Inhibitory Effects of Onion (Allium cepa L.) Extract on Proliferation of Cancer Cells and Adipocytes via Inhibiting Fatty Acid Synthase

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Abstract

Onions (Allium cepa L.) are widely used in the food industry for its nutritional and aromatic properties. Our studies showed that ethyl acetate extract of onion (EEO) had potent inhibitory effects on animal fatty acid synthase (FAS), and could induce apoptosis in FAS over-expressing human breast cancer MDA-MB-231 cells. Furthermore, this apoptosis was accompanied by reduction of intracellular FAS activity and could be rescued by 25 mM or 50 mM exogenous palmitic acids, the final product of FAS catalyzed synthesis. These results suggest that the apoptosis induced by EEO occurs via inhibition of FAS. We also found that EEO could suppress lipid accumulation during the differentiation of 3T3-L1 adipocytes, which was also related to its inhibition of intracellular FAS activity. Since obesity is closely related to breast cancer and obese patients are at elevated risk of developing various cancers, these findings suggested that onion might be useful for preventing obesity-related malignancy.

Keywords: Fatty acid synthase - onion - MDA-MB-231 cells - 3T3-L1 adipocytes - apoptosis

Introduction

Allium vegetables have been employed for a long time in traditional medical practice to treat a variety of diseases. Onion (Allium cepa L.), one of the representative Allium vegetables, has been used for centuries for its pungency, flavoring value, and medicinal properties. The bulb of onion is used medicinally and has been consumed as seasoning food for many centuries (Sengupta et al., 2004). Phytochemical research has proved that onion is rich in flavonols and organosulfur compounds, which have exhibited tumor inhibitory properties in laboratory studies (Virtanen and Matikkala, 1976; Block, 1985; Arabbi et al., 2004).

High intakes of onions have been directly associated with the management and prevention of obesity (Lee et al., 2008). Onion extract supplementation reduced the amounts of mesenteric fat and influenced the adipokine production at a transcriptional level in the high-fat induced obese animal model (Kim et al., 2012). Onion extracts reduced blood low-density lipoprotein cholesterol and increased high-density lipoprotein cholesterol of high-fat feeding Sprague-Dawley rats (Lee et al., 2012). Some researchers investigated the anti-obesity activity of a 70% ethanol extract from Allium fistulosum L. (commonly known as Chinese onion) in high-fat induced obese mice. The extracts significantly reduced body weight, white adipose tissue weight and adipocyte size of the treated mice compared to high-fat induced control mice (Sung et al., 2011).

In recent years, extensive research has focused on the anticarcinogenic potential of onion and its constituents. Onion and its organosulfur constituents are studied extensively for their chemopreventive potential against cancer (Le Bon and Siess, 2000). In a French epidemiological study, higher onion intake was correlated with lower risk of breast cancer (Challier et al., 1998). Furthermore, many experimental studies have demonstrated that organosulfur compounds and Allium extracts have inhibitory effects on carcinogenesis in animals.

Numerous studies suggest that obesity and excess weight can play a prominent role in the incidence and progression of various cancers (Prieto-Hontoria et al., 2011). Obesity has been associated with a higher risk and a poor prognosis of breast cancer in multiple studies (Tartter et al., 1981; van den Brandt et al., 2000; Lahmann et al., 2004; Croenke et al., 2005; Caan et al., 2008; Dawood et al., 2008). According to an American Cancer Society study, obesity can increase the mortality of patients with cancers of the breast, kidneys, liver, etc. (Calle et al., 2003).

Fatty acid synthase (FAS, EC 2.3.1.85), a metabolic enzyme that catalyzes the synthesis of long-chain fatty acids, is expressed at high levels in adipose tissues and a variety of human cancers, including breast (Alo et al., 1996; Milgraum et al., 1997), prostate (Epstein et al., 1995; Swinnen et al., 2002), endometrium (Pizer et al., 1998), ovary (Gansler et al., 1997), colon (Rashid et al., 1997), lung (Visca et al., 2004; Orita et al., 2008),
and pancreas cancer (Alo et al., 2007). Although the mechanism of FAS over expression is unknown, it seems to be up-regulated during the early stages of tumorigenesis (Rashid et al., 1997; Kuhajda, 2000). This differential expression between normal and neoplastic tissues makes FAS a potential diagnostic tumor marker (Walter et al., 2009). The fatty-acid synthesis as a target pathway for chemotherapy has been identified by the studies with FAS inhibitors (Kuhajda, 2006).

