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SELECTION OF REFERENCE GENES FOR NORMALIZATION OF REAL-TIME PCR DATA IN VISCERAL ADIPOSE TISSUE OF FEMALE RATS ON A FRUCTOSE-ENRICHED DIET

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Abstract - Considering the association of increased fructose intake with the rise in metabolic syndrome and gender differences in its incidence, this study was aimed at evaluating and identifying the optimal reference genes for the relative quantification of gene expression by real-time PCR in the adipose tissue of female rats exposed to a fructose-enriched diet as an animal model of metabolic syndrome. The transcription of four commonly used reference genes, BA, HPRT1, TBP and B2M, was quantified by TaqMan real-time PCR. The expression stability of these genes was evaluated by direct comparison of Ct values and by using the geNorm and NormFinder algorithms. NormFinder revealed HPRT1 as the most stable gene, and both programs identified HPRT1 and TBP as the most stable combination of genes in this experimental paradigm. In addition, HPRT1 was shown to be reliable enough as the sole reference gene.

*Key words***:** Reference genes, real-time PCR, fructose diet, adipose tissue, female rats.

INTRODUCTION

The consumption of fructose has dramatically increased over the past several decades, mainly in the form of added sugar in processed food and many caloric beverages (Lê and Tappy, 2006). There are overwhelming data from both animal models and human studies associating excessive fructose intake with an increased obesity, insulin resistance, hyperinsulinemia, dyslipidemia and hypertension (Bray et al., 2004; Basciano et al., 2005; Ruxton et al., 2010; Sobrecases et al., 2010; Welsh et al., 2010), all of which are pathologies recognized within the metabolic syndrome (Bray et al., 2004), a leading risk factor for type 2 diabetes, coronary artery disease and stroke. Apart from the rise in metabolic syndrome in the population, there is a significant impact of gender on its prevalence and distribution (Tonstad S, 2007; Ogbera, 2010; Novak et al., 2011). Metabolic perturbations associated with high fructose intake are mediated by modulation of the expression of genes involved in carbohydrate and lipid metabolism (Rutledge and Adeli, 2007). Adipose tissue is considered as a simple storage of energy-rich substrates. However, it is becoming more evident that this tissue is an active endocrine organ (Guzik TJ, 2006), playing an active role in the regulation and pathological deregulation of metabolic homeostasis in the brain, liver and other key metabolic tissues. In addition, the fructose utilization pathway in adipose tissue is largely different from its hepatic metabolism.

The most popular method in gene expression analysis is the Real-Time PCR, or quantitative polymerase chain reaction (qPCR) (Ginzinger, 2002; Walker, 2002; Valasek and Repa, 2005). qPCR is applied in a real-time setting, simultaneously amplifying targeted DNA molecules and quantifying specific transcripts. There are many advantages of this over other quantification methods. It is one of

the most sensitive and specific methods that can detect a single copy of a specific transcript (Palmer et al., 2003); it allows for the simultaneous analysis of gene expression in various samples and it does not require post-amplification manipulation. In addition, qPCR assays can reliably detect very small differences in gene expression between samples (Gentle et al., 2001), and have considerably low coefficients of variation (Schmittgen et al., 2000). However, being a very sensitive technique, qPCR requires a correction of the expression data for the differences between samples (sample-to-sample and non-biological variations). Numerous variations can arise from the differences in the quality and/or quantity of the RNAs used in PCR, especially when solid tissues are analyzed. Frequently the variations do not derive from the experimental design, including handling of tissues, cell number, RNA integrity, enzymatic efficiency, RNA extraction, storage of isolated RNA, efficiency of reverse transcription and amplification (Bustin and Mueller, 2005; Fleige and Pfaffl, 2006). To adjust for these variations, the normalization of qPCR data for the target gene against a suitable reference gene is necessary (Livak and Schmittgen, 2001; Huggett et al., 2005). The ideal reference gene is expected to be expressed constitutively at a constant level, regardless of the experimental conditions in different tissues or cell types, at all developmental stages (Huggett et al., 2005). In addition, it should be subjected to the same steps of analysis as the gene of interest. Nevertheless, the expression of the most commonly used reference genes considerably varies, not only with the experimental treatment, but also between sexes, tissues, environmental conditions, etc. (Thellin et al., 1999; Schmittgen and Zakrajsek, 2000; Derks et al., 2008). Since the results are expressed as a target/reference ratio, the accurate determination of target gene expression is dependent on the stability of the expression of the reference gene. Thus, the use of an inappropriate reference gene produces unreliable results, emphasizing the importance of the expression stability validation of a reference gene for the specific experiment prior to its use for normalization in qPCR (Schmittgen and Zakrajsek, 2000).

