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The differentiation of beige adipocyte in pericardial and epicardial adipose tissues induces atrial fibrillation development

Y. HE, N. MA, M. TANG, Z.-L. JIANG, H. LIU, J. MEI

Department of Cardiothoracic Surgery, Xinhua Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China

Yi He and Nan Ma contributed equally to this work

Abstract. – OBJECTIVE: Growing evidence has identified that excessive accumulation of pericardial adipose tissues (PAT) and epicardial adipose tissues (EAT) is associated with atrial fibrillation (AF) development. Moreover, beige adipocytes, present in PAT and EAT, have been proved beneficial in consumption of fatty acid and promotion of weight lose by nonshivering thermogenesis. The objective of this prospective, observational study was to reveal the potential association between beige adipocytes and AF development.

PATIENTS AND METHODS: Fat tissues from subcutaneous adipose tissue (SAT), PAT and EAT were obtained from 70 AF and 30 sinus rhythm patients. Hematoxylin and eosin (H&E) staining were performed to analyze morphological changes in fat tissues. Real-time PCR was performed to identify mRNA expression of unique uncoupling protein-1 (UCP-1). Western blotting and immunohistochemistry (IHC) were performed to determine protein expression of UCP-1.

RESULTS: Our results indicated that pericardial and epicardial adipocytes in AF patients demonstrated white-like change tendency and had lower expression of UCP-1 when compared to sinus rhythm patients. Additionally, the decrease of UCP-1 mRNA expression in PAT and EAT, together with LA enlargement, were independent risk factors of AF. Further, UCP-1 mRNA expression in EAT, but not in PAT, have a significant correlation with LA diameter. The function of nonshivering thermogenesis in PAT and EAT was impaired in AF patients, and this dysfunction in EAT had a great correlation with LA dilation.

CONCLUSIONS: Our data provide a new therapeutic target for LA remodeling and AF treatment.

Key Words:
Atrial fibrillation, Epicardial adipose, Immunohistochemistry, Beige adipocytes.

Introduction

Atrial fibrillation (AF) is the most common arrhythmia encountered in clinical practice. The presence of AF often leads to considerable morbidity and mortality from stroke and heart failure[1,2]. Obesity, along with its various co-morbidities have gradually emerged as an important factor contributing to AF[3,4]. Though the pathophysiological mechanisms of obesity involved in AF occurrence are not completely understood, the role of cardiac adipose tissue in the AF has been addressed recently. By using multi-detector computed tomography (CT) scanning, the Framingham heart study proved that increased epicardial adipose tissue (EAT) volume gave rise to prevalent AF, and this association remained significant even after adjusting for body mass index (BMI), age and sex[5]. Al Chekakie et al[6] revealed that compared to sinus rhythm (SR) patients, AF patients had a significantly richer EAT volume and that EAT volume was an independent risk factor of AF development. Sicari et al[7] compared the thickness of EAT and pericardial adipose tissue (PAT) in 49 healthy subjects by using echocardiography and magnetic resonance imaging, revealing that PAT rather than EAT, was a better cardio metabolic risk marker. In traditional opinion, adipose tissue can be classified into white adipose tissue (WAT) and brown adipose tissue (BAT) according to their different metabolic functions. WAT is usual yellow in color and contains large unilocular adipocytes, and its primary function is to store energy in the form of triglyceride. BAT is usual brow in color owing to its high mitochondrial content, and contains multiple-scattered smaller lipid droplets in adipocytes, and

Corresponding Author: Ju Mei, MD; e-mail: jumei147@hotmail.com
Hao Liu, MD; e-mail: liuhao1619@126.com

