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The relationship between carbonic anhydrase-III expression and oxidative stress in brown adipose tissue

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Abstract

Objective: High-fat foods increase adipose tissue size, and induce obesity. Although carbonic anhydrase III is abundantly found in brown adipose tissue, its function is not fully defined. In this study, we investigated the relationship between carbonic anhydrase III enzyme mRNA expression and malondialdehyde, oxidative stress marker, in brown adipose tissue of rats that were fed high-fat diets. In addition, we investigated potential effect of N-acetylcysteine as an antioxidant in this relationship.

Methods: In our study three experimental groups were formed and each contained 6 rats (control, obese, and antioxidant groups). The experimental groups were fed for a duration of 85 days with high fat diets. In these groups, carbonic anhydrase III mRNA expression, total carbonic anhydrase hydratase activitie, and malondialdehyde levels were measured in brown adipose tissues dissected from rat scapula regions.

Results: According to our findings, carbonic anhydrase III mRNA expression was higher in the obese group than in the control group ($p = 0.004$), and malondialdehyde levels were lower in the obese group than in the control group ($p = 0.03$). It was observed that carbonic anhydrase III mRNA expression was higher in the antioxidant group than in the control group ($p = 0.006$), and malondialdehyde levels were lower in the antioxidant group than in the control group ($p = 0.006$). In addition, in the obese group carbonic anhydrase III mRNA expression was higher than in the antioxidant group ($p=0.01$).

Conclusion: In brown adipose tissue of rats that were fed high-fat diets, this study showed that the carbonic anhydrase III mRNA expression increased and the malondialdehyde level decreased.

Keywords: Carbonic anhydrase III, brown adipose tissue, obesity, N-acetylcysteine, high-fat diet.

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Kahverengi yağ dokusundaki karbonik anhidraz III ekspresyonu ve oksidatif stress arasındaki ilişki

Öz

Amaç: Yüksek yağlı besinler yağ dokusu hacmini artırır ve obeziteyi indükler. Kahverengi yağ dokusunda karbonik anhidraz III bol miktarda bulunmasına rağmen fonksiyonu tam olarak bilinmemektedir. Bu çalışmada karbonik anhidraz III enzim mRNA ekspresyonu ve oksidatif stress markırı olan malondialdehit arasındaki ilişkinin incelenmesi amaçlandı. Ayrıca antioksidan molekül olan N-asetilsisteinin bu ilişkiyi nasıl etkilediği saptanmaya çalışıldı.

Yöntemler: Çalışmamız her grupta altı sıçan bulunan üç grup üzerinde (kontrol, obez ve antioksidan grup) yapıldı. Deney grupları, grupların özelliklerine göre belirlenmiş yüksek yağlı diyet veya kontrol diyetiyle 85 gün beslendi. Besleme süresinin sonunda scapula bölgesinden alınan kahverengi yağ dokusunda karbonik anhidraz III mRNA ekspresyonu, total karbonik anhidraz aktivitesi ve malondialdehit seviyesi ölçüldü.

Bulgular: Obez grubunda kontrol grubuna göre karbonik anhidraz III mRNA ekspresyonunun daha yüksek olduğu (p=0.004) ve malondialdehit seviyesinin daha düşük olduğu (p=0.03) saptandı. Antioksidan grupta kontrol grubuna göre karbonik anhidraz III mRNA ekspresyon seviyesinin daha yüksek olduğu (p=0.006) ve malondialdehit seviyesinin daha düşük olduğu (p=0.006) tesbit edildi. Bunlara ilaveten obez grubunda karbonik anhidraz III mRNA ekspresyonu antioksidan grubuna göre daha yüksek olduğu belirlendi (p= 0.01).

Sonuç: Bu çalışma yüksek yağlı diyetle beslenen sıçanların kahverengi yağ dokusunda karbonik anhidraz III mRNA ekspresyonunun arttığını ve malondialdehit seviyesinin azaldığını dolayısıyla oksidatif stresin kısmen baskılandığını gösterdi.

Anahtar kelimeler: Karbonik anhidraz III, kahverengi yağ dokusu, obesite, N-asetilsistein, yüksek yağlı diyet.

INTRODUCTION

High-fat foods increase adipose tissue size, and induce obesity. Obesity is expressed as a new world syndrome[1]. The most common causes of obesity are known as excess calorie intake by diet, reduction of physical activity, and genetic predisposition[2]. Obesity is defined as a medical condition which decreases life expectancy and increases health problems due to excessive adipose tissue volume in the body[3].