Onion is well-known for its benefits to weight control and cancer prevention. However, there is no “cross-talk” between its anti-obesity and cancer prevention activities. This study therefore aimed to examine whether the anti-cancer activity of onion is related to its anti-obesity effect. Our previous study has found that crude extracts of nine species of Allium vegetables including onion showed high inhibitory activity on FAS (Sun et al., 2009). In the present study, we further investigated the inhibitory effect of onion extracts on FAS over-expressed cancer cells and adipocytes.

Materials and Methods

Reagents and antibodies

Acetyl-CoA, Malonyl-CoA, Insulin (INS), Dexamethasone (DEX), Oil red O, NADPH, MTT dye, Hoechst-33258, 3-isobutyl-1-methylxanthine (IBMX), palmitic acid, EDTA and DTT were purchased from Sigma. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were purchased from GIBCO BRL. Antibodies against FAS and GAPDH were purchased from Cell Signaling Technologies.

Preparation of plant extracts

Dried bulb of onion (467.5 g) was extracted successively with petroleum (3 ×1500 ml, 30 min) and 50% ethanol (3 × 1500 ml, 30 min) at room temperature, to give 0.81g and 80.0 g of extract, respectively. Ethanol extract (80.0 g) was dissolved in 500 ml of water and then partitioned successively at room temperature with ethyl acetate (3 × 500 ml) to give 5.92 g of ethyl acetate extracts (EEO). The aqueous phase contained 72.69 g of residue. To make a 5 mg/ml stock solution for long storage at −20 °C.

Cell culture

Mouse 3T3-L1 preadipocytes and human breast cancer MDA-MB-231 cells were both obtained from the Cell Culture Center of the Institute of Basic Medical Sciences (IBMS), Chinese Academy of Medical Sciences (Beijing, China). 3T3-L1 preadipocytes were incubated in DMEM (low glucose) and MDA-MB-231 cells in DMEM (high glucose), 10% fetal bovine calf serum, 100 U/ml penicillin-streptomycin.

Cell lysis and immunoblotting

Cells were lysed as previously described (Uddin et al., 2004). Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore, Billerica, MA). Immunoblotting was done with different antibodies and visualized by the enhanced chemiluminescence (Amersham, Piscataway, NJ) method.

Differentiation of 3T3-L1 preadipocytes

Two days after confluence (Day 0, d0), the cell differentiation was induced in DMEM containing 10% FBS, 0.5 mM IBMX, 1 mM DEX, and 1.7 mM INS for two days (Day 2, d2, early stage), and another two days (Day 4, d4, middle stage) in DMEM containing 10% FBS and 1.7 mM INS. Cells were maintained in DMEM containing 10% FBS every other day for the following four days (Day 6–8, d6–8, late stage). All experiments were strictly carried out on cell lines between 5–20 passages. All cell culture condition was 37 °C in a humidified 5% CO2 incubator.

MTT assay

3T3-L1 preadipocytes were seeded in 96-well plate (5 ×103 cells/well) and then treated with EEO in different concentrations for the next 24 h. Thereafter, 20 ml of MTT solution (5 mg MTT/ml in PBS) was added into each well of a microtiter plate and incubated for 4 h at 37 °C. The resultant formazan product was dissolved in 200 ml DMSO/well, and its concentration was measured at 492 nm by a microplate spectrophotometer (Multiskan, MK3).

Hoechst-33258 staining

3T3-L1 preadipocytes were seeded in 12-well culture dishes (5 ×103 cells/well). After experimental treatment, cells were washed twice with PBS, and stained with Hoechst-33258 (5 mg/ml) for 5 min in the dark, and then followed by extensive washes. Nuclear staining was examined under the fluorescence microscope and images were captured using ImagePro Plus software (MediaCybernetics, Silver spring, MD).