This study was focused on the identification of the most stable reference gene for the relative quantification of gene expression by comparative qPCR in the adipose tissue of female rats on standard and fructose-enriched diets. The reference gene was selected from the panel of four candidates commonly used as endogenous controls: β-actin (BA), β_2-microglobulin (B2M), TATA box binding protein (TBP) and hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1). To address this issue, we compared the cycle threshold (Ct) values directly and used two mathematical models, NormFinder and geNorm, to analyze the data obtained by TaqMan qPCR. Taking into account the special significance of the glucocorticoid receptor (GR) for energy metabolism, we also performed a relative quantification of GR gene expression against the two most stable reference genes in order to test their suitability in our experimental paradigm.

MATERIALS AND METHODS

Animals and treatment

Female Wistar rats (21 days old), bred in our laboratory were randomly divided in two dietary-based experimental groups: a control group fed with commercial standard chow and drinking water, and a fructose group fed with the same chow and 10% (w/v) fructose solution in the drinking water. Both experimental groups had *ad libitum* access to food and drinking fluid during 9 weeks. The animals were kept under standard conditions, at 22°C and a 12 h light/dark cycle. The procedures complied with the European Communities Council Directive (86/609/EEC) and were approved by the Ethical Committee for the Use of Laboratory Animals according to the guidelines of the EU-registered Serbian Laboratory Animal Science Association (SLASA).

RNA extraction and reverse transcription

The animals were killed by rapid decapitation; visceral adipose tissue was excised and immediately stored in liquid nitrogen until use.

Total RNA was extracted from thawed visceral adipose tissue (100-200 mg) using TRIzol®Reagent (AmBion) following the manufacturer's protocol. RNA was dissolved in 30 µl of RNase-DNase free water (Eppendorf), and RNase inhibitor (Applied Biosystems) was added. The RNA concentration was determined in triplicate samples by measurement of the optical density at 260 nm. RNA purity was verified by an average A260/A280 ratio and values >1.8 were considered satisfactory. RNA integrity was confirmed by 1% agarose gel electrophoresis. All RNA samples were treated with RNase-free DNase I (Ferments) according to the manufacturer's instructions.

Reverse transcription was performed in 20 µl reactions with MultiScribe™ Reverse Transcriptase in the presence of Random Primers using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Reactions were incubated under RNase-free conditions at 25°C for 10 min, followed by 37°C for 2 h and a final denaturation at 85°C for 5 min. Each reverse transcription reaction was accompanied by a no-RT control in which the reverse transcriptase was replaced by DEPC water. The cDNA was stored at -80°C until further use.

Real-time PCR

The expression of GR and each candidate reference gene (BA, B2M, TBP and HPRT1) was analyzed by TaqMan qPCR using an AB Prism 7000 Sequence Detector System (Applied Biosystems). The TaqMan Gene Expression Assays consisted of unlabeled PCR primers for amplification of the target gene and TaqMan minor groove binder probe FAM™ dye-labeled. Primers spanning exon-exon junctions were used to exclude genomic DNA contamination. The Gene Expression Assay IDs and amplicon lengths are indicated in Table 1. All reactions were performed in 25 µl volumes in triplicate and the mean Ct value for each triplicate was used for further analysis. The reaction mix consisted of 1 × TaqMan® Universal PCR Master Mix, with AmpErase UNG, 1 × TaqMan® Gene Expression Assay primer-probe mix (Applied Biosystems) and cDNA template (20 ng of RNA converted to cDNA). The PCR reactions were initiated with a 2 min incubation at 50°C for UNG activation, and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s in accordance with the manufacturer's recommendations. The no-template control was included for each target gene to detect possible reagent contamination. To minimize experimental variation, the same batch of cDNA was used to quantify all genes for one sample, and the same gene was tested on the different samples in the same PCR run.

