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Effects of Obesity on Serum Concentration of Methylation and Oxidative/Nitrosative Stress Metabolites Following DMBA Treatment of Female Zucker Rats

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Abstract

Obesity has been an epidemic in the United States for more than two decades. Studies in humans and animals have suggested that obesity is major risk for breast cancer development. It is well established that increased oxidative stress and changes in DNA methylation can contribute to carcinogenesis. Low molecular weight antioxidant glutathione plays exceptional role in protection of cells from oxidative damage. S-adenosylmethionine, a metabolite of amino acid methionine, is a key methyl group donor for numerous of methylation reaction including DNA. The main objective of this experiment was to investigate the effects of obesity on serum concentration of oxidative stress markers and products of oxidative damage and methylation-reaction donor S-adenosylmethionine following 7,12-dimethylbenz(a) anthracene (DMBA) treatment. Obese and lean female Zucker rats were maintained on a regular chow diet for two weeks. All rats were orally gavaged at age 50 days with 65 mg/kg DMBA. Twenty four hours after DMBA treatment, all rats were sacrificed and serum was collected. Highly sensitive HPLC method with CoulArray electrochemical detector was utilized for determination of thiols, 3-NT and SAM, SAH, and Adenosine. The obese rats had a 40% increased level of oxidized glutathione (P=0.007), decreased GSH/GSSG ratio (P=0.003), increased SAH (P=0.025) and also a 30% increased level of Ado (P=0.005) compared to lean rats. The lean animals showed a slightly higher (20%) concentration of SAM and 50% higher SAM: SAH ratio (P=0.02) compared to obese animals. These results showed that by using DMBA-induced mammary tumor development obesity can contribute to a significant imbalance in oxidative/reduction homeostasis in serum and depression in serum SAM: SAH ratio, known as "methylation ratio", and increase SAH level, known as potent inhibitor methylases. These data suggest that changes plasma concentrations of glutathione, SAH and SAM reflect intracellular changes that possibly can contribute to carcinogenesis.

Keywords: Obesity; Zucker rats; Oxidative/Nitrosative stress; DMBA-induced mammary tumors; Breast cancer

Introduction

Obesity has been an epidemic in the US for more than two decades. The proportion of overweight and obese adults in the population continues to increase. Breast cancer is the second leading cause of death in women in US. The American Cancer Society has estimated that 229,315 women will be diagnosed with new cases of breast cancer (29% of total new cancer cases) and 38,552 women will die from breast cancer, which is 14% of estimated total cancer deaths in 2012 [1].

A recent report suggests that obesity is a major risk factor for both premenopausal and postmenopausal women. They reported data from two large chemoprevention trials that had enrolled women at a high risk of breast cancer. Obesity was associated with only a modest, non-significantly increased risk of postmenopausal breast cancer but a significant 70% increased risk of premenopausal breast cancer compared to normal weight [2].

An investigation of the role of overweight and obesity in carcinogenesis documented not only an association between body mass index (BMI) and mortality from various types of cancer, but it also provided a reliable estimate of the contribution of overweight and obesity to the total mortality from cancer. The study reported that women with the highest BMI (40 kg/m^2) had mortality rates from all types of cancer combined that were 62% higher (with a relative risk of death of 1.62) than the rates of women of normal weight [3].

Chronic oxidative stress plays a critical role in etiology and pathogenesis of many diseases in human and animals. One of the aspects of this problem is the role of oxidative stress in the genesis of obesity or their interaction. It has been established in the literature that chronic oxidative stress plays important role in pathogenesis of obesity [4-6].

The sulfur-containing amino acid methionine not only is one of the essential amino acids participating in a protein synthesis, but also has been a source of incredibly important metabolites of S-adenosyl methionine and S-adenosyl homocysteine [7-9]. S-adenosylhomocysteine (SAM) is an intermediate product of methionine after addition of ATP by enzyme methionine adenosyltransferrase (MAT) [10]. It can be synthesized in many mammals cells and stored or used extensively for biochemical reactions donating one carbon (CH_3) group which is known as methyl group, in a variety (approximately 100) of methylation reactions, including DNA, RNA, proteins, phospholipids, hormones, neurotransmitters and many others products. Methylation reaction plays important regulatory and biosynthetic roles intracellularly under physiological homeostatic conditions and in the genesis and development of many diseases [11,12]. After donation of a methyl group, SAM becomes S-adenosylhomocysteine (SAH), another

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intermediate product of methionine. It has been established that SAH plays an appositive roll to SAM not activating but the inhibiting activity of methylases and decreasing of the methylation of many subtracts. Deficiency of a methyl group or inappropriate methylation potentially has harmful effect on genomic stability. SAM: SAH ratio, known as "methylation ratio", plays extremely important role in balanced methylation capacity [13,14].