Adipose tissue is categorized as white adipose tissue (WAT) and brown adipose tissue (BAT)[4]. While WAT is found abundantly in subcutan, perirenal, and visceral regions of adults, brown adipose tissue is especially found in neonatal (in interscapula, perirenal, axilla, and paravertabral regions[5]), and its amount decreases over time[6-7]. As WAT deposits primarily triglycerides as fat droplets in adipocytes, BAT causes energy consumption by heat making[4]. Like WAT, BAT performs its other functions with various biomolecules that it releases such as leptin, adiponectin, etc. In addition, it converts troid hormone to triiodothyronine form thyroxine thanks to deiyodinase type II enzyme[8].

BAT consists of brown fat cells, nerves, and blood vessels[9]. Plenty of mitochondria are located in brown fat cells. Mitochondria perform a thermogenic function by uncoupling protein 1 (UCP-1) located in the inner mitochondrial membrane. UCP-1 is particularly expressed in these cells[5,10]. Homologous proteins such as UCP-2 and UCP-3 are expressed in BAT as well[5].

Carbonic anhydrase (CA) (EC 4.2.1.1) is an enzyme that catalyzes the reversible hydration of carbondioxyde. It has a zinc atom at the active site. In humans, 16 CA isoenzymes have been identified with different tissue distribution and cellular localization. These isoenzymes have roles in physiological

processes such as calcification, pH regulation, ion secretion, and transport of CO_2[11].

CA III is a cytosolic enzyme. It is particularly found in the liver, skeletal muscle, WAT, and BAT. CA III isoenzymes differ from the other isozymes with various characteristics. It has low hydratase activity[12], and it is resistant to inhibition by sulphonamides[13]. It constitutes amount in the range of 8% to 25% in the soluble protein fraction (cytosolic proteins) of myocytes and adipocytes[12]. Although CA III is abundantly found in the body (particularly adipocytes), its function is not fully defined[14]. Previous studies have shown that CA III expression made cells more resistant against cytotoxic H_2O_2 concentration, protected the cells from oxidative damage, and exhibited antioxidant effects[15]. Also, very few studies were done regarding the effects of CA III in BAT. In this study, we investigated the relationship between CA III enzyme mRNA expression and malondialdehyde (MDA), oxidative stress marker, in BAT of rats that were fed high fat diets. Furthermore, we investigated the potential effect of N-acetylcysteine (NAC) as an antioxidant in this relationship.

METHODS

Animals and experimental design: All of the experiments were performed with the approval of the Karadeniz Technical University Animal Experimentation Ethics Committee protocol numbered 2014/7 application.

Rats used in this study were supplied from Karadeniz Technical University (KTU) Surgical Application and Research Center. They were kept in the same place and were provided 12:12 hour light-darkness cycle. 18 male Sprague-Dawley rats, aged 6-8 weeks old and weighing 150-200 g, were used. All dietary food used in the study (standard rat diet (D12450B) and high-fat diet (D12451)) was purchased from Research Diets. Diet ingredients are presented in Table 1 and Table 2 of this paper.

Table 1: Ingredients of standard rat diet

D12450B		
Product	**g%**	**kcal %**
Protein	19.2	20
Carbohydrate	67.3	70
Fat	4.3	10
Total		100
kcal/g	3.89	

Table 2: Ingredients of high-fat diet

D12451		
Product	**g%**	**kcal %**
Protein	24	20
Carbohydrate	41	35
Fat	24	45
Total		100
kcal/g	4.73	

Initially, all rats were fed standard rat diet for 15 days, and then they were weighed. Rats were randomly selected and divided into groups of three where each group contained six rats. While the control group was fed with standard rat diet, the obese group was fed with a high -fat diet. As for the antioxidant group, it was fed with a high -fat diet and NAC, an antioxidant molecule, was added to this group's drinking water (2 g/L). Rat diets and water were given ad libitum to all groups. At the end of the 85 day feeding period, the rats were weighed and then euthanized.

Table 3: GAPDH and CA III primers

Gene names	Sequence
CA III forward primer (Iontek).	5' TGCTGTGGTTGGCATTTT TC 3'
CA III reverse primer (Iontek).	5' AGGCTGCGCAGCTTGGCCAT 3'
GAPDH forward primer (Integrated DNA technologies)	5' AGATGGTGAAGGTCGGTGTG 3'
GAPDH reverse primer (Integrated DNA technologies)	5'CATTCTCAGCCTTGACTGTGC 3'

BAT was extracted from the scapula regions of the rats and then frozen on dry CO2. The specimens were immediately placed in microcentrifuge tubes, and stored at -80 ° C until the tests were done.

