Arachidonic acid inhibits the production of angiotensin-converting enzyme in human primary adipocytes via a NF-κB-dependent pathway

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Background: The modulating mechanism of fatty acids on angiotensin-converting enzyme production (ACE) in human adipocytes is still elusive. Diet-induced regulation of the renin angiotensin system is thought to be involved in obesity and hypertension, and several previous studies have used mouse cell lines such as 3T3-L1 to investigate this. This study was carried out in human subcutaneous adipocytes for better understanding of the mechanism.

Methods: Human adipose stem cells were isolated from subcutaneous adipose tissue biopsies collected from four patients during bariatric surgery and differentiated into mature adipocytes. The mRNA expression and the activity of ACE were measured under different stimuli in cell cultures.

Results: Arachidonic acid (AA) decreased ACE mRNA expression and ACE activity in a dose-dependent manner while palmitic acid had no effect. The decrease of ACE by 100 μM AA was reversed by the addition of 5 μM nuclear factor-κB (NF-κB) inhibitor. Furthermore, when the production of 20-hydroxyeicosatetraenoic acid, a metabolite of AA, was stopped by the specific inhibitor HET0016 (10 μM) in the culture media, the effect of AA was blocked.

Conclusions: This study indicated that AA can decrease the expression and activity of ACE in cultured human adipocytes, via an inflammatory NF-κB-dependent pathway. Blocking 20-hydroxyeicosatetraenoic acid attenuated the ACE-decreasing effects of AA.

Keywords: Fatty acids; human adipose stem cells; 20-hydroxyeicosatetraenoic acid

Introduction

The World Health Organization defines hypertension as a pathological status in which "the blood vessels have persistently raised pressure". The deleterious consequence of hypertension is the damage it causes to affected organs, leading to an increased risk of nephropathy, vasculopathy, cardiovascular, or cerebrovascular events (1). Pathophysiologically, risk factors for hypertension include obesity, smoking, family history, high salt food intake and an overall unhealthy lifestyle and diet. Among the currently available medications for treating hypertension, angiotensin-converting enzyme (ACE) inhibitors and

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angiotensin receptor blockers are recommended as first choices (2).

ACE is a membrane-bound protein that indirectly increases blood pressure by converting angiotensin I to the active angiotensin II, a vasoconstrictor. ACE is most commonly found in endothelial and epithelial cells. ACE, in association with the other renin angiotensin system (RAS) components, was reported to be expressed in adipose tissue and cultured adipocytes (3). It is estimated that nearly 30% of the circulating angiotensin is produced by adipose tissue (4,5).

Obesity is a well-known risk factor for hypertension, and obesity-related hypertension has been ascribed to an over-activated RAS (6). The activity of the adipose tissue RAS contributes to systemic high blood pressure and chronic inflammation in adipose tissue (7,8). We recently reported that a high fat diet induced an increase in ACE expression in individuals with a genetic susceptibility and was associated with increased blood pressure and elevated blood glucose in a clinical human study (9,10).

Arachidonic acid (AA) is a long-chain omega-6 (n-6) polyunsaturated fatty acid (PUFA) which is obtained from food or by stepwise desaturation and chain elongation of linoleic acid (LA), an essential fatty acid (EFA) (11). Marine fish, animal tissues and eggs are the major supply of AA, algae and some plants were also reported as potential sources of AA (12-14). Because of the lack of biosynthesis enzymes, humans and other mammals cannot directly synthesize AA. Therefore, they have to obtain enough AA via food or dietary intake of its precursors (15). AA is the main component of membrane lipids, and mainly metabolized by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450). AA could be converted into various metabolites such as inflammatory lipids or eicosanoids (16). AA can be converted into prostaglandins (PGs) and thromboxanes (TXs) by the cyclooxygenase (COX) pathway, the metabolites of this pathway play an important role in vessel tone regulation, mediating platelet aggregation and immune response (17-19). Through the lipoxygenase (LOX) pathway, AA can be metabolized into leukotrienes (LTs) and lipoxins (LXs). Lipoxins mainly exhibit anti-inflammatory properties (20). Besides, epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs) are generated through the cytochrome P450 (CYP450) pathway. And these compounds play a main part in the modulating of kidney, lung, and cardiovascular function (21). 20-HETE is considered a potent vasoconstrictor by various means.