its primary function is to generate heat via uncoupled oxidative phosphorylation[8]. The unique uncoupling protein-1 (UCP-1) is the main marker of BAT, which uncouples the mitochondrial respiration from ATP production, thus enabling the adipocytes to generate heat through nonshivering thermogenesis[9]. Recently, a third type of adipocyte named beige adipocyte was discovered besides white and brow adipocytes[10]. Beige adipocyte is brown-like adipocyte characterized by high UCP-1 expression but resided in WAT[10,11]. Recent studies have confirmed that compared to subcutaneous adipose tissue (SAT), both PAT and EAT contained beige adipocytes and expressed higher level of UCP-1 as well[12-14]. However, the decreased amount and weakened function of beige adipocytes were found in obese and overweight subjects while inflammatory cytokines and pathological vasoconstriction factors were increased[15-18]. Researchers have explored approaches to stimulate white adipocytes transformation into beige adipocytes in animal models, which provided a potential therapeutic targets for obesity associated cardiovascular diseases in future[19,20]. Though beige adipocytes have been confirmed to play an important role in obesity, and obesity was positively associated with a high risk for AF, the correlation between beige adipocytes and AF development has not been studied. In present study, we collected fat samples from lone AF patients and patients with SR, and then identified the relationship between the UCP-1 expression of fat sample and clinical data, attempting to delineate the potential role of beige adipose tissue in the development of AF.

Patients and Methods

Study Population and Data Collection

The protocol was approved by the Ethics Committee of Xinhua Hospital, and all patients signed their written informed consent before sample collection. From September 2014 to June 2016, a total of 70 consecutive patients with lone AF (56 paroxysmal AF and 14 persistent AF) underwent the complete thoracoscopic ablation were enrolled in this study. Surgical criteria and procedures were performed as previously described[21]. Another 30 patients who received open-heart surgery (including valvular replacement (n=16), ventricular (n=8) and atrial (n=6) septal defects repair surgery) with no history of arrhythmia (SR group) were collected as the control group. Potential predictors of AF were chosen based on a review of the literature. We collected data prospectively from patient medical records including: age, sex, body mass index (BMI), preoperative comorbidities (diabetes mellitus, hypertension, stroke, and coronary artery disease), left atrial (LA) diameter (measuring by echocardiography), fasting glucose and blood fat (cholesterol, triglyceride, high density lipoprotein (HDL) cholesterol and low density lipoprotein (LDL) cholesterol).

Collection of Human fat Tissues and Preparation

Fat samples (1 to 2 g) were obtained from three different locations during surgery for all patients: (1) SAT from thoracoscopic ports or sternal wall; (2) PAT from the parietal pericardium; (3) EAT was harvested adjacent to the left-interventricular groove. For quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western-blot experiment, samples were snap-frozen in liquid nitrogen and stored at -80°C for subsequent analysis. For immunohistochemistry (IHC) experiment, tissues were cut into appropriate cubes and immersed into 4% paraformaldehyde.

Morphological Assessment and Immunohistochemistry

The fat tissues obtained from surgery were immersed in 4% paraformaldehyde for 24 h to complete fixation. Thereafter, the tissues were dehydrated, embedded in paraffin, and sectioned into 4- atients rom 70 lted that μm sections. After deparaffinization, sections were stained with hematoxylin and eosin (H&E) for morphological assessment. To evaluate the UCP-1 expression in fat tissues, sections were processed for Immunohistochemistry (IHC) staining with the anti-human UCP-1 polyclonal antibody (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. In brief, sections were baked at 65°C for 30 min and deparaffinized in xylene and then rehydrated in a series of ethanol solutions with increasing concentration. Then, sections were pretreated in microwave in retrieval buffer for 10 min and peroxidase activity was removed with 3% H_2O_2-- methanol for 30 min. After incubating with normal goat serum for 30 min to eliminate nonspecific staining, sections were incubated with anti-human UCP-1polyclonal antibodies (Abcam, Cambridge, MA, USA) overnight at 4°C .The next day, the sections were washed with phosphate-buffered saline (PBS) for 3 times

and incubated with secondary antibody (Abcam, Cambridge, MA, USA) for another 30 min at room temperature. Staining was completed after incubation with the DAB solution for 10 min and then rinsed with phosphate buffered saline (PBS). Finally, sections were counterstained with 0.1% hematoxylin and cover-slipped.