CA III Expression: RNA isolation was performed by using TRIzol reagent (Invitrogen). For cDNA synthesis, the Roche transcriptor first strand cDNA synthesis kit (Cat. No: 04 896 866 001) was used. SYBR green dye was used to determine the expression of related genes. RT-PCR procedure was performed with the Roche Light Cycler 480 II devices according to the Roche kit (LightCycler FastStart DNA master SYBR green I qRT-PCR, Cat. No: 12 239 264 001) procedure. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene. GAPDH and CA III primers are presented in Table 3 of this paper. Results were calculated according to efficiency method in the advanced relative quantification software of the Roche LightCycler Systems[16,17]. They were expressed as arbitrary unit (CA III mRNA / GAPDH mRNA).

Measurement of tissue MDA: MDA levels were measured according to the methods of Mihara and Uchiyama with slight modifications[18]. This method in acidic conditions is based on the complex formation of MDA and thiobarbituric acid (TBA). This complex can be measured spectrophotometrically at 532 nm.

Approximately 150 mg of BAT were homogenized in 2 ml of cold homogenization solution (1.15% KCl, 0.05% triton X 100). The homogenates were centrifuged at 3000 rpm for 10 min. Following which the fat layer on the upper part of the tube was removed, and the infranatant was used. 3 ml of 1% H3PO4 and 1 ml of TBA (0.046 M acetic acid: water 1/1) were added to 0.5 ml of homogenate. The reaction mixture was incubated at 100 °C for 45 min. 2 ml of n-butanol was added to the mixture, and centrifuged at 4 000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm. 1,1,3,3 tetramethoxypropane were used as a standard. The MDA results were expressed as nmol / g tissue.

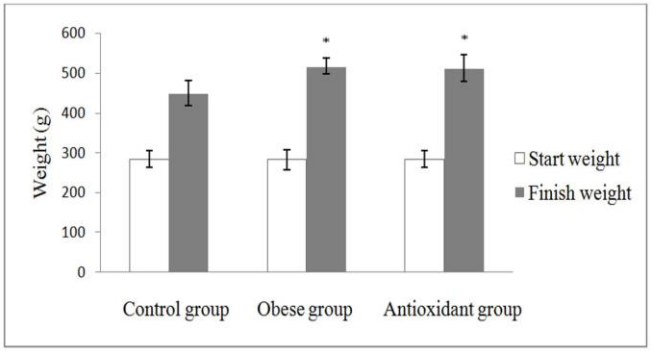
Measurement of CA hydratase activity: Total CA (CA III and other CA isoenzymes) hydratase activity was measured according to the modified Wilbur-Anderson method[19]. This method is based on observing the rate in which the pH falls from 8.2 to 7.0 potentiometrily. Approximately 150 mg of BAT were homogenized in 2 ml of ice cold homogenization solution (1.15% KCl, 0.05% triton X 100). The homogenates were centrifuged at 3000 rpm for 10 min. In the upper part of the tube, the fat layer was removed, and the infranatant was used. Usage of infranatant was made according to the method of Alver and colleagues[20]. Protein concentration of infranatants were measured according to the modified Bradford method[21]. CA activity was stated as EU/g protein.

Statistical analysis: Our results were stated as mean ± standard deviation (SD). The Kruskal-Wallis test was used to compare the means of the groups and then the Mann-Whitney U-test was used. Statistical analyses were performed via SPSS 16.0 software. $P<0.05$ was assumed to be statistically important.

RESULTS

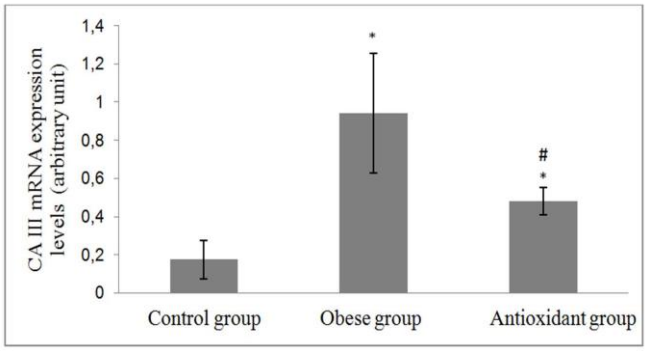
The rats were fed ad libitum in a surgical research center for a total of 85 days. At the end of the nutrition program, the weight was determined to be higher in the obese and antioxidant groups with respect to the control group (p = 0.018) (Figure 1).