However, it shows a potential conflicting role in regulating renal hypertension (22).

In human endothelial cells, angiotensin-converting enzyme (ACE) mRNA expression and ACE activity are increased via nuclear factor-κB (NF-κB) pathway (23), many genes involved in vascular physiopathology are regulated by NF-κB. NF-κB activation was diminished *in vivo* in injured vessels by angiotensin-converting enzyme inhibitors (24). However, the mechanism involved in modulating ACE expression in adipose tissue remains elusive, and this study therefore aimed to investigate the effects of fatty acids on ACE expression and activity in human adipocytes.

In this study, primary human adipocytes were differentiated and cultured in the presence of unsaturated and saturated fatty acids. The ACE expression in the cells and ACE activity in the culture media were measured. The potential relationship between ACE and fatty acids was assessed via artificial perturbation of the NF-κB inflammatory pathway.

We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/atm-20-7514).

Methods

Cell isolation and culture

Human adipose stem cells (ASCs) were isolated from four patients who were participants in the LEMBAS study (diet-induced changes in Liver fat and Energy Metabolism prior to Bariatric Surgery) (25). This clinical intervention study was approved by the Ethics Committee of the Charité University Medicine in Berlin (Application no.EA4/006/15) in accordance with the Declaration of Helsinki and registered at www.drks.de (DRKS00009509). All four study participants were morbidly obese with a body mass index (BMI) above 40 kg/m². Their subcutaneous adipose tissue biopsies were collected during the Roux-en-Y gastric bypass operation. Informed consent was acquired from each individual prior to surgery.

ASCs were isolated following the method modified from Lee *et al.* (26). Briefly, 5 mg (2–3 mm³) of adipose tissue (AT) was minced into small pieces. The minced tissue was rinsed by phosphate buffer saline (PBS), passed through a 250 μm funnel-shaped mesh and then digested in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Thermo Fisher Scientific, Waltham, MA, USA) containing collagenase I (1 mg/mL) (Sigma Aldrich

Chemie, Steinheim, Germany) at 37 ℃ for 2 hours with constant shaking (100 rpm). Subsequently, the digested AT was filtered through a 250 μm mesh and the filtrate, which contains the ASCs, was collected and centrifuged at 500 ×g for 10 minutes. Afterwards, the upper fat layer and the middle medium layer above the cell pellet in the tube were removed. The cells were resuspended after adding red blood cell (RBC) lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) to diminish the RBC cells for better attachment. After centrifugation at 500 ×g for 5 minutes, the cells were resuspended again with Preadipocyte Growth Medium containing Preadipocyte Growth Medium SupplementMix (Promo Cell, Heidelberg, Germany) and 1% penicillin/streptomycin (Sigma Aldrich Chemie, Steinheim, Germany) and cultured in a 15 mL flask. Cells were maintained in a 37 ℃ incubator with 5% CO_2 and refed every 2–3 days until 80–90% confluence, and then cultured for 14 days in 12-well plates in differentiation medium which contained DMEM/F12, 1% penicillin/streptomycin, 500 μM IBMX, 25 nM dexamethasone, 0.2 nM triiodothyronine (T3), 33 μM D-biotin, 15 mM pantothenate, 20 nM human insulin, 0.01 mg/mL transferrin, and 2 μM rosiglitazone (all chemicals were purchased from Sigma Aldrich Chemie, Steinheim, Germany). Differentiation of the mature adipocytes was confirmed by oil red O stain. NF-κB inhibitor BAY117082 and 20-hydroxyeicosatetraenoic acid (20-HETE) production inhibitor HET0016 (27) were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Oil red O staining

After 14 days of adipogenesis, differentiated adipocytes were washed with sterile PBS and fixed in 10% formaldehyde (Sigma Aldrich Chemie, Steinheim, Germany) for 1 hour of incubation at room temperature. Oil red O was prepared by mixing oil red O stock solution with deionized water in the ratio of 3:2. Thereafter, adipocytes were gently rinsed with water, and 60% isopropanol (Sigma Aldrich Chemie, Steinheim, Germany) was added for 5 minutes. Finally, the adipogenic cultures were incubated in oil red O for 10 minutes, rinsed with tap water until the water ran clear, and analyzed under the microscope.

