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Title Page

Drug Metabolizing and Antioxidant Enzymes in Monosodium L-Glutamate Obese Mice

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Running Title Page

Running title: Drug Metabolizing and Antioxidant Enzymes in Obese Mice

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Abbreviations:

AKR, aldo-keto reductases; ARE, antioxidant response element; CAT, catalase; CBR1, carbonyl reductase 1; CDNB, 1-chloro-2,4-dinitrobenzene; DME, drug-metabolizing enzyme; GPx, glutathione peroxidase; GST, glutathione S-transferase; GR, glutathione reductase; HPRT1, hypoxanthine-guanine phosphoribosyltransferase 1; MSG, monosodium l-glutamate; NCBI, National Center for Biotechnology Information; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, NF-E2-related factor 2; qPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species; RPlP0, ribosomal protein, large P0; SI, small intestine; SOD, superoxide dismutase; TR, thioredoxin reductase; UGT1A, UDP-glucuronosyltransferases 1A

Abstract

The prevalence of obesity is rapidly increasing across the world. Physiological alterations associated with obesity are known to alter enzymes expression and/or activities. As drug-metabolizing and antioxidant enzymes serve as defense system against potentially toxic compounds, their modulation might have serious consequences. Here we studied selected antioxidant and drug-metabolizing enzymes (DME) in monosodium glutamate (MSG)-mouse model of obesity. Specific activities, protein and mRNA expressions of these enzymes in liver as well as in small intestine were compared in obese male mice and in their lean counterparts. Furthermore, expression of the NF-E2-related factor 2 (Nrf2) and its relation to obesity was tested. Obtained results showed that obesity affects expression and/or activities of some DME and antioxidant enzymes. In obese mice, up-regulation of UDP-glucuronosyltransferases 1A (UGT1A), NAD(P)H: quinone oxidoreductase 1(NQO1), nuclear transcription factor Nrf2 and down regulation of some isoforms of glutathione S-transferases (GST) were observed. Most of these changes were tissue and/or isoform specific. NQO1 seems to be regulated transcriptionally via Nrf2, but other enzymes might be regulated post-transcriptionally and/or post-translationally. Enhanced expression of Nrf2 in livers of obese mice is expected to play a role in protective adaptation. On the other hand, elevated activities of NQO1 and UGT1A may cause alterations in drug pharmacokinetics in obese individuals. Moreover, decreased capacity of GST in obese animals indicates potentially reduced antioxidant defense and weaker chemoprotection.

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Introduction

Obesity, disease characterized as a condition resulting from the excess accumulation of body fat, has become one of the most important public health problems worldwide. The importance of obesity as a risk factor for a number of conditions, including type 2 diabetes, cardiovascular diseases, hypertension, certain types of cancer and metabolic syndrome, is well documented (Pi-Sunyer, 2003). A number of studies have revealed the link between obesity, type 2 diabetes and oxidative stress. In obese organism generation of excessive reactive oxygen species (ROS) increases, which may trigger pro-inflammatory signaling pathways and consequently modulate gene expression of several pro-inflammatory cytokines. In addition, increased production of ROS can lead to activation of stress-sensitive signaling pathways, which in turn impair insulin secretion and action (Houstis et al., 2006). ROS increase can also alter the total antioxidant capacity of the cell. Even though numerous studies exist, available information regarding antioxidant enzymes in obese status are far from being complete.

Obesity and associated pathologies are often treated by different therapeutic agents, metabolized by drug metabolizing enzymes (DMEs). The levels of DMEs are influenced by a variety of factors, such as age, gender, nutritional status, exposure to drugs or environmental chemicals as well as diseases and other pathologies. Over the past two decades various studies have been conducted in order to identify the effect of obesity on drug metabolism. Many studies using different nutritional and genetic rodent models of obesity identified differences in the expression and activity of DMEs in obese and lean animals. However, most reports were focused only on hepatic cytochromes P450 activities (Roe et al., 1999; Kim et al., 2004; Yoshinari et al., 2006; Cheng et al., 2008; Kudo et al., 2009; Ghose et al., 2011) and information about other DMEs is insufficient.

Due to lacking information, present study was designed to test activities, protein and gene expression levels of other DMEs and antioxidant enzymes. For this purpose, model of murine obesity, in which the subcutaneous injection of monosodium l-glutamate (MSG) to newborn mice leads to central obesity and type 2 diabetes resembling hypothalamic obesity (Olney, 1969; Perello et al., 2004; Balbo et al., 2007), was used. Apart from stunted body growth, disturbances in body weight control

and increased adiposity, adult MSG mice show hyperleptinemia, hyperglycemia, hyperinsulinemia and insulin resistance (Shapiro et al., 1993; Balbo et al., 2000; Macho et al., 2000; Matyskova et al., 2008) making it a useful model of the metabolic syndrome. Development of obesity in this model is explained by lower metabolic rate and lack of physical activity (Martins et al., 2001; Maletinska et al., 2006) rather than excessive energy intake. Low intensity swimming exercise at early age can improve or attenuate onset of obesity in this model (Scomparin et al., 2006; Andreazzi et al., 2009). Indispensable advantage of this model is that obesity develops without hyperphagia, in contrast to most of the genetic models and also there is no need of high-fat diet, which may compromise various nutritional intervention studies. MSG-obese mice have been also reported as a suitable model for diabetes (Nagata et al., 2006; Sasaki et al., 2009) and non-alcoholic liver steatosis (Sasaki et al., 2011; Franca et al., 2014; Fujimoto et al., 2014), conditions often treated by various drugs. Moreover, this model is used for nutritional intervention studies, such as effect of *Hibiscus sabdariffa* (Alarcon-Aguilar et al., 2007), Red yeast rice (Fujimoto et al., 2012b) or Spirulina (Fujimoto et al., 2012a).