Intracellular fatty acids assay

The amount of intracellular fatty acid was determined by Fatty Acid Assay Kit. Briefly, 3T3-L1 preadipocytes were seeded in 100 mm cell culture dishes. After experimental treatment, cells were washed twice with PBS, and then extracted by homogenization with 200 ml of chloroform-Triton X-100 (1% Triton X-100 in PBS) in a microhomogenizer. Then spun the extract 5–10 min at top speed in a microcentrifuge. Collected organic phase (lower phase), air dried at 50 °C to remove chloroform. Vacuum dried 30 min to remove trace chloroform. Dissolved the dried lipids in 200 ml of Fatty Acid Assay Buffer by vortexing extensively for 5 min. Added 2 ml ACS Reagent into all sample wells and incubated the reaction at 37 °C for 30 min. Added 50 ml of the Reaction Mix containing 44 ml Assay Buffer, 2 ml Fatty Acid Probe, 2 ml Enzyme Mix and 2 ml Enhancer to the test samples. Incubated the reaction for 30 min at 37 °C, protected from light. The colorimetric assay was measured at 570 nm by a microplate spectrophotometer.

Oil Red O staining

To investigate the effects of EEO on lipid accumulation in 3T3-L1 preadipocytes, the cells were differentiated
The fast-binding inhibition was determined by adding FAS inhibition assays

The preparation, storage and use of FAS isolated from chicken liver were performed as described previously (Soulié et al., 1984). The final purified enzyme was determined to be homogeneous by polyacrylamide gel electrophoresis (PAGE) analysis in the presence and absence of SDS. FAS activity was determined by monitoring the decrease in absorbency at 340 nm resulting from the oxidation of NADPH using an Amersham Pharmacia Ultraspec 4300 pro UV-Vis spectrophotometer at 37 °C. The assay mixture contained 100 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 2.5 μM acetyl-CoA, 10 μM malonyl-CoA, 32 μM NADPH, and 10 μg chicken liver FAS in a total volume of 2.0 ml (Tian et al., 2004). After cells were harvested, pelleted by centrifugation, resuspended in cold assay buffer (100 mM potassium phosphate buffer, 1 mM EDTA, 0.6 mM PMSF and 1 mM dithiothreitol, pH 7.0) ultrasonically disrupted and centrifuged at 12000 rpm for 30 min at 4 °C, the supernatant was collected for the overall reaction assay. 25 ml supernatant was added into the reaction mix contained 25 mM KH2PO4-K2HPO4 buffer, 0.25 mM EDTA, 0.25 mM dithiothreitol, 30 mM acetyl-CoA, 100 mM malonyl-CoA, 350 mM NADPH (pH 7.0) in a total volume of 200 ml. Protein content in the supernatant was determined using a bicinchoninic acid (BCA) assay (Pierce) and results were expressed as the specific activity in mg of FAS at the same protein concentration.

Results

Inhibitory effect of EEO on FAS in vitro

Four fractions (petroleum ether, ethanol, ethyl acetate and water) of onion were tested to determine their inhibitory activities on FAS. It indicated that EEO showed the highest activity to inhibit FAS with IC50 of 2.4±0.3 μg/ml (Table 1). So EEO was chosen for the further cell level research.

Expression of FAS in MDA-MB-231 cells and 3T3-L1 adipocytes

FAS levels of MDA-MB-231 and 3T3-L1 cells were determined by Western blotting analysis with specific antibodies against the targeted proteins and GAPDH as control. As shown in Figure 1A, both the two cells expressed FAS in high levels. And FAS was higher expressed in MDA-MB-231 cells than in 3T3-L1 cells.

EEO inhibited intracellular FAS activity in MDA-MB-231 cells and 3T3-L1 adipocytes

Compared with control, EEO significantly inhibited the intracellular FAS activity with a dose-dependent manner. As shown in Figure 2A, after treated with EEO at the concentrations of 25 μg/ml and 50 μg/ml for 24 h, the intracellular FAS activities of MDA-MB-231 cells were reduced to 56.3% and 32.1%, separately, compared with control. Figure 3A showed that 20, 40 and 60 μg/ml EEO inhibited 37.7%, 69.8% and 73.6% intracellular FAS activities of MDA-MB-231 cells and 3T3-L1 adipocytes, respectively.
Differentiated adipocytes were stained in red, while a lot of lipids, which can be stained red by Oil Red O.