Free fatty acids preparation and treatment

Palmitate (Sigma Aldrich Chemie, Steinheim, Germany) was dissolved completely in ethanol at 70 ℃ and then complexed with fatty acid-free bovine serum albumin (BSA) (Sigma Aldrich Chemie, Steinheim, Germany) at 55 ℃ for 10 minutes yielding the final palmitic acid (PA) stock solution of 5 mM. The working solution of 500 μM (28-30) was prepared with DMEM before experiments.

Arachidonic acid (AA) (Cayman Chemical, Ann Arbor, Mi, USA) was complexed with fatty acid-free BSA. The working solutions of 50, 100, or 200 μM were prepared with DMEM before the experiments.

After differentiation for 7 days (approximately 80% differentiation as estimated by lipid droplets), the cell culture medium was changed to DMEM with 1% BSA and without fatty acids, or with PA 500, AA 50, 100, or 200 μM, for the purpose of determining the effects of saturated and unsaturated fatty acids. Subsequently, AA 100 μM was used in further experiments (see results section) and other research reports (31-33). To explore the effects of NF-κB pathway inhibition, adipocytes were pretreated with 5 μM BAY117082 for 1 hour and then treated with either DMEM + 0.1% BSA or DMEM + 0.1% BSA + 100 μM AA for 24 hours. In order to ascertain the effects of 20-HETE, adipocytes were pretreated with 10 μM HET0016, the specific inhibitor of 20-HETE (27), for 1 hour and then treated with either DMEM + 0.1% BSA or DMEM + 0.1% BSA + 100 μM AA for 24 hours.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted by using the Nucleospin® RNA II Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's protocol. cDNA was synthesized from 1 μg of RNA of each sample by using the high capacity cDNA reverse transcription kit (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). QRT-PCR was performed using the power SYBR Green PCR master mix (Applied Biosystems by Life Technologies, Carlsbad, CA, USA) and detected in triplicates with the ABI ViiA™7 Real time PCR system (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). The samples were normalized to a ribosomal protein large P0 (RPLP0). The primers synthesized by Thermo Fisher Scientific (Waltham, MA, USA) are shown in *Table 1*.

Assessment of ACE activity

ACE activity was determined in triplicate by measuring the fluorescence of the product generated by the specific

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Table 1 Primers used in the study

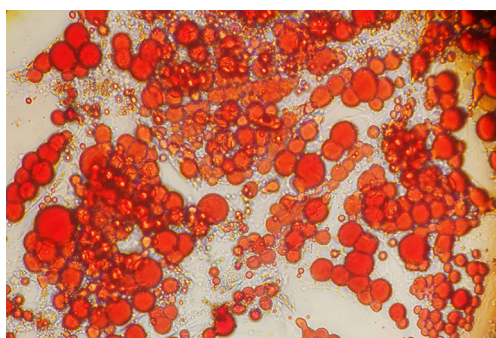| Gene	Forward	Reverse
RPLP0	5'-GCTTCCTGGAGGGTGTCC-3'	5'-GGACTCGTTTGTACCCGTTG-3'
ACE	5'-CAGAACACCACTATCAAGCGGA-3'	5'-CACGCTGTAGGTGGTTTCCAT-3'

ACE, angiotensin-converting enzyme.

Figure 1 The oil red O staining of differentiated cells photographed using a microscope (×100). Lipid droplets are stained in red.

substrate Abz-Gly-Phe (NO$_2$)-Pro (Bachem, Bubendorf, Switzerland, cat. no. M-1100). The protocol designed by Sentandreu *et al.* was followed (34). Briefly, 50 µL testing samples and standard solutions of eight different concentrations were added into a 96-well microplate. Captopril-inhibition and blank-contrast wells were also included. A 200 µL substrate working solution, which was mixed by diluting M1100 in 150 mM Tris-base buffer (pH 8.3) with 1.125 M NaCl, was added into each well to initiate the enzyme reaction. Fifty µL of samples were incubated in a final volume of 300 µL with 0.45 mM of specific substrate. The plate was read two times with a Tecan infinite M200 microplate reader at an excitation wavelength of 365 nm, and an emission wavelength of 425 nm. The first measurement was performed at room temperature when the reaction was initiated, and the second after incubating the solution at 37 ℃ for 30 minutes. ACE activity was determined by the fluorescence emission differences between the incubation periods.