Owing to all these facts it is of considerable importance to analyze DMEs just in MSG-obese mice but the only previous study of DMEs in MSG-obese mice reportedsuppression of sex-dependent hepatic cytochromes P450 2C11, 2A2 and 3A2 via blocked growth hormone secretion (Pampori and Shapiro, 1994). Present complex study was focused on analysis of other DMEs, namely carbonyl reductase 1 (CBR1), NAD(P)H:quinone oxidoreductase 1 (NQO1), aldo-keto reductases (AKR), UDP-glucuronosyltransferases 1A (UGT1A), glutathione S-transferases (GST) and antioxidant enzymes as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), thioredoxin reductase (TR). DMEs and antioxidant enzymes were studied together as both groups of enzymes serve as defense system against potentially toxic compounds. Above that, some of these enzymes (e.g. GST, NQO1) are considered to be drug-metabolizing as well as antioxidant enzymes. In addition, we also tested expression of transcriptional factor NF-E2-related factor 2 (Nrf2), a central regulator of antioxidant and detoxification gene expression in response to electrophilic or oxidative stress.

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Materials and Methods

Chemicals and Reagents.

Protease inhibitor cocktail tablets (EDTA free Complete Protease Inhibitor Cocktail Tablets) were supplied by Roche (Mannheim, Germany), SOD Assay Kit-WST (Dojindo, Tabaru, Japan) was purchased from Probior (München, Germany). Protein standard for electrophoresis and non-fat dry milky were obtained from Bio-Rad (Hercules, CA, USA), RNAlater from Qiagen (Austin, Texas, USA), TriReagent from Molecular research center (Cincinnati, OH, USA), DNase I and ProtoScript II reverse transcriptase from NEB (Whitby, ON, USA), qPCR Core kit for SYBR Green I from Eurogentec (Seraing, Belgium), SYBR Safe DNA gel stain from Invitrogen (Carlsbad, CA, USA). All primers were synthesized by Generi Biotech (Hradec Králové, Czech Republic). Leptin and insulin ELISA assay kits were supplied by BioVendor (Brno, Czech Republic).

Primary antibodies for detection of proteins (diluted as follows in brackets) were purchased from Abcam (Cambridge, UK):β-actin, AKR1A1, GSTA, GSTM (1:3000), AKR1C3, CBR1, GSTP (1:5000), calnexin, SOD, CAT, GPx2 (1:2000); Novus Biologicals (Cambridge, UK):GR, TR (1:2000), NQO1(1:3000). Primary antibodies for detection of UGT1A (1:300) and secondary antibodies (anit-mouse, anti-rabbit and anti-goat) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used in dilution 1:3000. Western blotting was done using a chemiluminescence kit from GE Healthcare (Buckinghamshire, UK).

All other chemicals, which were of HPLC or analytical grade, were obtained from Sigma-Aldrich (Prague, Czech Republic).

Animals and Treatments

NMRI mice obtained from BioTest (Konárovice, Czech Republic) were housed in air-conditioned animal quarters with a 12 h light/dark cycle at 23 °C. Food (standard chow diet ST-1, Velaz, Czech Republic) and tap water were provided *ad libitum*. The mice were cared for and used in accordance with the Guide for the Care and Use of Laboratory Animals (Protection of Animals from Cruelty Act No. 246/92, Czech Republic). Ethical Committee of Charles University in Prague, Faculty

of Pharmacy in Hradec Králové approved all animal experimental procedures (Permit Number: 34354/2010-30).

Newborn male mice were divided into two groups: 1) mice with MSG-induced obesity and 2) control mice. For hypothalamic lesion-induced obesity, MSG (4 mg/g body weight, s.c.) was administered to newborn mice daily from postnatal day 2 to 8 (from day 2 to 6 mice received 10 mg/day, 2 following days 20 mg/day). Controls were treated with saline of osmolality corresponding to the MSG solution (Matyskova 2008). Both groups (10-12 animals per group) were fed *ad libitum*, lean mice consumed on an average 5.0 g/day, while obese mice ate only 4.3 g daily. Body weight and food intake were monitored once a week. At 8 month of age, mice were fasted for 12 h and sacrificed by cervical dislocation. Blood samples were collected into K_3EDTA coated plastic tubes, plasma and erythrocytes were separated immediately by centrifugation (3000 rpm, 10 min, 10 °C). Liver was dissected, washed with saline buffer containing protease inhibitor cocktail tablets and immediately frozen in liquid nitrogen, and the entire small intestine (SI) was similarly washed from the inside content and frozen. Small pieces of each tissue were separately placed in RNAlater solution. All biological samples were stored in freezer at -80°C until further use.

Plasma Leptin and Insulin Levels

Leptin and insulin concentrations in plasma were quantified by ELISA assay kits according to manufacturer's instructions.

Preparation of Subcellular Fractions

Microsomal and cytosolic fractions were obtained from liquid nitrogen-frozen liver and SI of mice. Pieces of entire SI or liver were individually homogenized in 0.1 M sodium phosphate buffer (pH 7.4) at the ratio of 1:6 (w/v; liver) or 1:5 (w/v; SI), using a Potter-Elvehjem homogenizer and sonication with Sonopuls (Bandelin, Germany). The subcellular fractions were isolated by differential centrifugation of the tissue homogenate. The supernatant from first centrifugation (5000g, 20 min) was centrifuged at 20000g for 60 min. Resulting supernatant was further processed by the centrifugation at 105000g (60 min, 4°C). Supernatant and sediment from this step correspond to cytosol and

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microsomes, respectively. A rewashing step in a 0.1 M sodium phosphate buffer, pH 7.4 (followed by the second ultracentrifugation) was incorporated in the preparation of microsomes, which were finally resuspended in 0.1 M sodium phosphate buffer (pH 7.4) containing 20% glycerol (v/v). All fractions were stored at −80°C. Protein concentrations in subcellular fractions were assayed using the bicinchoninic acid assay (BCA) according Sigma-Aldrich protocol.