RNA Extraction and Quantitative Real-time PCR

Samples were collected for detecting UCP-1 mRNA expression by qRT-PCR using an ABI PRISM 7500 sequence detector system (Applied Biosystems, Foster City, CA, USA). In brief, freshly frozen tissues (100 mg) were sonicated in 1 ml of Trizol (TaKaRa Biotechnology, Dalian, China) and left on ice for 15 min. Total RNA was extracted by phenol-chloroform extraction, followed by precipitation with isopropanol and washed with 75% ethanol. Concentrations of RNAs were determined by reading the absorbance at 260 nm. Reverse transcription of RNA (1 μg) was performed with the use of a PrimeScript RT Master Mix (Perfect Real-time) kit (TaKaRa Biotechnology, Dalian, China). Gene-transcript levels of UCP-1 were quantified by Real-time polymerase chain reaction (PCR) with the use of a SYBR Premix Ex TaqTMII (Perfect Real-time) kit, TaKaRa Biotechnology (Dalian, China). The primers used were as follows:

– UCP-1 forward primer: 5'-AGGTCCAAGGTGAATGCCC-3';
– Reverse primer: 5'-TTACCACAGCGGTGATTGTTC-3'.
– β-actin forward primer, 5'-CTACAATGAGCTGCGTGTGG-3';
– Reverse primer: 5'-CGTGAGGAAGGTCGGAAGGAA-3'.

The conditions used for PCR involved 30 s incubation at 95°C, followed by 40 cycles, which involved heating to 95°C for 5 s and then to 64°C for 34 s. 2-ΔΔCt method was applied to analyze gene expression

Western Blot

Frozen fat tissue was ground in RIPA buffer and protein concentrations were determined using the BCA assay (Beyotime, Shanghai, China). Equal amounts of protein (40 mg) was loaded and run on a 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA), blocked by incubation in 5% nonfat milk (Millipore, Billerica, MA, USA) for 1 h, and incubated overnight with anti-human UCP-1 antibody (Abcam, Cambridge, MA, USA) at 4°C. The next day, membranes were washed and incubated for 1.5 h at room temperature with the appropriate secondary antibody linked to horseradish peroxidase (HRP) (Abcam, Cambridge, MA, USA). The signal was visualized using an enhanced chemiluminescence fluid.

Statistical Analysis

Statistical analysis was performed by using SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA). Dichotomous data were evaluated using Pearson's χ^2 test or Fisher's exact test, whereas unpaired t-test and Mann-Whitney U test were used for continuous variables with normal or non-normal distribution. In addition, univariate analysis was performed to examine the influence of baseline characteristics on AF, followed by binary logistic regression analysis to identify independent risk factors of AF. Explanatory variables with $p<0.2$ upon univariate analysis were entered into the regression model. Finally, linear regression and correlation analysis to exam the association between these risk factors.

Results

Baseline Characteristics

A total of 100 patients were studied: 70 patients with AF and 30 patients with SR as controls. The baseline characteristics of the AF and SR groups are shown in Table I. Univariate analysis demonstrated that there was no significant difference in age, sex, and preoperative comorbidities. The BMI was significantly greater in the AF patients compared to SR controls ($p=0.015$), but no significant differences were identified in the blood fat and fasting glucose. Furthermore, the LA diameter was significantly greater in the patients with AF ($p<0.01$).

Adipose Tissues Displayed Brown-like Adipose Characteristics in SR Group But not in AF group

Microscopic analysis revealed that the structural discrepancy between white adipocytes (in SAT) and beige adipocytes (in PAT and EAT) in AF group was not so apparent as it was in SR group (Figure 1A and IB). Though the regions of multilocular adipocytes were scarce, we still found the multilocular adipocytes in PAT and EAT biopsies from SR group, while in PAT and

Table I. Baseline characteristics of the AF and SR groups.

Characteristics	SR (n=30)	AF (n=70)	*p*-value
Male gender (%)	40%	60%	0.082
Age (years)	57.36±6.76	58.23 ± 5.63	0.115
BMI (kg/m2)	27.86±1.42	28.65 ± 1.47	0.015
Diabetes mellitus (%)	30%	31.4%	1.00
Hypertension (%)	10%	14.3%	0.75
History of stroke (%)	6.7%	12.9%	0.50
Coronary artery disease (%)	26.7%	34.3%	0.49
Left atrial diameter, mm	44.33±5.80	51.60 ± 4.49	<0.01
Fasting glucose (mmol/l)	5.57±1.13	5.79 ± 1.08	0.37
Cholesterol (mmol/L)	7.23±2.05	7.49 ± 2.00	0.55
Triglyceride (mmol/L)	1.74±0.35	1.66 ± 0.33	0.33
HDLcholesterol (mmol/L)	1.98±0.32	1.91 ± 0.31	0.32
LDLcholesterol (mmol/L)	2.97±4.00	2.91 ± 0.42	0.53

EAT from AF patients, only uni-locular lipid droplets were observed. Also, adipocyte size enlargement and lipid droplets coalescence were visually inspected in AF group.