Statistical analysis

Statistical data were processed using SPSS 20.0 (IBM SPSS, Chicago, IL, USA). Results are expressed as the mean ± SD and data were analyzed using student's *t*-test for unpaired samples or the one-way ANOVA for multiple comparisons among groups.

Results

Evaluation of the cultured cells

In order to ascertain the success of the adipocyte differentiation, oil red O staining was performed. The cultured cells were characterized with typical red oil-stained lipid drops distributed in the cytosol (*Figure 1*). At least 80% of the cells were fat cells according to the staining. Combined with some morphological features, the cultured cells qualified for the current study according to the optimal protocol (26).

ACE expression in the presence of different fatty acids

Two kinds of fatty acids were selected to stimulate the adipocytes. For the saturated fatty acids, PA was selected as the example. Neither the mRNA expression of ACE nor the ACE activity in the culture media were affected by the addition of PA (*Figure 2A,B*). On the contrary, in the presence of AA, an inflammation-related unsaturated fatty acid, both the mRNA expression of ACE and the ACE activity in the culture media decreased dose-dependently (*Figure 2C,D*). At the dose of 100 µM, AA inhibited the expression of ACE in adipocytes, which in turn resulted in lowered ACE activity in the media (*Figure 2E,F*). The concentration of 100 µM was chosen for the subsequent reversal experiments of AA effects.

Involvement of the NF-κB pathway in the suppression of ACE

In adipose tissue, free fatty acids have been found to activate the RAS through the NF-κB pathway (35). In light of this, we investigated whether inhibiting the NF-κB pathway could rescue the expression of ACE. Compared to control cells, culture of the adipocytes in the presence of the NF-κB
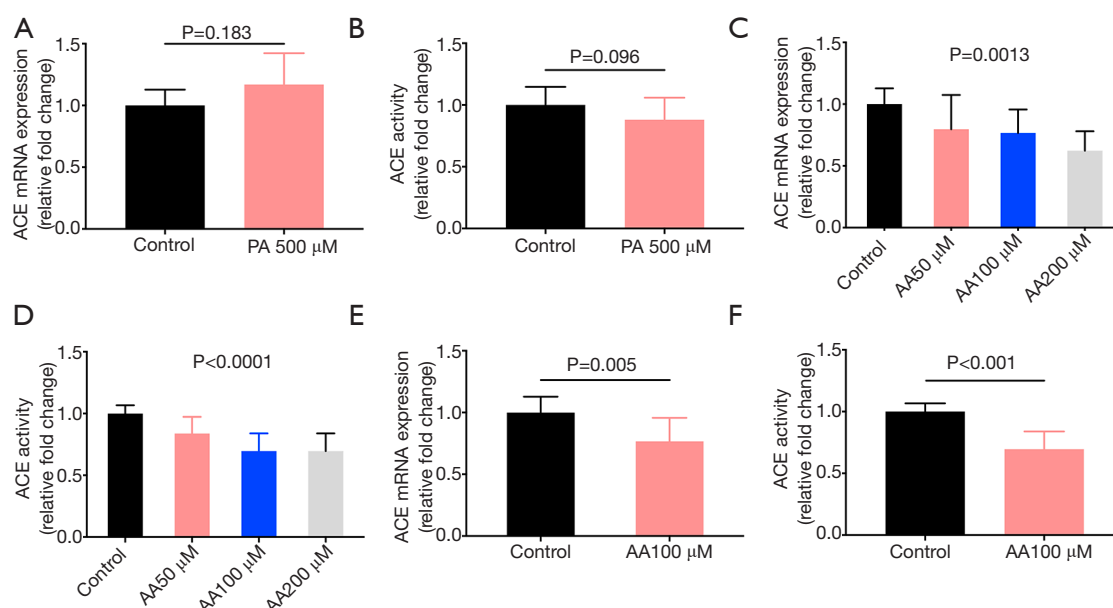
Figure 2 The angiotensin-converting enzyme (ACE) mRNA level in the cell extract and ACE activity in the culture media are measured under the stimuli of palmic acid (PA, 500 μM) or arachidonic acid (AA, 50, 100, 200 μM), respectively. (A) In the presence of 500 μM PA, the mRNA expression of ACE in the fat cell extract is not affected compared with controls (P=0.183). (B) Similar results are obtained for ACE activity in the culture media treated with the same PA concentration (P=0.096). (C) In the presence of AA at different concentrations, the expression of ACE mRNA in the culture media shows a statistical difference (P=0.0013). (D) ACE activity shows a similar response under the same condition (P<0.0001). As the dosage of AA is increased, the ACE expression and activity show trends of decline (n=3). The effect of 100 μM AA on ACE expression and ACE activity are displayed in part (E) and (F), respectively (P=0.005, P<0.001). All values are expressed as mean ± SD of 3 experiments, and each experiment is conducted in triplicate.