Enzyme Assays

Enzyme activities were assayed in the cytosolic and microsomal fractions obtained from homogenate of mouse liver and SI. The enzyme activities assays (each performed in 4-8 replicates) were repeated three times. The amount of organic solvents in the final reaction mixtures did not exceed 1% (v/v). The assays of all enzymes were based on spectrophotometric detection of product formed or detection of decreasing substrate/cofactor levels using microplate reader Tecan Infinite M200 (Tecan; Männedorf, Switzerland). Selected parameters for evaluation of oxidative stress were determined using slight modifications of previously published methods.

Activity of GPx was assayed by a coupled reaction with GR. GPx mediated reduction of *tert*-butyl peroxide leads to oxidation of glutathione, whose subsequent reduction by GR is accompanied by decrease in NADPH, which is monitored as a decrease in absorbance at 340 nm (ε_{NADPH} = 6,22 $mM^{-1}.cm^{-1}$) (Flohe and Gunzler, 1984; Handy et al., 2009).

CAT activity was assayed according to the method described by Goth (Goth, 1991), which is based on the detection of remaining H_2O_2 in reaction mixture. H_2O_2 forms a yellow complex with ammonium molybdate, which was measured at 405 nm.

The assay of SOD was performed using SOD Assay Kit-WST according to the general protocol. The inhibition activity of SOD on superoxide anion radical formation leads to lower formazan production, which can be determined by a colorimetric method. The activity of SOD is expressed as the decrease ($nmol \cdot min^{-1} \cdot mg^{-1}$) in formazan production.

TR and GR activities were measured according to Bonilla et al. (Bonilla et al., 2008). TR activity was assayed by the method using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). TR reduces

DTNB by NADPH into yellow 5′-thionitrobenzoic acid, whose formation was followed at 412 nm for 5 min. The method for assay of GR activity is based on the NADPH-dependent reduction of oxidized glutathione. Resulting decrease in absorbance due to the consumption of NADPH was observed for 6 min at 340 nm.

Activity of NQO1 was assayed by measuring cytochrome c reduction in the presence of NADH and menadione as an intermediate electron acceptor. The increase in reduced cytochrome c (ε=28 mM^{-1}. cm^{-1}) was observed for 5 min at 550 nm. The activity corresponding to NQO1 activity in the cytosol was that which was inhibited by dicoumarol, a known inhibitor of NQO1 (Fitzsimmons et al., 1996; Cullen et al., 2003).

The activities of carbonyl reducing enzymes (AKR1A, AKR1C and CBR1) were assessed in cytosol using the following substrates: 4-pyridinecarboxaldehyde (1 mM; AKR1A), acenaphthenol (1 mM; AKR1C) and menadione (0.5 mM; CBR1).Spectrophotometric determination (detection wavelength 340 nm, 25°C) of NADPH consumption (in the case of acenaphthenol NADPH formation) in the reaction mixture served for the assessment of reductase/dehydrogenase activities (Maser, 1995; Ohara et al., 1995; Palackal et al., 2001; Mate et al., 2008).

The UGT1A activity was assayed using *p*-nitrophenol as a specific substrate towards UGT1A according to Mizuma et al. (Mizuma et al., 1982). The final concentration of *p*-nitrophenol was 0.5 mM. The decrease in concentration of *p*-nitrophenol (ε = 18.3 $mM^{-1}.cm^{-1}$) was measured after 20 min of incubation at 405 nm.

Cytosolic GST activity was determined using 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrates. The increase of absorbance attributing to formation of glutathione conjugate was measured at 340 nm (Habig et al., 1974).

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting

Microsomal or cytosolic proteins of mice liver and SI were separated by SDS-PAGE (10% stacking gel) and subsequently transferred onto nitrocellulose membranes (450 nm) using Trans-Blot® TurboTM Transfer System (Bio-Rad, Hercules, CA, USA). The membranes were blocked for 2h in

5% non-fat dry milk dissolved in TBS-Tween-20 (150 mM NaCl, 10 mM Tris; pH 8.0 and 0,3% Tween 20).

For immunodetection, the membranes were probed overnight with primary antibodies at dilutions described in Chemicals and Reagents section in TBS–Tween 20 supplemented with 1% BSA, washed four times with TBS–Tween 20 buffer and probed with the complementary secondary antibodies for 1 hour. The membranes were rinsed four times with TBS-Tween 20, and the signal was detected using enhanced chemiluminescence kit according to the manufacturer´s instructions. β-Actin and calnexin served as the loading controls. Intensity of bands was evaluated using a C-DiGit™ Blot Scanner (Li-Cor, Bad Homburg, Germany).

Total RNA Extraction and cDNA Synthesis

Approximately 50 mg of liver or SI tissue were used for total RNA extraction using TriReagent according to manufacturer´s instructions. The homogenization of the samples was performed with a pestle microhomogenizer in 1.5 ml Eppendorf tube using 1 ml of TriReagent per 50 mg of tissue. RNA yields and purity were determined measuring the absorbance at 260 and 280 nm using NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). All samples had absorption ratio A260/A280 greater than 1.8. The quality of RNA was checked by agarose gel electrophoresis and the integrity by 3´:5´assay according to Nolan et al. (Nolan et al., 2006). Ten µg of RNA were treated with DNase I to avoid genomic DNA contamination for 20 min at 37°C, inactivated by heat (10 min at 75°C) and diluted to concentration 0.2 µg/µl. RNA was stored at −80°C until further analyses. First strand cDNA synthesis was carried out using ProtoScript II reverse transcriptase and random hexamers (or oligo-dT for 3´:5´assay) following the manufacturer's protocol. After initial heat denaturation of 1 µg of total RNA (65°C for 5 min), the reactions (20 µl) were incubated for 10 min at 25°C, for 50 min at 42°C and for 15 min at 75°C. Obtained cDNAs were diluted 10x (or 36000x for 18S analyses) prior to quantitative polymerase chain reaction (qPCR). All cDNAs were stored at −20°C until qPCR assay.