Figure 1. Weight change of the experiment groups.
*Statistically significant with respect to control group p<0.05.
Values are expressed as mean ± SD.



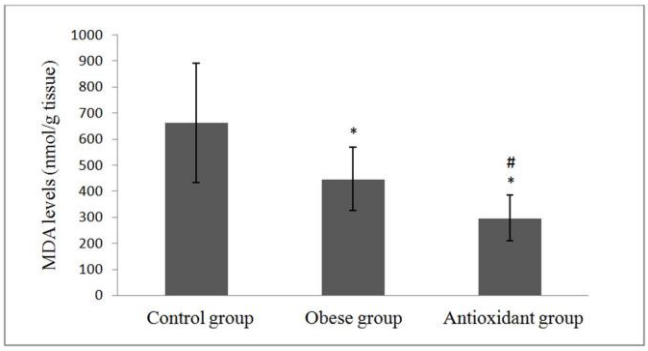
CA III mRNA levels in the BAT of the control, obese, and antioxidant groups were measured. There were significant differences among the groups (p = 0.001) (Figure 2). When the control and obese groups were compared, CA III mRNA levels were determined to be higher in the obese group than in the control group (respectively, 0.94±0.31, 0.17±0.10 arbitrary unit, p = 0.004) (Figure 2). When the control and antioxidant groups were compared, CA III mRNA levels were determined to be higher in the antioxidant group than in the control group (respectively, 0.48±0.07, 0.17±0.10 arbitrary unit, p = 0.006) (Figure 2). As for the obese and antioxidant groups, CA III mRNA levels were determined to be higher in the obese group than in the antioxidant group (respectively, 0.94±0.31, 0.48±0.07 arbitrary unit, p = 0.01) (Figure 2).

Figure 2. CA III mRNA expression levels in the BAT.
*Statistically significant with respect to control group p<0.05.
Statistically significant with respect to obese group p< 0.05.
Values are expressed as mean ± SD.



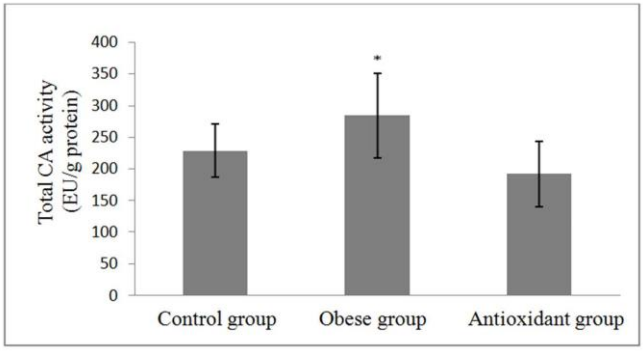
Similarly, a significant difference was observed among the MDA levels in the BAT of the control, obese, and antioxidant groups (p = 0.004) (Figure 3). When the control and obese groups were compared, MDA levels were higher in the control group than in the obese group (respectively, 664±229, 420±140nmol/g tissue, p = 0.037) (Figure 3). When the control and antioxidant groups were compared, MDA levels were significantly higher in the control group than in the antioxidant groups (respectively, 664±229, 334±128nmol/g tissue, p = 0.004) (Figure 3). As for the obese and antioxidant groups, MDA levels were significantly higher in the obese group than in the antioxidant group (respectively, 420±140, 334±128nmol/g tissue, p = 0.037) (Figure 3).

Figure 3. MDA levels in the BAT.
*Statistically significant with respect to control group p<0.05.
Statistically significant with respect to obese group p<0.05.
Values are expressed as mean ± SD.



Total CA (CA III and other CA isoenzymes) hydratase activity levels in the BAT specimens of the control, obese, and antioxidant groups were measured. There were significant differences among the groups (p = 0.04) (Figure 4). Total CA hydratase activity was found to be higher in the obese group than in the control group, but the difference was not statistically significant (respectively, 284±67, 229±42 EU/g protein, p = 0.4) (Figure 4). Total CA hydratase activity was found to be higher in the control group than in the antioxidant group, but it was not statistically significant (respectively, 229±42, 192±51 EU/g protein, p = 0.08) (Figure 4). As for the obese and antioxidant groups, Total CA hydratase activity levels were determined to be significantly higher in the obese group than in the antioxidant group (respectively, 284±67, 192±51 EU/g protein, p = 0.03) (Figure 4).