Glutathione is the major intracellular and most abundant intracellular antioxidant involved in regulation of intracellular redox environment and is essential in complex process of detoxification. Concentration and intracellular distribution of glutathione are directly proportional to the capacity to detoxify pro-oxidant exposure, and conditions with low GSH reserves would express a vulnerability phenotype that is less able to detoxified and resolve oxidative stress. The GSH:GSSG ratio are essential for redox-sensitive cell signaling and homeostasis [15-17].

Figure 1 is an overview of the 3 interdependent pathways: folate cycle, methionine cycle, and transsulfuration cycle involved in folate-dependent methionine transmethylation and transsulfuration to gluthathione. The vital importance of these three interconnected pathway is often underscored by their essentiality for error-free DNA synthesis, for cellular methylation capacity, and for the maintenance of glutathione redox homeostasis [18].

Previously, we reported that obesity increases incidences of mammary tumor development using obese Zucker rat DMBA-induced mammary tumor model [19]. In this model, we use lean and obese Zucker rats, and at day 50 of age, we gavage them with 65 mg DMBA/kg of body weight. DMBA treatment has been known for the past 60 years to induce mammary tumors in rats, and breast cancer researchers, including us, have used this model for past 20 years. We have used this DMBA-induced mammary tumor model to investigate the effects of obesity on serum concentration of oxidative stress markers and products of oxidative damage and methylation-reaction donor S-adenosylmethionine following 7,12-dimethylbenz (a)

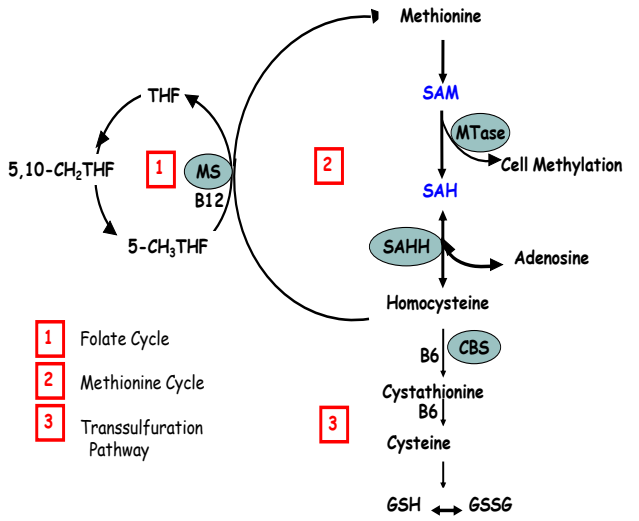

Figure 1: Methionine metabolism. The three interdependent pathways, the folate cycle, methionine cycle, and transsulfuration, are involved in folate-dependent methionine transmethylation to gluthathione redox metabolism.

anthracene (DMBA) treatment. We found that obesity can contribute to significant imbalances in oxidative/reduction homeostasis in serum and depression in serum SAM: SAH ratio, known as "methylation ratio", and increase SAH level, known as potent inhibitor methylases after DMBA treatment.

Experimental design

Obese *fa/fa* and lean Zucker rats were purchased at 5 weeks of age (Harlan Industries, Indianapolis, IN). Animals were housed two per cage and allowed *ad libitum* access to water and a regular chow diet (Harlan-Teklad, Madison, WI). All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas for Medical Sciences. Since it has been shown for the past several years that the DMBA adducts can be detected after 24 hrs post-DMBA treatment, we therefore used 24 hrs as the marker to detect the DNA methylation status [20,21]. At age 50 days, all rats were orally gavaged with 65 mg/kg DMBA (Sigma Chemical Co., St. Louis, MO) in sesame oil. Rats were weighed twice per week (Monday and Thursday at 9:00 AM). Rats were sacrificed 24 hours post-DMBA treatment; serum was collected and stored until analysis.