Primer design, Quantitative Real-Time PCR

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Relative mRNA expression levels were measured for GR, SOD, CAT, TR, GPx2, GSTA1/2 (because of>90% similarity, one primer set was designed to recognize both GSTA1 and GSTA2 isoforms), GSTA3, GSTA4, GSTM1, GSTM3, GSTP1/2 (because of >90% similarity, one primer set was designed to recognize both GSTP1 and GSTP2 isoforms), AKR1A, AKR1C6, AKR1C20, CBR1, NQO1, UGT1A (primer set designed against 5th exon common for UGT1A subfamily), Nrf2, and three reference genes 18S, hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) and ribosomal protein, large P0 (RPlP0) selected based on previous experiments with MSG-obese mice (Matouskova et al., 2014).

The primers were designed manually using Primer3 software (Untergasser et al., 2012) and the specificity of the primers was checked by NCBI Blast tool. Analyzed genes (with their corresponding NCBI accession numbers), the primer sequences and amplicon sizes are listed in Table 1.

The qPCR analyses were performed in iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using SYBR Green I detection in a final volume of 20 µl. The reaction mixture consisted of components from qPCR Core kit for SYBR Green I as specified by manufacturer, both forward and reverse primers (final concentration 100 nM) and 5 µl of diluted cDNA. Two batches of diluted cDNA (5 µl, corresponding to 50 ng of reverse transcribed RNA) were subjected to qPCR to amplify all target genes, for mRNA normalization reference genes were measured in both batches separately.

The PCR reactions were initiated by the denaturation step of 10 min at 95°C, followed by 40 cycles of amplification, which were performed according to the following thermo cycling profile: denaturation for 10 sec at 95°C, annealing for 20 sec at 60°C and extension for 20 sec at 72°C. Fluorescence data were acquired during the last step. Dissociation protocol with a gradient (0.5°C every 30 s) from 65°C to 95°C was used to investigate the specificity of the qPCR reaction and presence of primer dimers. Gene-specific amplification was confirmed by a single peak in the melting curve analysis. The size of all amplicons was confirmed by 2% agarose gel electrophoresis stained with SYBR Safe DNA gel stain. The sample maximization method criterion was used to establish the run layout.

The absence of contamination from either genomic DNA amplification or primers dimers formation was ensured using two types of controls, the first one without reverse transcriptase (no-RT control, one for each RNA), and the second one with no DNA template (NTC control, one for each primer pair). All qPCRs were run in duplicates, the average standard deviation within duplicates of all samples studied was 0.15 cycles. qPCR efficiencies in the exponential phase were calculated for each primer pair by standard curves (5point 5-fold dilution series of pooled cDNA), the mean quantification cycle (Cq) values for each serial dilution were plotted against the logarithm of the cDNA dilution factor and calculated according to the equation E=10[-1/slope] (Bustin et al., 2009). The amplification efficiencies for all studied genes ranged from 91% to 109%. Calculations were based on the "Delta-Delta Ct method" (Livak and Schmittgen, 2001). The relative mRNA levels were normalized to geometric mean of Cq values of two reference genes; 18S and HPRT1 or 18S and RPlP0 in liver or SI, respectively (Matouskova et al., 2014). The data were expressed as fold change of the obesity groups relative to the control.

Statistical Analysis

All calculations were done using Microsoft Excel and GraphPad Prism 6.0. Nonparametric Mann-Whitney test was used for the statistical evaluation of differences between obese and control group. The differences were regarded as significant when $P < 0.05$.

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Results

Body Weight, Plasma Insulin and Leptin levels

MSG mice were significantly heavier than control mice at the age of eight months. As previously reported, MSG mice were hypophagic, they consumed less food than control mice. In addition, MSG mice developed hyperleptinemia (28x higher amount of leptin than control mice) and hyperinsulinemia (7x higher amount of insulin than control mice).Table 2 illustrates the MSG-induced obese status, *i.e.* body average weight and food consumption (measured in 4 last weeks), plasma insulin and leptin levels.

Antioxidant Enzymes Activity, Protein and mRNA.

Enzymatic activities, assayed using specific substrates, and protein expressions of several antioxidant enzymes (CAT, SOD, GPx, GR, and TR) were determined in cytosolic fraction of pooled samples obtained from mouse liver and SI, while corresponding levels of mRNA were measured in samples from individual animals. The results are summarized in Table 3.

In liver, GPx activity was significantly lower in obese animals compared to control mice, while in the SI the GPx activity was markedly increased. In mice, GPx comprises eight isoforms (Brigelius-Flohe and Maiorino, 2013), here we tested mRNA level of GPx2 isoform only, which is prevalent in intestinal epithelium. Although GPx activity in SI was elevated, mRNA of GPx2 isoform was down-regulated in obese mice. Similarly, immunoblotting using specific anti-GPx2 polyclonal antibody showed lower amount of the respective protein isoform in obese mice. In liver, immunoblotting did not detect any GPx2 protein, however GPx2 mRNA was detectable in liver and the amount of GPx2 mRNA was higher in obese mice compared to lean controls. The specific activities of GR, TR and CAT in the SI were significantly lower. Immunoblotting showed higher relative amount of GR and CAT proteins in obese animals, whereas mRNA quantity of these enzymes remained unchanged. Despite the TR protein amount was unchanged, activity as well as relative TR mRNA quantity were lower in SI from obese mice in comparison to control mice.