The UCP-1 Expression was Significantly Decreased in AF Group Compared with SR Group

UCP-1 is an important marker of beige adipocytes distinguished from white adipocytes. Compared with SR patients, mRNA expression of UCP-1 in three locations of AF patients was significantly declined, as assessed by Real-time PCR (Figure 2A). Furthermore, the results of UCP-1 protein expression performed by Western blot and IHC were consistent with the mRNA results. As shown in Figure 2 B, the protein expression of UCP-1 in three different locations from AF patients was all significantly decreased compared with their corresponding counterparts in SR patients. What's more, the result of IHC further confirmed the protein expression tendency of UCP-1. In SR patients, the regions of pericardial and epicardial adipose tissue that from contained multilocular adipocytes, which were strongly positively stained by UCP1, were observed. Also, in unilocular adipocytes of AF patients, no positive or only weak positive UCP1 staining was observed. Taken together, the expression of UCP-1 was significantly decreased at both mRNA and protein levels in AF patients compared with SR patients, suggesting that adipose tissues from AF patients have lost the capacity of thermogenesis

The UCP-1 Expression was an Independent Risk Factor for AF

According to the univariate analysis, multiple risk factors have been associated with the development of AF, including gender, age, BMI, LA diameter, and adipose tissue UCP-1 mRNA level in four locations. In order to study the relationship between UCP-1 and AF presence, binary logistic regression analysis was performed. Results showed that LA diameter, UCP-1 mRNA in PAT and in EAT were independent risk factors of AF after adjusting for age, sex and BMI (Table II). In view of a potential confounding relationship of LA diameter and UCP-1 mRNA levels, we found that

Table II. Binary logistic regression analysis.

Characteristics	Odds Ratio (95% CI)	*p*-value
Age (year)	1.11 (0.97 to 1.26)	0.12
Male gender (%)	3.01 (0.70 to 13.35)	0.14
BMI (kg/m2)	1.16 (0.73 to 1.84)	0.52
Left atrial diameter (mm)	1.43 (1.18 to 1.73)	<0.01
UCP-1 mRNA in SAT	0.66 (0.43 to 1.00)	0.50
UCP-1 mRNA in PAT	0.85 (0.74 to 0.98)	0.03
UCP-1 mRNA in EAT	0.71 (0.52 to 0.96)	0.03

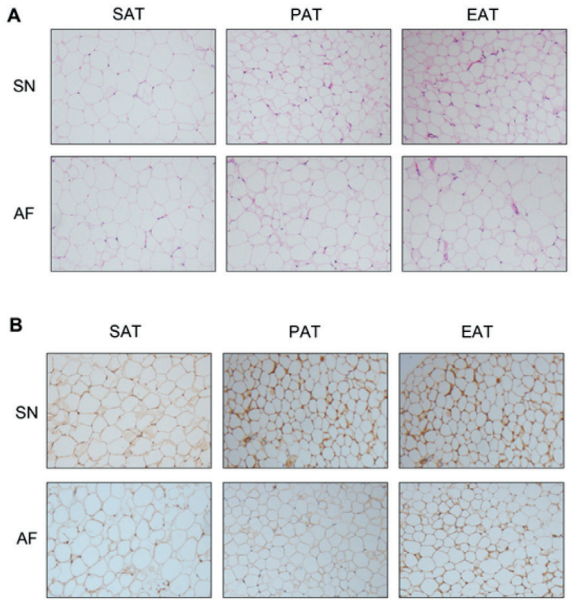

Figure 1. The adipocytes have increased lipid deposition and beige adipocytes transformed into white in AF patients. ***(A)*** The representative images of hematoxylin and eosin (H&E) staining and ***(B)*** UCP-1 immunohistochemistry staining at 200 X magnification.

there was a strong correlation between LA diameter and UCP-1 mRNA in EAT (Figure 2C), which implied the dysfunction of beige adipocytes in EAT played an important role in LA remodeling.