pathway inhibitor (BAY117082) increased ACE expression in the presence of AA (*Figure 3A*). In other words, the introduction of the NF-κB inhibitor completely blocked the effects of AA on ACE expression.

20-Hydroxy-5, 8, 11, 14-eicosatetraenoic acid (20-HETE) is a cytochrome P450 (CYP)-derived metabolite of AA which was shown to play a complex role in blood pressure and also blood sugar regulation (36,37). Activation of RAS was reported to be 20-HETE dependent (38,39). The effects from BAY117082 led us to postulate that the response to the NF-κB pathway inhibition might be related to 20-HETE metabolism of AA. We found that the inclusion of 20-HETE inhibitor HET0016 could compromise the effects of AA (*Figure 3B*). Activity of ACE in the media was also elevated compared to that of the AA-treated group (*Figure 3C*). When a mere 10 nM 20-HETE was added in the culture media of adipocytes, ACE activity was suppressed (*Figure 3D*).

Discussion

Dietary fat, in the form of fatty acids, has been found to exert different effects on preadipocyte differentiation and proliferation (40). Excessive saturated fatty acid intake and accumulation have been found to have detrimental effects on metabolism, resulting in insulin resistance, obesity, vascular disease, and hypertension. Obesity is known to activate the RAS pathway (41), and we previously reported that the introduction of a diet high in saturated fat elevates ACE even in normal weight healthy young study participants and leads to increases in blood pressure and blood glucose levels (9,10). The adipose tissue ACE gene expression was increased significantly in response to 6 weeks of a high fat diet, and energy consumption from total fat and saturated fat was 45% and 18%, respectively. We assumed that the high fat diet activated the renin-angiotensin system with increased ACE expression in human adipose tissue. In this study, PA stimulation seemed
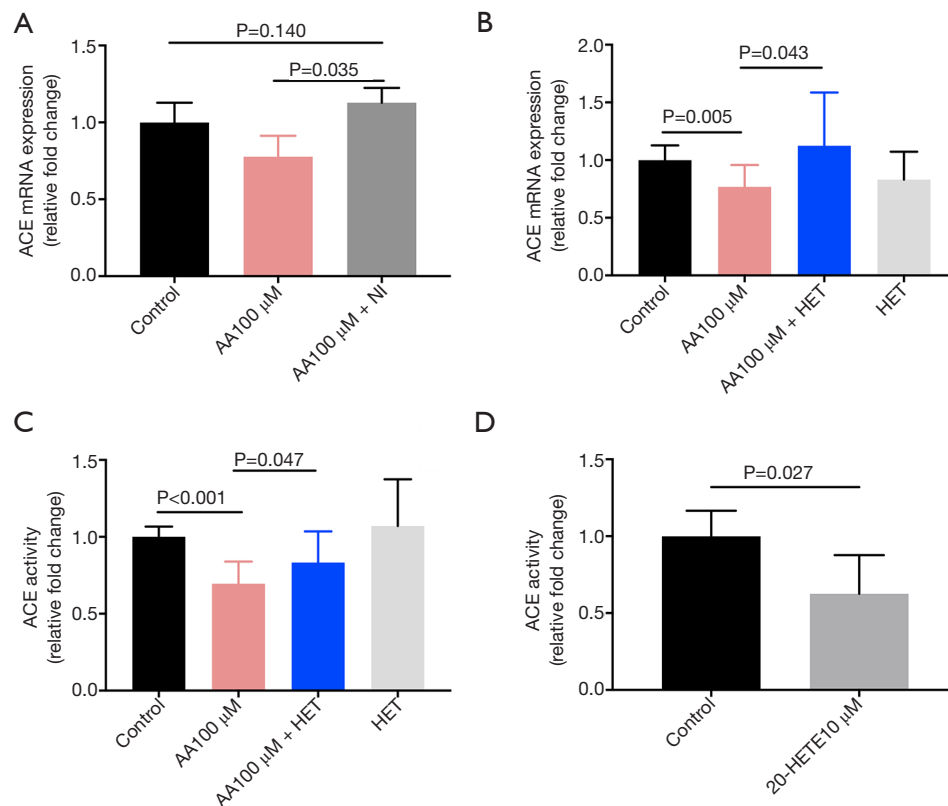