3.6. EEO reduced lipid accumulation in 3T3-L1 adipocytes. The intracellular lipid content was measured by Oil Red O staining. 3T3-L1 preadipocytes were treated with EEO at the indicated concentrations for 24 h. The intracellular lipid content was determined by Oil Red O staining. 3T3-L1 preadipocytes were treated with EEO at the indicated concentrations for 24 h. (A) The intracellular lipid content was measured by Oil Red O staining. 3T3-L1 preadipocytes were treated with EEO at the indicated concentrations for 24 h. (B) The intracellular lipid content was measured by Oil Red O staining. 3T3-L1 preadipocytes were treated with EEO at the indicated concentrations for 24 h. (C) The effect of exogenous palmitic acid on 3T3-L1 cells. The relative cell viability was determined by MTT. Data were expressed as means ± S.D. (n = 3). ** p<0.01 significantly different from respective control.

EEO reduced intracellular fatty acids in MDA-MB-231 cells

The amount of intracellular fatty acids in MDA-MB-231 cells treated with 25 μg/ml and 50 μg/ml EEO were measured. Results (Figure 2B) showed that the level of intracellular fatty acids in treated cells decreased by 30.6% and 40.8%, compared with the control (2.06 μM). 3.6. EEO reduced lipid accumulation in 3T3-L1 adipocytes. Oil Red O staining to visualize lipid after 3T3-L1 cells were incubated with EEO. Mature adipocytes store a lot of lipids, which can be stained red by Oil Red O. Differentiated adipocytes were stained in red, while undifferentiated cells were not. The representative images demonstrated that the 3T3-L1 cells treated with EEO obviously reduced their lipid accumulation, since they were less stained than differentiated cells (Figure 3B).

Palmitic acid rescued cells apoptosis induced by EEO

To confirm that the cell apoptosis induced by EEO was related to FAS inhibition, MDA-MB-231 and 3T3-L1 cells were exposed for 24 h to different concentrations of EEO (0 μg/ml, 25 μg/ml and 50 μg/ml) in presence of exogenous palmitic acid (0 μM, 25 μM, 50 μM), the end product of FAS reaction. Palmitic acid reduced the cytotoxic effects of EEO (0 μg/ml, 25 μg/ml and 50 μg/ml) for 24 h, apoptosis of MDA-MB-231 cells was demonstrated by Hoechst-33258 staining, revealed cell membrane permeability increasement and nuclear condensation (Figure 2C).

Discussion

Obesity is a growing health problem in both developed nations and some developing countries like China. It is linked with several health disorders such as hypertension, cardiovascular diseases, type 2 diabetes and certain cancers. Obesity-related cancers of the breast, prostate and colon are the leading cancers in the industrialized countries.

Recent emerging epidemiological data further reveal that obesity is also associated with poor prognosis in patients with breast cancer. It was reported that 48% of breast cancers are diagnosed among obese women and...
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that 23% of these breast cancers are attributable to obesity (Colditz, 1992). Excess adipose tissue leads to metabolic changes such as reduced high-density lipoprotein cholesterol, elevated triglycerides, hypertension, and insulin resistance (Khondekar et al., 2011). Obesity-induced esophageal reflux, hypertension, insulin resistance, and hormone alterations could contribute to an increased risk in esophageal, kidney, colorectal, and breast cancers, respectively (Wang and Dubois, 2012).

Although studies are inconclusive at this time, preliminary evidence suggests that onion may play a role in decreasing the risk of obesity-related cancers. Several epidemiological studies have shown a reduction of the risk of different cancers via consumption of onion (Hsing et al., 2002; Setiawan et al., 2005; Galeone et al., 2006). The supporting mechanisms including inhibition of carcinogenic formation, modulation of carcinogen metabolism, inhibition of mutagenesis and genotoxicity, inhibition of cell proliferation and increase of apoptosis, inhibition of angiogenesis, and immune system enhancement. These mechanisms, however, didn't include the fatty acid synthesis pathway.