Discussion

In present study we demonstrated for the first time that UCP-1 reduction in cardiac adipose tissues and LA enlargement were independent factors for AF development. In addition, the decreased UCP-1 expression in adipose tissues not only accompanied with the white-like adipose conversion in PAT and EAT adipocytes, but also with the LA enlargement, by which EAT possibly contributes to the genesis and maintenance of AF. A plenty of evidence indicated that obesity was a risk factor for AF, and even the epidemic of obesity appears to feed the epidemic of AF[3,22,23]. Indeed, obesity increased almost 50% of the risk for AF when compared to normal individuals[24]. Though adipose tissue around the heart only accounted for 3% of the whole organ mass, its excessive accumulation did have the pro-arrhythmia effects[5-7]. The imbalance between energy intake and calories expend causes the obesity and overweigh. Therefore, adipose tissue, which stores the most of fatty acid in human body, plays a key role in energy homeostasis. In fact, obesity depends on the number of the white adipocytes of the mounts of WAT, which store excess energy in the form of triglycerides, while brown and beige adipocytes have remarkable capacity to dissipate energy via UCP-1, and the trans-differentiation of white adipocytes into brown adipocytes may offer a potential therapeutic target for preventing obesity and metabolic diseases[8,25]. Recently, mouse models have become available with specific genetic manipulations that produce a lean phenotype with more BAT mass and enhanced insulin sensitivity[26,27]. Adipose tissue of obese mice exhibits decreased mitochondrial gene expression and mitochondrial mass that can be reversed by treatment with the peroxisome proliferator-activated receptor γ (PPARγ) agonist rosiglitazone[28]. These findings indicate that mitochondrial remodeling and increased energy expenditure in WAT also can affect whole-body energy homeostasis. Therefore, a more comprehensive understanding about the association between AF and beige adipocytes may provide a new aspect to recognize the obesity-associated AF. Our result revealed that pericardial and epicardial adipocytes in AF patients did turn out to be hypertrophy, lipid droplet coalescence and fat deposits. Also, the UCP-1 mRNA expression level in these three fat depots of AF group was significantly lower when compared to SR group. The degree of decline in the level of UCP-1 mRNA in PAT and EAT was discrepant significantly, where should have consisted of mounts of beige adipocytes as previous reported previously[13,14]. Western blot analysis and IHC results were completely in accord with the mRNA expression. Taken together, these data suggest that AF patients may have difficulty in dissipating heat, for the activity of beige adipocytes is significantly impaired. LA dilation as a hallmark of atrial structural remodeling, is also associated with electrical remodeling, which finally favors arrhythmogenesis and perpetuates AF[29]. Iacobellis et al[30] compared the 30 obese individuals with 20 healthy normal weigh volunteers in EAT thickness and cardiac function by echocardiography, and found that atrial dilatation and diastolic dysfunction were common findings in obese individuals. Moreover, EAT, as a particular form of visceral fat deposited around the heart, could directly contact with the surface of the atrium and pulmonary veins. Growing evidence has suggested that EAT is an important source of inflammatory mediators. Researchers confirmed that EAT secrets a plenty