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Figure 3 The inhibitory effect of arachidonic acid on ACE production in human subcutaneous adipocytes was reversed by NF-ƙB inhibitor (NI) and 20-HETE inhibitor HET0016 (HET). (A) The reversal of the inhibition of angiotensin-converting enzyme (ACE) mRNA expression in the presence of 100 µM arachidonic acid (AA100) by the 5 µM NF-ƙB inhibitor BAY117082 (NI) in cultured adipocytes (n=3). (B) 10 µM 20-HETE inhibitor HET0016 (HET) competes off the inhibition of ACE mRNA expression by 100 µM AA (AA100) (n=3). (C) Reversal of the AA-induced (100 µM, AA100) inhibition of ACE activity by 10 µM HET0016 (HET) (n=3). (D) ACE activity is down-regulated when a mere 10 nM 20-HETE is added in the culture media of adipocytes. All values are expressed as mean ± SD of 3 experiments, and each experiment is conducted in triplicate.

to have no effect on the cultured adipocytes (*Figure 2*). This finding might be ascribed to the fact that saturated fatty acids need to be deposited in adipocytes in an esterified form. The data suggests that the increase in ACE seen in the presence of a high fat diet is not a direct consequence of the interaction of PA with fat cells.

AA is a typical n-6 polyunsaturated fatty acid found in daily food which can be generated by elongation and desaturation from linoleic acid, a predominant n-6 fatty acid in some plant oils, although increased intake may not necessarily result in increased levels of AA in humans (42). It was reported to stimulate preadipocyte differentiation in a cyclooxygenase-dependent manner (43). In this assay, AA was selected as the example of an unsaturated fatty acid to stimulate adipocytes. We found that in the presence of AA,

cultured adipocytes exhibited lowered expression of ACE (*Figure 2E*) which, in turn, resulted in the lowered ACE activity in the media and might be considered a protective effect (*Figure 2F*).

Free AA can be metabolized through four enzymatic and one non-enzymatic pathway. The enzymatic pathways include cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome p450 (CYP 450). The CYP 450 pathway involves two enzymes: CYP450 epoxygenase, and CYP450 ω-hydroxylase, giving rise to EETs and 20-HETE, respectively (31,44). Other metabolites include prostaglandins (PGs), prostacyclin, thromboxane (Tx), hydroperoxyeicosatetraenoic acid (HPETE), leukotrienes (LTs), lipoxins, hypoxins, and anandamide. The non-enzymatic pathway is important for the production of

isoprostanes and nitroeicosatetraenoic acid. Most of the AA metabolites are highly bioactive and involved in various crucial vital processes.