Phase I Drug-Metabolizing Enzymes Activity, Protein and mRNA.

The specific activities of several Phase I biotransformation enzymes were tested (CBR1, NQO1, AKR1A and AKR1C). The results are summarized in Table 4.

In liver of obese animals, the activity of CBR1 was up-regulated, but on protein and mRNA level the up-regulation was not significant. In case of hepatic NQO1, a significant increase of specific activity, protein and mRNA levels was found in obese mice. In SI, no differences for both CBR1 and NQO1 were detected; their activity, mRNA and protein levels remained unchanged. AKR1C subfamily comprises eight isoforms, here we tested only liver specific isoforms AKR1C6 and AKR1C20 and significant up-regulation at the mRNA levels of both isoforms was detected. However, no change in AKR1C activity and protein expression was observed. AKR1A specific activity, assayed using 4-pyridinecarboxaldehyde as a model substrate, did not differ between lean and obese mice. At the mRNA and protein level the expression was unchanged in liver, however, in SI, both mRNA and protein amounts, were increased.

Phase II Drug-Metabolizing Enzymes Activity, Protein and mRNA.

The specific activities, mRNA and protein levels of two types of conjugating enzymes were determined in microsomal (UGT1A) and cytosolic (GST) fractions obtained from murine liver and SI. The results are summarized in Table 5. In liver of obese mice, the specific activity of total UGT1As was higher compared to lean controls. Relative mRNA quantity of UGT1A family (primer set was designed to recognize all members of the UGT1A family) was also increased in liver of obese mice. Correspondingly, UGT1A protein levels in obese mice were higher in comparison to lean mice. In the SI, the activity of UGT1A was under the detection limit and UGT1A mRNA and protein levels were similar in obese and lean mice.

Total activity of hepatic **GSTs,** measured using universal substrate CDNB, was lower in obese mice than in lean ones, but in SI remained unchanged. Specific activity towards CDNB substrate was approximately four times lower in SI than in liver (data not shown). Immunoblotting using class-specific polyclonal antibodies showed significant changes in all GST classes studied; GST proteins from M- and A-classes were in both tissues increased in obese mice, while P-class GST protein level was lower in liver and higher in SI. In mice, GST superfamily consists of 19 isoforms (Cui et al.,

2010). In our study, mRNA levels of seven selected isoforms were measured. Some significant differences on the mRNA level in obese and lean animals were observed in both tissues. For example, amount of GSTM3 isoform was higher in liver but lower in SI from obese mice (not statistically significant). GSTP1/2 mRNA was significantly decreased in liver from obese mice, but not affected in SI.

Nrf2 mRNA

Considering the transcription factor Nrf2 as a prime regulator of antioxidant and detoxification enzymes upon oxidative stress, we tested its relative mRNA quantity in both tissues. In liver of obese mice, Nrf2 mRNA was significantly increased (fold change: 2.25 ± 0.42), while in SI it was not changed significantly.

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Discussion

There are many indications that pathologies including obesity may be a source of inter-individual variability in the expression and activities of DMEs and antioxidant enzymes. Decrease in antioxidant enzymes activities may lead to oxidative stress with many possible consequences, the changes in the activity of DMEs might modulate the pharmacokinetics and/or pharmacodynamics of therapeutic agents that are commonly administered to obese individuals for the treatment of various comorbidities associated with obesity (Blouin and Warren, 1999).

The aim of present study was to evaluate the effect of obesity on expression and activity of antioxidant enzymes and DMEs in obese mice. Hepatic and small intestinal enzymes were studied concurrently to obtain more complex information about possible changes in drug metabolism of obese mice. Animals with MSG-induced obesity were chosen, as this animal model is often used for various obesity-related applications, from disease based studies (*e.g.* diabetes, non-alcoholic liver steatosis and metabolic syndrome) to drug toxicity and nutritional intervention testing.

In our study, application of MSG to newborn mice resulted in the increased body weight (by 50%) as well as in hyperleptinemia and hyperinsulinemia which confirmed a disease status as previously published (Matyskova et al., 2008).

Similarly as Yang et al. (Yang et al., 2000), we observed reduced hepatic GPx activity in obese mice, however SOD and CAT activities remained unchanged. Furukawa et al. (Furukawa et al., 2004) observed reduced SOD, GPx and CAT activities in adipose tissue, but not in the liver or muscle of obese mice. Noeman et al. (Noeman et al., 2011) reported decrease in the activity of GPx, but not CAT in the hepatic tissue of obese rats. Such discrepancy in activities of antioxidant enzymes in obese state can be caused by different obesity models and differences between early stages of obesity and chronic obesity (Vincent and Taylor, 2006). Lower enzymatic activities (GR, TR), higher protein amounts, whereas unchanged mRNA levels were observed in SI of obese mice. Such inconsistency is often seen, since correlation between these biological molecules can be weak, possibly due to different regulation, slower protein degradation, enzyme inactivation, etc. In the case of NQO1, significant

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increase in mRNA and protein levels leading to elevated activity of NQO1was observed in livers of MSG-obese mice. Likewise Cheng et al. (Cheng et al., 2008) observed 3.6-fold NQO1 mRNA increase in livers of male ob/ob mice. NQO1, a multifunctional flavoprotein that detoxifies quinones by two-electron reduction to hydroquinones, has been extensively studied for its chemoprotective and antioxidant properties (Ross and Siegel, 2004). Apart from reduction and detoxification of exogenous quinones, this enzyme also participates in reduction of endogenous quinones such as vitamin E quinone and ubiquinone, which provides increased protection against lipid peroxidation (Kohar et al., 1995). Hence observed increased NQO1 activity could be a protective response to induced obesity.