HPLC method: All methodological details for determination of SAM, SAH, adenosine, and thiols are previously published [22,23]. Briefly, 100 µl of 10% meta-phosphoric acid was added to 200 µl of plasma to precipitate protein; the solution was mixed well, and incubated on ice for 30 minutes. After centrifugation for 15 minutes at 18,000g at 4°C, supernatants were passed through a 0.2 µm nylon membrane filter and were injected into the HPLC system. The analyses were performed using HPLC with a Shimadzu solvent delivery system (ESA model 580) and a reverse phase C18 column (5 µm; 4.6 × 150 mm, MCM, Inc., Tokyo, Japan) obtained from ESA, Inc. (Chemsford, MA). A 20 µl aliquot of plasma extract was directly injected onto the column using Beckman Auto sampler (model 507E). All plasma metabolites were quantified using a model 5200A Coulochem II and CoulArray electrochemical detection systems (ESA, Inc., Chelmsford, MA) equipped with a dual analytical cell (model 5010), a 4-channel analytical cell (model 6210), and a guard cell (model 5020). The concentrations of plasma metabolites were calculated from peak areas and standard calibration curves using HPLC software.

Statistical analysis

The data are presented as mean ± SD and were assessed by two-way analysis of variances (ANOVA). A P-value of<0.05 was considered as significant.

Results

The average body weight at the beginning of the experiment for lean rats was 74.83g, and for obese rats, the average body weight was 109.83g. At the end of the experiments, the average body weights (mean + standard error) were 127.33 g + 3.7 g for lean rats and 217.83 g + 7.51 g for obese rats. The lean rats gained more than 52 g and the obese rats gained more than 90 g during course of this experiment.

Obesity changed the biochemical profiles in serum for both methylation-related and oxidative stress-related metabolites. As shown in Table 1, obese rats had a significantly (P=0.024) lower level of free reduced glutathione (fGSH) compared to lean rats. On the other hand, obese rats have a much higher (P=0.007) level of free oxidized glutathione (fGSSG) compared to lean rats. Obesity lowered the

concentration of reduced free glutathione, and increased concentration of free oxidized glutathione (P=0.003) reduced the fGSH:fGSSG ratio, or "intracellular oxidative ratio," compared to lean rats. A serum level of glutathione are reflecting and representing intracellular changes. In addition to changes of glutathione concentration in serum, obese rats also had a significantly (P=0.002) lowered level of free cysteine and a significantly (P=0.001) higher concentration of free cystine (the oxidized form of cysteine) compared to lean rats. All of these changes, were accompanied with a significant (p=0.001) increase of fCystine:fCysteine ratio, known as "extracellular oxidative ratio," in obese rats.

We observed a significantly (P=0.041) lower level of Glutamyl-Cysteine (a metabolic precursor in synthesis GSH) in the serum of obeserats compared to lean group. Meanwhile, serum concentration of Cystein-Glycine (a product of catabolism of GSH) in obese rats was significantly (P=0.031) higher compared to lean rats. As a consequence of the increases in both oxidative ratios (fGSH:fGSSG and fCystine:fCysteine) in the serum of obese rats, we observed a significantly (P=0.04) increased concentration of 3-nitro-Tyrosine in obese rats compared to the lean group. 3-nitro-Tyrosine is a modified amino acid Tyrosine that can be formed under oxidative-reduction imbalance in obese rats.

As shown in Table 2, obesity significantly (P=0.042) lowered the serum concentration of methionine compared to the lean rats. The serum concentration of free reduced homocysteine was significantly higher (P=0.034) in obese animals compared to lean rats. Both methionine and homocysteine alternative changes are followed by an increased concentration of adenosine (P=0.005) in obese rats compared to lean rats. Despite a decrease of serum concentration of S-adenosylmethionine (SAM) that was not significant between obese and lean rats, serum concentration of the product of S-adenosylhomocysteine (SAH) was significantly higher (P=0.025) in obese rats compare to lean rats leading to a decrease of almost twice (P=0.023) of the SAM: SAH ratio, known as "methylation ratio".

	Lean	Obese	P
fGSH, µmol/L	1.571 ± 0.116	1.402 ± 0.111	0.024
fGSSG, µmol/L	0.232 ± 0.0411	0.327 ± 0.055	0.007
fGSH:fGSSG	6.93 ± 1.23	4.41 ± 0.95	0.003
fCystine, µmol/L	26.79 ± 4.43	38.02 ± 3.21	0.001
fCysteine, µmol/L	36.94 ± 5.27	26.32 ± 3.08	0.002
fCystine:fCysteine	0.752 ± 0.214	1.47 ± 0.273	0.001
Glu-Cys, nmol/ml	0.31 ± 0.05	0.25 ± 0.04	0.041
Cys-Gly, nmol/ml	4.64 ± 0.72	5.67 ± 0.79	0.031
3-nitro-Tyrosine, pmol/ml	25.98 ± 7.324	35.65 ± 8.092	0.04

Mean ± SD

Table1: Concentration of oxidative stress related thiols in serum of obese and lean rats.