FAS may play a junction role between obesity and cancer risk: on the one hand, increased de novo lipogenesis contributes to increased fat mass (Lenhard, 2011), on the other hand, high expression of FAS in human breast, colorectal, prostate, endometrial, ovary, and thyroid cancers supports the hypothesis that FAS is essential for generating cell membranes during tumor cell proliferation (Menendez and Lupu, 2007). In this study, we found that EEO not only showed a high inhibitory activity on FAS (Table 1), but also influenced the normal cell cycle of both cancer and fat cells (Figure 1B, 1C). These results suggested that FAS, the target for cancer and obesity, was also an acting site of EEO.

The reduction of intracellular FAS activity (Figure 2A) and fatty acids amount (Figure 2B) in MDA-MB-231 cells revealed that FAS was the target where EEO acted on. The activity of FAS in cells was important to the amount of intracellular fatty acids because the FAS plays the key role of de novo fatty acid biosynthesis. It was reported that most normal human tissues, except liver and adipose tissue, exhibit low levels of FAS expression. However, the expression of FAS is surprisingly high in a variety of human cancers, such as cancer of the breast, prostate, ovary and lung (Shurbaei et al., 1996; Gansler et al., 1997; Alo et al., 1999; Kuhajda, 2000; Visca et al., 2004). Figure 1A showed that high levels of FAS expression in MDA-MB-231 and 3T3-L1 cells. Interestingly, the level of FAS expression had close relation to the cell viabilities treated with EEO. EEO reduced the viabilities of both MDA-MB-231 and 3T3-L1 cells, but the IC_{50} of 3T3-L1 cells was higher than that of MDA-MB-231 cells. It revealed that EEO had lower toxicity on normal 3T3-L1 cells (relatively low level of FAS expression) than on cancer MDA-MB-231 cells. So EEO would prevent cancer and have no cytotoxic to normal cells in an appropriate concentration. This result provided the possibility for the utility of onions to explore effective drugs for preventing and treating cancer. In this study, EEO was obtained from dried bulb, the edible parts, of onions. Considering that onion has been used as a food source since prehistoric times, we believe that high intake of EEO is safe.

Like reported FAS inhibitors such as C75 and cerulenin (Kuhajda, 2006), EEO could induce apoptosis in cancer cells (Figure 2C). Previous studies suggested that the mechanism of the apoptosis through inhibiting FAS could be explained by the accumulation of malonyl-CoA, which was likely to trigger cancer cell death and induce apoptosis (Pizer et al., 2000; Zhou et al., 2003). It was presented that some signal pathways in cell apoptosis had close relation to the inhibition of FAS, which might be an ideal reason for why FAS inhibitors could treat cancer cells.

Some research, however, showed that palmitic acid, the final product of FAS, was important for the formation of cell membrane. So the reduction of synthesized palmitic acid might be another reason to explain why the inhibition of FAS could induce apoptosis. In this study, we found that the EEO treated cell viabilities could be rescued by adding exogenous palmitic acid, which provided strong evidence for the cell membrane thesis (Figure 2D and Figure 3C).

In mature adipocytes, the accumulation of fatty acids significantly reduced after adding EEO (Figure 3B), along with the declined FAS activity (Figure 3A). Since the size of fat cells depended on the lipid accumulation, adipose tissue mass could be reduced by preventing lipid accumulation inside adipocytes. Although low concentration (20 µg/ml) of EEO failed to influence the viability of 3T3-L1 cells, it did reduce the intracellular lipid accumulation. It revealed that EEO had the application potential in preventing and/or treating obesity. Obesity-related health problems consume about 7% of the US health-care budget in direct medical costs and about 1-5% in Europe (Pan et al., 2004). Obesity represents a major avoidable contribution to the costs of illness in the developed countries. Onion, as a worldwide edible plant, is cheap and convenient to get. If EEO is developed into a new drug for treating obesity-related cancers, the money saved will be considerable.

In conclusion, EEO could induce MDA-MB-231 cells apoptosis and reduce intracellular lipid accumulation of 3T3-L1 adipocytes via inhibiting intracellular FAS activity. The result of palmitic acid rescued EEO induced apoptosis in cancer cells confirmed that the apoptosis was related to inhibition of FAS. Since EEO showed potent inhibition on the proliferation of MDA-MB-231 and 3T3-L1 cells, it had the potential to be developed into a drug candidate for treating obesity-related cancers.

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