UGT1A is a complex family of enzymes with distinct but overlapping substrate specifities. These enzymes play an essential role in enhancing elimination of eobiotic and xenobiotic compounds, containing expanding amounts of clinically relevant drugs. In our study, higher glucuronidation activity towards *p*-nitrophenol and elevated levels of expression of UGT1A family were observed in obese mice. Similarly Xu et al. (Xu et al., 2012) observed obesity-induced isoform specific expression of UGT1A1,-1A6, -1A7 and 1A9 and higher (47%) acetaminophen glucuronidation in ob/ob mouse liver. These findings suggest that obesity may on one hand provide higher detoxification capacity of liver, *e.g.*, protection from acetamoniphen-induced hepatotoxicity, but on the other hand may enhance deactivation of many drugs or conversion of certain drugs to highly reactive metabolites (*e.g.* non-steroidal anti-inflammatory drugs) (Ritter, 2000). All clinical studies using obese subjects show significant increase in UGT-mediated biotransformation (Brill et al., 2012), e.g. acetaminophen and oxazepam, drugs undergoing glucuronidation, showed higher clearance in obese compared to non-obese individuals (Abernethy et al., 1983).

GSTs belong to crucial phase II enzymes, defending cells against oxidative stress and variety of toxic chemicals and metabolites. In the current study, catalytic activity detected using CDNB, the universal GST substrate, was lower in liver from obese animals than from lean mice. On the contrary Koide et al. (2011) reported higher total GST activity in obese/diabetic mice. Similarly as our results Roe et al. (1999) reported lower GST activity in genetically obese (ob/ob) male mice than in lean counterparts. In obese children and adults, GST-mediated biotransformation of busulfan was slowed

down compared to non-obese individuals when normalized for body weight (Gibbs et al., 1999). Transcriptomic analysis showed much lower ($P<0.001$) relative expression of GSTP1/2 in liver of obese mice, which corresponds to lower GSTP-class protein amount and might be responsible for lower catalytic activity. Human GSTP1 polymorphism was recently associated with increased susceptibility to diabetes and abdominal obesity (Amer et al., 2012). Moreover, diminished GSTP1/2 expression in liver suggests that obese individuals are more prone to cancer (Esteller et al., 1998; Helzlsouer et al., 1998). On the other hand, ΔPMT mice strain lacking three GST classes (GSTP, M, T) were surprisingly healthy and fertile animals with normal life expectancy (Xiang et al., 2014).

With aim to bring deeper information, the possible regulation mechanism of enzyme expression was also studied in MSG-obese mice. It is known that expression of antioxidant and DME genes is regulated by many transcription factors and nuclear receptors as thoroughly reviewed by Nakata et al. (2006). Here we focused on Nrf2, which is a transcription factor that has a central role in maintaining cellular homeostasis in response to oxidative stress. Under basal conditions, Nrf2 is maintained at a low level because it is targeted constitutively for proteasomal degradation by ubiquitinylation. Upon exposure to oxidative or electrophilic stress, Nrf2 accumulates in the nucleus where it binds to antioxidant response elements (AREs) of antioxidant and detoxification genes, inducing their expression (Hayes and Dinkova-Kostova, 2014). Recently, a role of Nrf2 in obesity has also been described, using mainly Nrf2-knockout mouse model. Deletion of Nrf2 protected mice from onset of diet-induced obesity and insulin resistance (Pi et al., 2010; Chartoumpekis et al., 2013). On the other hand, Nrf2-deficient mice were more susceptible to environmental chemical-induced damages (Aoki et al., 2001; Enomoto et al., 2001) and prone to develop tumors following chemical carcinogen exposure (reviewed in Jung et al., 2013).

Similarly as Kim et al. (2004), we also observed increased level of Nrf2 mRNA (2.25-fold change) in obese mice. Accordingly, NQO1 as a prototypical Nrf2 target gene (Nioi et al., 2003) was up-regulated in obese mice. In addition to NQO1, transcriptional activation of some GST genes was associated with Nrf2-mediated mechanism (Aleksunes and Manautou, 2007). Knight et al. (2008) identified 10 GST isoforms (GSTA1/2, A4, M1, M2, M3, M4, M6, O1, T1) inducible by Nrf2. Later,

it was shown that hepatic GSTP1 and P2 gene expressions were induced inNrf2-dependent manner (Yeager et al., 2009). In our study, only GSTM3 isoform corresponds to elevated Nrf2 expression in liver. GSTA1/2 showed high inter-individual variations in liver of obese mice, which can be possibly due to the fact that only one promoter contains ARE consensus sequence and hence only one isoform is induced by up-regulated Nrf2. Recently, AKRs were also identified as Nrf2-target genes (Jung et al., 2013) and in accordance, elevated expression of AKRs in our obese animals was observed, but their activity remained unchanged. AKRs, can serve as an example of two different regulation pathways. In liver AKR1C6 and AKR1C20 mRNA were up-regulated, but protein level and activity remained unchanged, hence post-transcriptional regulation must have occurred. In SI, AKR1A was elevated both on mRNA and protein levels, but activity remained unchanged, which means the further regulation probably occurs later, after protein synthesis, *i. e.* post-translationally. In the case of most other enzymes, no correlation among activity, mRNA and protein expression levels suggests a substantial role for regulatory post-transcriptional, translational and protein degradation processes.