20-HETE induces oxidative stress, increases BMI, and is related to metabolic syndrome. In human mesenchymal stem cell-derived adipocytes, the expression levels of major 20-HETE synthases, CYP4F2, decreased during adipocyte differentiation which means lower levels of endogenous 20-HETE exist; however, exogenous administration of 20-HETE (0.1–1 μM) increased adipogenesis in a dose-dependent manner (45). 20-HETE has been proven to be one of the activators of NF-κB (46). In this study, we found that blocking the 20-HETE synthesis using HET0016 reversed the AA-suppressed ACE expression and ACE activity (*Figure 3A,B,C*), and subsequently, we found that adding a mere 10 nM 20-HETE into the culture media down-regulated the ACE activity of unpretreated adipocytes (*Figure 3D*). Thus, we postulate that AA exerts at least part of its ACE-suppressing effect through its metabolite 20-HETE, and inhibiting 20-HETE synthesis can reverse the ACE-suppressing effect. This is not in accordance with a previous report which reported that 20-HETE activates ACE expression through the NF-κB pathway (39). Thus, in this study, a NF-κB inhibitor was also employed to block the activation of this pathway. Clearly, the decreased expression of ACE was also reversed by this perturbation (*Figure 3*).

Notably, our findings have been obtained in cultured human primary adipocytes, therefore differences might be due to tissue and cell type specificities. Human preadipocytes have been shown no differences between DM and non–diabetes mellitus (NDM) patients in accumulating cytoplasmic lipid and upregulating expression of adipogenic genes (47). We can not exclude that the morbid patients undergo epigenetic changes due to metabolic conditions. On the other hand, we suppose that the effects of fatty acids on ACE would be more expressed in adipocytes from obese patients and patients with T2D. Due to the high costs and complexity of this kind of cell culture, ASCs of 3–4 patients are usually used to investigate the pathophysiological mechanisms (48).

Previous reports utilized endothelial cells or adipocytes from other tissues. To our knowledge, this is the first use of adipocytes originating from human subcutaneous stem cells to evaluate the effects of AA relevant to the RAS. In addition, the ASCs were isolated by biopsies from four morbidly obese patients who each had a BMI above 40 kg/m^2, potentially resulting in an abnormal cell state.

These ASCs may also be influenced by obesity-related sources in terms of differentiation and physiological function.

Even for adipocytes, the regulation of RAS was reported to be depot specific. For example, according to some reports, angiotensin can only increase fat mass, fat cell sizes, adipose and systemic inflammation in visceral adipose depots but not in subcutaneous depots (6). Thus, the findings described in this study might imply that cell type selection is crucial to data interpretation in the study of RAS functions. However, the current findings may be limited by the cell culture study, and more animal or human studies are needed for confirmation.

The result suggests that the inflammatory NF-κB pathway exerts beneficial effects by lowering ACE expression. Indeed, postprandial inflammation is a common phenomenon and may exert beneficial regulatory effects in adipose tissue as suggested by previous studies (49). Indeed, we observed long-term metabolic improvements upon increasing dietary intake of n-6 fatty acids despite acute increases in adipose inflammatory responses (50). Moreover, elevated levels of AA are not linked to increased risks of cardiovascular disease in large epidemiological meta-analyses (51). Our data, therefore, may help to explain the long-term beneficial effects of increased intake of n-6 fatty acids (52).

The limitations of this study relate to the *in vitro* nature of the investigation which differs from an *in vivo* situation. Moreover, the primary adipocytes were obtained from four morbidly obese individuals and may have undergone epigenetic changes due to the metabolic condition of the patients. Also, larger sample size trails will bring more evidence.

Conclusions

This study primarily demonstrated that subcutaneous adipocytes responded differentially to saturated and unsaturated fatty acids in ACE production. We have provided initial evidence that interfering with adipocyte metabolism might be a potential method to modulate the RAS and its subsequent pathological and physiological events.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at http://dx.doi.org/10.21037/atm-20-7514

Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-20-7514

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-20-7514). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This clinical intervention study was approved by the Ethics Committee of the Charité University Medicine in Berlin (Application no. EA4/006/15) in accordance with the Declaration of Helsinki and registered at www.drks.de (DRKS00009509). Informed consent was acquired from each individual prior to surgery.

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