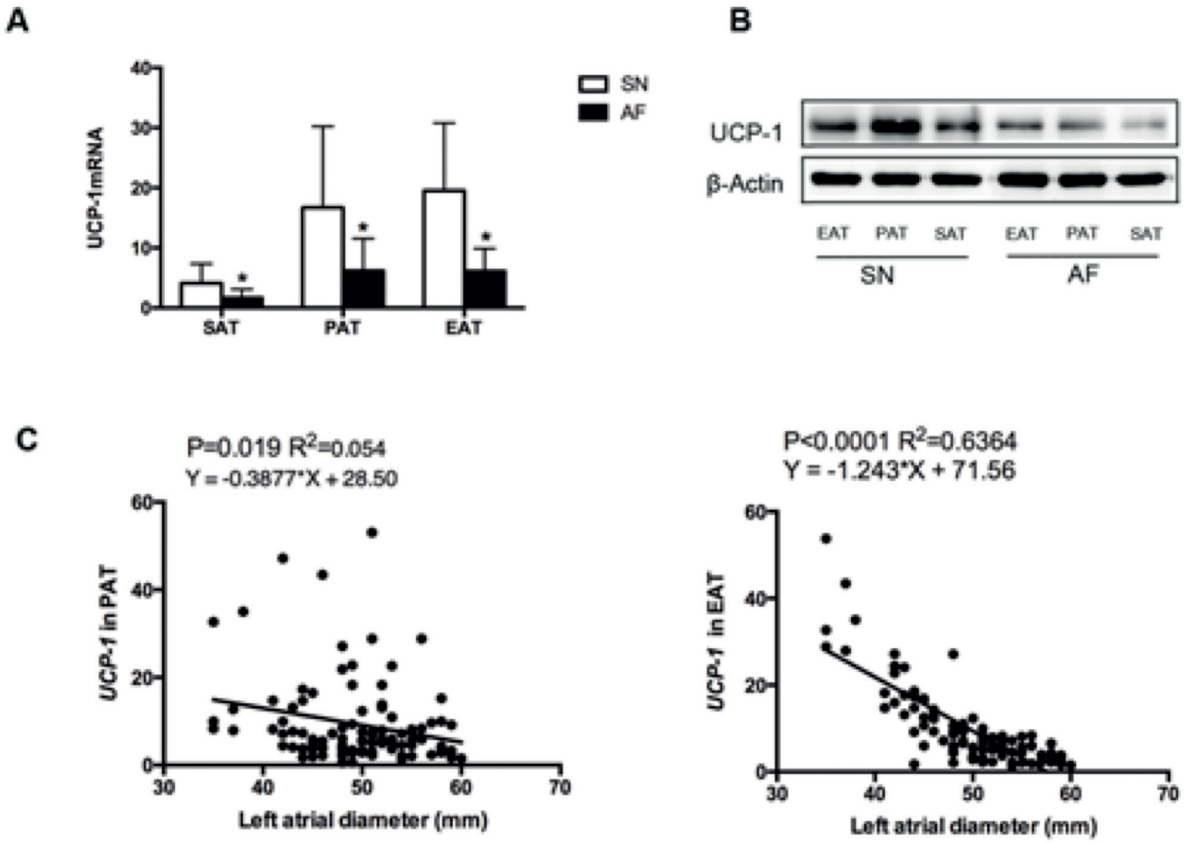

Figure 2. The expression of UCP-1 was significantly decreased in AF patients compared with SR patients. A typical ***(A)*** UCP-1 mRNA in the adipocytes was measured by quantitative Real-time, and ***(B)*** Detection UCP-1 protien expression was measured by Western blotting. ***(C)*** The positive correlation of UCP-1 mRNA in PAT and EAT with LA dilation. The UCP-1 mRNA decrease in EAT had strong correlation with LA enlargement in AF group.

of pro-inflammatory and pro-fibrotic cytokines, which reflect atrial structural remodeling through paracrine mechanism[31,32]. Consistent with previous studies, our study found LA diameter was significantly larger in AF group compared with SR group. Additionally we confirmed for the first time the idea that the decrease of UCP-1 in EAT may cause the adjacent LA dilation, which may provide a novel and valuable insights about the EAT effect on LA remodelling.

Conclusions

Taken together, the purpose of this study was to compare the morphological changes and UCP-1 expression of adipocytes tissue between long AF patients and SR individuals. We found that the unilocular adipocytes were hypertrophy, lipid droplets coalescence in pericardial, and epicardial adipose tissues from AF patients. Additionally, we confirmed that mRNA expression of UCP-1 decline in pericardial and epicardial adipose tissue was strongly associated with AF development and LA enlargement, which probably provided a new prevention and treatment target for AF. However, the present study may suggest a pathogenic role for cardiac adipose tissues in the development of AF, particularly in metabolic disturbance in PAT and EAT. Further studies are required to identify more precise role of these fat tissues in the progression of AF.

Conflict of interest

The authors declare no conflicts of interest.

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