In summary, our current study shows that activities, mRNA and/or protein expression levels of several antioxidant or drug-metabolizing enzymes were different in MSG-obese mice and in lean counterparts. Enhanced expression of Nrf2 in livers of obese mice is expected to play a role in protective adaptation. Accordingly, elevated activities of several studied enzymes (e.g. NQO1, UGT1A or AKR) may cause alterations in drug pharmacokinetics in obese or diabetic patients. Moreover, decreased capacity of GST in liver of obese mice and down-regulation of GSTP1/2 at mRNA and protein levels are consistent with potentially reduced antioxidant defense, and moreover may have major implications in xenobiotic and drug metabolism. Since the MSG-obese mouse model is being more and more used as a model of various complications of metabolic syndrome, these findings can be useful in elucidating the therapeutic use of anti-diabetic and/or anti-obesity drugs and management of type II diabetes in chronic diabetic obese patients.

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Footnotes

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Table 1

Target and Reference Genes Selected for qPCR. National Center for Biotechnology Information (NCBI) reference sequences, primers and amplicon sizes.

Gene	NCBI Accession No.	Forward primer	Reverse primer	Amplicon size
GR	NM_010344	AGCAGTGCACTCGGAATTCA	CGAATGTTGCATAGCCGTGG	182
SOD	NM_011434	AACCAGTTGTGTTGTCAGGAC	CCACCATGTTTCTTAGAGTGAGG	139
CAT	NM_009804	AGCGACCAGATGAAGCAGTG	TCCGCTCTCTGTCAAAGTGTG	181
TR	NM_0011042523	CTATGAGAATGCTTACGGGAGGT	GGAACCGCTCTGCTGAATAGAT	95
GPX2	NM_030677	CCCTACCGGCCATTTCCTTT	ACCTACCCCAGACTTAGAGCC	74
GSTM1	NM_010358	AATTGGGATTGGTGCAGGGT	ACTGACCTGTGTGTTTGGAGT	101
GSTM3	NM_010359	GCTCATGATAGTCTGCTGCAG	GCTTCATTTTCTCAGGGATGGC	80
GSTA1/2	NM_008181 / NM_008182	GATTGGGCAATTGGTATTATGTC	CCTGTTGCCCACAAGGTAGT	142
GSTA3	NM_010356	GACCTGGCAAGGTTACGAAG	TATCTCCAGATCCGCCACTC	195
GSTA4	NM_010357	CCTCGCTGCCAAGTACAACT	TTGCCAACGAGAAAAGCCTC	231
GSTP1/2	NM_013541 / NM_181796	AGCCTTTTGAGACCCTGCTG	CGGCAAAGGAGATCTGGTCA	75
AKR1A	NM_021473	AGCCTGGTCAGGTGAAAGC	GGCCTCCCCAATCTCAGTT	104
AKR1C6	NM_030611	TGTCACTGCATTGGAAGAGTG	TCAGAAGCTTGGATTAGGGTGA	68
AKR1C20	NM_054080	GGCAAAGCTAAGAGTTGCAGA	TGGTAAACGTCACATGGGTTCA	100
CBR1	NM_007620	GCTGCTCCCTCTAATAAAACCC	CCTCTGTGATGGTCTCGCTTC	125
NQ01	NM_008706	GTCCATTCCAGCTGACAACC	TCCTTTTCCCATCCTCGTGG	142
UGT1A	*a*	CTTCCTCCTGGCCATTGTGT	TTCTTCACTCGCCCCTTTCC	99
Nrf2	NM_010902	TGCCCACATTCCCAAACAAG	CTGCCAAACTTGCTCCATGT	229
HPRT1	NM_013556	CAGTCCCAGCGTCGTGATTA	GGCCTCCCATCTCCTTCATG	167
RPlP0	NM_007475	GATGGGCAACTGTACCTGACTG	CTGGGCTCCTCTTGGAATG	136
18S	NR_003278	GGCCGTTCTTAGTTGGTGGAGCG	CTGAACGCCACTTGTCCCTC	133

Footnotes: *a* Set of primers designed for all UGT1A isoforms

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Table 2

Body Weight, Plasma Insulin and Leptin Levels.

	control mice	**MSG mice**
final body weight (g)	44.0 ± 1.0	61.0 ± 3.0*
average food intake, (g)	4.98 ± 0.08	4.35 ± 0.09*
insulin (ng/ml)	0.31 ± 0.04	2.26 ± 0.24*
leptin (ng/ml)	0.72 ± 0.03	19.97 ± 0.72*

Footnotes: Food intake was monitored last four weeks of the experiment. Blood samples were collected after 12h fasting and pooled from 11 animals. Results are mean ± standard deviation (SD), * statistically significant ($P<0.05$).

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Table 3

Antioxidant Enzymes. Activities, protein and mRNA relative levels.

	catalytic activities		**immunoblotting**		**mRNA quantity**	
	control	**obese**	**control**	**obese**	**control**	**obese**
liver						
SOD1	100.0 ± 1.5	104.8 ± 1.4	100.0 ± 6.9	114.5 ± 11.7	1.00 ± 0.46	1.36 ± 0.33
CAT	100.0 ± 1.4	99.3 ± 37.7	100.0 ± 3.2	88.7 ± 5.0*	1.00 ± 0.37	1.19 ± 0.20
GPx[a]	100.0 ± 0.5	78.3 ± 1.7*	n.d.	n.d.	1.00 ± 0.31	3.21 ± 1.15*
GR	100.0 ± 7.3	103.4 ± 2.6	100.0 ± 16.9	118.3 ± 14.0	1.00 ± 0.43	1.31 ± 0.48
TR	100.0 ± 12.3	107.2 ± 16.5	100.0 ± 13.8	66.40 ± 5.0*	1.00 ± 0.46	0.79 ± 0.23
small intestine						
SOD1	100.0 ± 4.5	106.0 ± 4.7	100.0 ± 10.0	151.4 ± 10.7*	1.00 ± 0.44	0.52 ± 0.08
CAT	100.0 ± 7.7	61.2 ± 1.7*	100.0 ± 10.6	160.4 ± 8.1*	1.00 ± 0.66	1.15 ± 0.42
GPx[a]	100.0 ± 3.8	119.0 ± 7.0*	100.0 ± 9.1	89.0 ± 7.2	1.00 ± 0.17	0.30 ± 0.12*
GR	100.0 ± 2.6	89.8 ± 1.5*	100.0 ± 7.3	147.7 ± 9.0*	1.00 ± 0.07	0.94 ± 0.25
TR	100.0 ± 2.0	86.9 ± 7.5*	100.0 ± 18.1	102.7 ± 11.9	1.00 ± 0.13	0.63 ± 0.12*