	Lean	Obese	P
Methionine, nmol/ml	43.68 ± 8.41	34.55 ± 6.01	0.042
fHomocysteine, nmol/ml	1.284 ± 0.237	1.644 ± 0.299	0.034
Adenosine, pmol/ml	305.3 ± 63.5	438.1 ± 63.3	0.005
SAM, pmol/ml	221.5 ± 68.2	179.7 ± 35.3	0.13
SAH, pmol/ml	14.77 ± 3.99	24.88 ± 8.94	0.025
SAM:SAH	15.83 ± 6.79	7.98 ± 3.02	0.023

Mean ± SD

Table 2: Concentration of methylation related metabolites in serum of obese/ treated and lean/treated rats.

Discussion

Oxidative stress plays a major role in the lives of cells under physiological and pathological conditions. Oxidative/reduced balance in intracellular and extracellular compartments of cells is equally important for cells homeostasis. Changes in this balance can have negative consequences on biochemical and morphological stability of the cell [24-26].

Free reduced glutathione is a major intracellular antioxidant. When reduced glutathione becomes oxidized, glutathione is transported in extracellular compartments including blood, and blood can reflect of intracellular status of these metabolites. We found that obesity decreased the fGSH:fGSSG ratio. Moreover, the ratio of cystine: cysteine, which is known as the extracellular reflection of oxidative stress, increases in obese rats which additionally support our finding with free glutathione. Based on changes of these two ratios, a decrease intracellular and extracellular antioxidative capacity in obese animals makes them a potentially more vulnerable target for reactive oxygen species (ROS) and nitrogen oxygen species (NOS) to attack and harm cellular homeostasis resulting in hard consequences on many intracellular reactions, including involvement of proteins, lipids, DNA, and RNA. As evidence of this much higher pro-oxidative environment in obese rats, one of the possible candidate molecules representing protein damage is 3-nitrotyrosine [27,28]. 3-nitrotyrosine (3-NT) is modified (damaged) amino acid by NOS and capable change proteins properties which will lead to change enzymes activity. The significantly increased serum level of 3-NT in obese rats compared to lean rats confirms and supports our finding and conclusions about negative influence of pro-oxidative state in obese rats compared to lean rats. Moreover, we can speculate that pro-oxidative intracellular conditions in obese rats is also capable of changing the status of the sulfur-containing amino acid cysteine and makes it potentially more oxidized which can be additionally to modify by NOS and damage proteins.

As we have shown earlier, a decrease in the serum concentration of cysteine and an increase of the cystine:cysteine ratio in obese rats is capable of having a potentially negative effect on glutathione synthesis because cysteine is a rate limiting amino acid. To support this statement, we analyzed serum concentrations of two intermediate metabolites involved in glutathione synthesis; free Glu-Cys and glutathione catabolism free Cys-Gly. The serum concentration of Glu-Cys in obese rats was lower and the serum concentration of Cys-Gly was higher in obese rats compared to lean rats leading to a deficiency of GSH and intracellular antioxidant capacity.

Methylation is an important biological reaction to keep metabolic balance and structural integrity of cells. One of the important aspects of cell life and genetic stability is DNA methylation. DNA uses SAM as a methyl group donor. Despite the fact that obese rats did not develop significant SAM deficiency, they had a higher level of SAH and a SAM/SAH methylation ratio almost two times lower that has suppressive methylation properties [29,30]. Moreover, we observed an increasing level of homocysteine in obese rats, which is a key point in the methionine metabolism cycle. If homocysteine is not converted to methionine through homocysteine remethylation using folate as a methyl group donor, it contributes to decreased levels of serum methionine in obese animals. Also, an increased homocysteine

concentration in obese animals decreases cysteine concentration through the transsulfuration pathway and decreases the synthesis of glutathione. Homocysteine plays a key role in interconnection of both methylation and transsulfuration reactions producing profound consequences for cells integrity and homeostasis.

In summary, we observed that obese rats have a significant impairment in antioxidative capacity accompanied by a methylation deficiency and genetic instability that can potentially have a Procarcinogenic effect.

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