable knockdown MDA-MB-231 cells (Figure 5C). Rescued the expression of SIRT3 inhibited the formation of tubular networks in SIRT3 stable knockdown MDA-MB-231 cells. In contrast, 4-HNE had significant enhanced effects on HUVECs tubular networks formation after the rescued expression of SIRT3 (Figure 5C). These results strongly support the functional mechanism of 4-HNE to promote angiogenesis induced by breast cancer cells through SIRT3-HIF-1α-VEGF axis.

Discussion

The current study demonstrates that 4-HNE promoted the cell growth, invasion and angiogenesis of breast cancer cells. 4-HNE could up-regulate the expression and secretion of VEGF in breast cancer cells. Moreover, HIF-1α, which activates the transcription of VEGF, was also found to be up-regulated by 4-HNE. Knockdown of Sirtuin-3 (SIRT3), a major mitochondria NAD+-dependent deacetylase, attenuated the regulation of 4-HNE to the levels of HIF-1α and VEGF in breast cancer cells. Additionally, the enhancing effect of 4-HNE to the cell growth, invasion and angiogenesis of breast cancer cells was proved to depend on SIRT3.

4-Hydroxynonenal (4-HNE) is α/β-unsaturated aldehyde released from the peroxidation of n-6 polyunsaturated fatty acids (Patrick et al., 2005). 4-HNE can bind to thiol (SH) and amino (NH2) groups of cysteine, lysine and histidine residues by Michael addition reaction and form a covalent bond between 4-HNE and the amino acid (Usatyuk and Natarajan, 2012). Thus, 4-HNE can increase the oxidative post translational modification (PTM) and alter the function of protein. 4-HNE was reported to modify and inhibit PTEN (a well-known tumor suppressor deleted or mutated in various types of cancer) activity in the hepatocytes (Shearn et al., 2011). Modification of AKT2 by 4-HNE also inhibits insulin-dependent AKT signaling (Shearn et al., 2011). The phosphorylation of DAXX at Ser668 and Ser671, triggering nuclear export of DAXX, is enhanced by the modification of 4-HNE (Sharma et al., 2008). Furthermore, 4-HNE causes induction, phosphorylation, and nuclear accumulation of p53 (Patrick et al., 2005). SIRT3 and SIRT1 were reported to be covalently modified by 4-HNE (Fritz et al., 2011). This modification by 4-HNE can inhibit the catalytic activity of SIRT3 and SIRT1.

HIF1 is a heterodimeric basic helix-loop-helix structure composed of an alpha (HIF-1α) and a beta (HIF-1β) subunit (Ke and Costa, 2006). HIF-1 functions as a major regulator of cellular and systemic homeostatic response to hypoxia by activating transcription of many genes that are involved in energy metabolism, angiogenesis, cell growth, survival, invasion or migration (Pugh and Ratcliffe, 2003). HIF-1α is degraded by the ubiquitin-proteasome pathway by binding of the von Hippel-Lindau tumor suppressor protein (pVHL) to the oxygen-dependent degradation domain (ODD) under the normoxic conditions (Pugh and Ratcliffe, 2003). The activity of the transcription factor for HIF-1α is also known to be increased by hypoxia, but various factors can also activate HIF-1α even under normoxic conditions (Lopez-Lazaro, 2006). VEGF, a well-known target gene of HIF-1α, is one of the major inducers of tumor angiogenesis (Kim et al., 2006). Moreover, VEGF also promotes the cell growth, survival, invasion or migration of cancer cells and the endothelial cells (Lee et al., 2007). Actually, 4-HNE was reported to stimulate the growth of HeLa cells (Sovic et al., 2001). Here, 4-HNE was proved to stimulate the cell growth of breast cancer cells. Furthermore, we first found that 4-HNE could promote the invasion and angiogenesis of breast cancer cells. We also found that 4-HNE increased angiogenesis and the levels of expression of VEGF of breast cancer cell. Although, the overexpression of VEGF in breast cancer can reflect the potential of angiogenesis (including blood flow, blood volume and permeability surface) in the tumor tissue (Xu et al., 2013), further research is required to determine whether the levels of 4-HNE was correlated with VEGF expression and angiogenesis in breast cancer.

HIF-1α protein is rapidly degraded and difficult to detect from cell lysates under normoxic conditions. However, HIF-1α is detectable from isolated nuclei under normoxic conditions (Finley et al., 2011). This provides the possibility to study HIF-1α protein under normoxic conditions. During the treatment with 4-HNE of breast cancer cells, increased levels HIF-1α were observed. Multidrug resistance (MDR) is a major cause of cancer chemotherapy failure (Szakacs et al., 2006). Hypoxia was reported to induce multidrug resistance of cancer cells through up-regulated HIF-1α (Li et al., 2013). Here, we found that 4-HNE, an oxidation products of polyunsaturated fatty acids, also up-regulated the levels of HIF-1α. Thus, 4-HNE may be involved in the multidrug resistance of cancer chemotherapy.

Sirtuin-3 (SIRT3), a major mitochondria NAD+-dependent deacetylase, was reported to destabilize HIF-1α (Chen et al., 2014). The activity of SIRT3 can be inhibited by 4-HNE (Fritz et al., 2011), we then investigated whether 4-HNE affects the levels of HIF-1α through SIRT3. Knockdown of SIRT3 increased the levels of HIF-1α and VEGF in MDA-MB-231 cells. Importantly, 4-HNE could not significantly affect the levels of HIF-1α and VEGF in SIRT3-knockdown MDA-MB-231 cells. These results support a role for 4-HNE in the regulation of the cell growth, invasion and VEGF expression through inhibiting SIRT3 induced destabilization of HIF-1α. Hypoxia induced multidrug resistance was reported to

depends on HIF-1α.

Acknowledgements

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