Footnotes: *a* –relative specific activity measured for all isoforms, protein and mRNA GPx2 specific isoform only. Results are mean ± standard deviation (SD), controls set to 100%, n=3 for catalytic activities and immunoblotting, n=4 for mRNA quantification, * statistically significant ($P<0.05$), n.d.- not detected. mRNA quantity expressed as a fold change.

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Table 4

Phase I DMEs. Activities, protein and mRNA relative levels.

	catalytic activities		**immunoblotting**			**mRNA quantity**	
	control	**obese**	**control**	**obese**		**control**	**obese**
liver							
CBR1[a]	100.0 ± 6.8	176.6 ± 12.7*	100.0 ± 2.2	130.25 ± 33.4		1.00 ± 0.19	1.25 ± 0.30
NQO1	100.0 ± 3.4	310.4 ± 21.2*	100.0 ± 12.9	177.9 ± 11.1*		1.00 ± 0.26	3.13 ± 1.19*
AKR1A[b]	100.0 ± 2.4	109.5 ± 2.8	100.0 ± 9.9	90.0 ± 2.2		1.00 ± 0.24	1.03 ± 0.23
AKR1C[c]	100.0 ± 2.5	113.0 ± 2.5	100.0 ± 10.9	94.3 ± 4.4	AKR1C6	1.00 ± 0.38	2.37 ± 0.21*
					AKR1C20	1.00 ± 0.13	3.54 ± 0.99*
small intestine							
CBR1[a]	100.0 ± 16.6	94.2 ± 24.7	100.0 ± 7.8	102.44 ± 26.4		1.00 ± 0.30	1.07 ± 0.50
NQO1	100.0 ± 19.8	97.4 ± 9.8	100.0 ± 5.5	109.1 ± 18.1		1.00 ± 0.25	0.51 ± 0.31
AKR1A[b]	100.0 ± 1.1	105.9 ± 7.7	100.0 ± 5.5	157.5 ± 6.4*		1.00 ± 0.31	1.58 ± 0.31*
AKR1C[c]	100.0 ± 4.0	46.2 ± 1.9*	n.d.	n.d.		n.d.	n.d.

Footnotes: Activities correspond to reducing equivalents obtained using following substrates: a- CBR1 activity tested using menadione , b – AKR1A activity was tested using 4-pyridinecarboxaldehyde and c-AKR1C activity corresponds to acenaphthenol reduction. Results are mean ± standard deviation (SD), controls set to 100%, n=3 for catalytic activities and immunoblotting, n=4 for mRNA quantification , * statistically significant ($P<0.05$), n.d.-not detected. mRNA quantity expressed as a fold change.

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Table 5

Phase II DMEs. Activities, protein and mRNA relative levels.

	catalytic activities		**immunoblotting**			**mRNA quantity**		
	control	**obese**		**control**	**obese**		**control**	**obese**
enzyme			class			isoform		
liver								
UGT1A	100.0 ± 1.1	167.8 ± 6.7*		100.0 ± 8.7	232.7 ± 50.8*		1.00 ± 0.41	2.71 ± 0.41*
GST	100.0 ± 4.0	77.7 ± 4.2*	GSTA	100.0 ± 13.4	127.9 ± 9.1*	GSTA1/2[a]	1.00 ± 0.10	2.32 ± 1.80
						GSTA3	1.00 ± 0.20	0.97 ± 0.30
						GSTA4	1.00 ± 0.29	1.03 ± 0.23
			GSTM	100.0 ± 10.4	127.7 ± 7.8*	GSTM1	1.00 ± 0.30	1.47 ± 0.30
						GSTM3	1.00 ± 0.46	2.38 ± 0.63*
			GSTP	100.0 ± 6.4	77.6 ± 13.4*	GSTP1/2[a]	1.00 ± 0.30	0.20 ± 0.10*
small intestine								
UGT1A	n.d.	n.d.		100.0 ± 26.3	118.2 ± 43.1		1.00 ± 0.47	1.02 ± 0.39
GST	100.0 ± 10.4	99.4 ± 13.0	GSTA	100.0 ± 5.0	162.8 ± 16.5*	GSTA1/2[a]	1.00 ± 0.22	0.75 ± 0.24
						GSTA3	1.00 ± 0.40	1.50 ± 0.33
						GSTA4	1.00 ± 0.45	0.84 ± 0.48
			GSTM	100.0 ± 10.6	125.1 ± 15.4	GSTM1	1.00 ± 0.25	0.86 ± 0.18
						GSTM3	1.00 ± 0.06	0.81 ± 0.11
			GSTP	100.0 ± 7.2	198.8 ± 65.4*	GSTP1/2[a]	1.00 ± 0.29	0.91 ± 0.12

Notes: Results are relative mean ± standard deviation (SD), controls set to 100%, n=3 for catalytic activities and immunoblotting, n=4 for mRNA quantification , * statistically significant ($P<0.05$), n.d.- not detected. mRNA quantity expressed as a fold change. a -two isoforms analyzed together.