Figure 4. Total CA activity levels in the BAT.
*Statistically significant in the obese group with respect to antioxidant group p<0.05.
Values are expressed as mean ± SD.



DISCUSSION

Increased systemic oxidative stress is observed in obesity. It is suggested that in obese people hyperleptinaemia, hyperglycemia, antioxidant defense failure, chronic inflammation, increased endothelium-derived reactive oxygen species (ROS), blood lipid level increasement, and muscle respiration rate elevation is caused by oxidative stress[22]. In addition, obesity models induced by high fat or high carbohydrate intake, oxidative stress and inflammation increases due to the macrophage activation and mitochondrial dysfunction, as well as peroxisomal fatty acid oxidation in the WAT. In the BAT, the results obtained from either diet induced or genetically obese models are controversial[23,24].

Depending on thermogenic mechanisms, the number and the activity of the mitochondria are higher in the BAT which implies a higher ROS production rate. However, Berlohr et al. reported higher aldehydes, which are the end products of lipid peroxidation, in the epididymal fat tissue of the obese mice induced with high -fat diets but not such an increase in the BAT[24]. Another experimental study in which obese and normal Zucker rats were used, showed that brown fat tissue had a specific redox state and protected itself from oxidative stress in the course of obesity by varying levels of enzymatic and non-enzymatic antioxidant molecules[23].

In the present study, we found that the MDA levels, which are a sensitive indicator of lipid peroxidation and oxidative stress, decreased in the BAT of the obese rats fed high -fat diets (p = 0.03 Figure 3).

NAC is a strong antioxidant that dissolves in water. It is used as an effective antioxidant in many oxidative stress related studies. NAC directly and indirectly exhibits antioxidant properties by scavenging ROS[25]. In our study, the MDA of the NAC used in the antioxidant group was lower than both the control and obese groups.

CA III is an isoenzyme which has properties such as low catalytic activity and resistance to inhibition by sulfonamides. In rodents, it is abundantly present in tissues involved in energy metabolism such as the liver, skeletal muscle, and fat tissue (both in white and brown fat tissues)[12]. Although, the exact function of CA III is not known, it has been suggested that CA III may have various roles in cellular functions beyond its catalytic activity[14-15]. CA III protects cells from oxidative stress by two reactive

sulfhydryl groups in its structure[12,26,27]. CA III is expressed in the BAT, and its expression seriously decreases in mice exposed to the cold[14]. In our study, we determined that CA III expression increased 5 fold in the BAT of the rats which were fed high -fat diets (p = 0.004 Figure 2).

Although the rats were fed high -fat diets, interpreting decreased MDA and increased CA III expression is difficult, since CA III might be an antioxidant molecule that is used by the cell against oxidative damage.

In this context, in the obese group MDA levels were found to be lower. It is known that increased CA III protects the cells against oxidative stress induced by H2O2[15]. Despite the same food and similar weight increase, in the antioxidant group it is observed that CA III expression was lower compared to the obese group (p=0.01). The NAC contained diet may have partially prevented the tissue from oxidative stress, and correspondingly caused to reduction in the CA III expression. In this state, CA III in the BAT acts as an oxidative stress sensor. In the WAT of rats, CA III is regulated with obesity. However, in the WAT, and in the early periods of adipogenesis, it was reported that CA III expression increased and protected the cell against peroxisome proliferator-activated receptor gamma (PPAR□2) mediated oxidative stress[28]. This difference between WAT and BAT may be a result of different metabolisms and differentiation of pathways.

Total CA (CA III and other CA isoenzymes) hydratase activity levels measured in the BAT of the obese group was determined to be higher in the obese group than in the antioxidant group (p = 0.03) (Figure 4). In addition, total CA hydratase activity was found to be higher in the obese group than in the control group, but the difference was not statistically significant (p = 0.4) (Figure 4). In general, changes in CA III mRNA levels do not fully represent the changes of total CA activity (Figure 4). This situation may derive from two possible reasons. The first, the co-existance of other CA isoenzymes in the same tissue may influence total CA activity. The second, it has been shown that specific activity of CA III isoenzymes is approximately up to 3% of the activity of CA II[12,29,30]. Consequently, the increased CA III isoenzyme barely influences total CA activity.

In the BAT of rats which were fed high -fat diets, this study showed that the CA III mRNA expression increased and the MDA levels decreased. In the BAT, in contrast to the WAT, low MDA levels were determined in high -fat diets. In this MDA decrease, increase of CA III, which owned a specific antioxidant role may be effective.

Declaration of Conflicting Interests: The authors declare that they have no conflict of interest.

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