The qPCR efficiency of GR and the four different endogenous control genes was evaluated using standard curves. Five-fold dilutions (100 ng–0.16 ng) of one randomly chosen cDNA sample were assessed in triplicate with GR, and each of the four endogenous control primer/probe systems and standard curves were generated. The corresponding amplification efficacy was calculated using the fallowing equation: $E=(10^{(-1/slope)}-1)x100$, where the slope is the value from the standard curve plot generated by the AB 7000 data analysis software (Table 2).

Determination of reference gene expression stability

To assess the expression stability of the candidate reference genes across all samples, a direct comparison of Ct values was performed and two different statistical algorithms: geNorm 3.5 (http://medgen.ugent.be/~jvdesomp/genorm) (Vandesompele et al., 2002) and NormFinder (http://www.mdl.dk/publication-snormfinder. htm) (Andersen et al., 2004), were used according to the developer's recommendations. The geNorm is a statistical algorithm that determines the two most stable reference genes from a set of tested genes. It is based on the pairwise stability measure ranking which relies on the principle that the two ideal candidate-normalizing genes should have an equal expression ratio in all samples, regardless of the experimental conditions. The program applies a statistical algorithm to calculate the average expression stability value (M) for each candidate reference gene as the average pairwise variation (V) for that gene with all other tested genes. The reference genes are then ranked by stepwise exclusion of the least stable gene (the highest M value) (Vandesompele et al., 2002).

Table 1. Four endogenous control genes evaluated in this study.

Gene symbol	Gene name	Molecular function	Applied Biosystems assay identifier	Amplicon Length (bp)
BA	β-Actin	cytoskeletal structure	Rn00667869_m1	91
B2M	β2-Microglobulin	defence/immunity	Rn00560865_m1	58
TBP	TATA box binding protein	transcription factor	Rn01455646_m1	75
HPRT1	hypoxanthine guanine phosphoribosyl transferase1	glycosyl transferase	Rn01527840_m1	64
GR	glucocorticoid receptor	transcription factor	Rn00561369_m1	73

Table 2. PCR amplification efficiencies four candidate endogenous control genes.

Gene symbol	Slope of inhibition curve	PCR Amplification efficiency (%)	R^2
BA	-3,455	95	0,998
B2M	-3,460	95	0,999
HPRT	-3,328	100	0,998
TBP	-3,259	103	0,998
GR	-3,342	99	0,998

Table 3. Cycle threshold (Ct) values of candidate endogenous control genes.

Gene symbol	Mean Ct ± s.e.m.	Ct Min	Ct Max	Ct Range	SD	CV (%)
B2M	23.51 ± 0.61	20,81	25,88	5,08	1,52	6,48
BA	25.43 ± 0.45	23,83	27,08	3,25	1,14	4,48
HPRT1	29.86 ± 0.42	27,41	31,14	3,74	1,06	3,54
TBP	32.30 ± 0.42	30,22	34,10	3,88	1,04	3,23

Table 4. Stability ranking of candidate endogenous control genes determined by Normfinder and GeNorm analyses.

Rank	NormFinder		geNorm	
	Gene Simbol	Stability	Gene Simbol	Stability
1	HPRT	0,058	HPRT	0,456
2	BA	0,079	TBP	0,461
3	TBP	0,084	BA	0,503
4	B2M	0,163	B2M	0,629

Note: High expression stability is indicated by a low stability value. Normfinder ranks genes based on combined inter- and intra-group variation analysis, while GeNorm uses pairwise analysis of expression stability.

NormFinder is another freely available algorithm for identifying the optimal reference gene among a set of candidates. It estimates expression variation and ranks genes according to their expression stability in a given experimental design (Andersen et al., 2004). In addition to identifying the most stable pair of reference genes, NormFinder identifies the best overall reference gene. In contrast to geNorm, this applet calculates both intragroup variance, describing the stability of the gene expressions within each group, and intergroup variance, which describes the stability of the gene expression between the groups.

Relative quantitation of target gene expression

Relative quantification of GR gene expression was performed using a comparative $2^{-\Delta\Delta Ct}$ method where ΔΔCt = (Ct target gene, test sample – Ct endogenous control, test sample) – (Ct target gene, calibrator sample – Ct endogenous control, calibrator sample). The amount of GR mRNA was normalized to each of the two best reference genes alone (chosen by the geNorm and NormFinder), as well as to normalization factor (NF) calculated as the geometric average of these two genes.

RESULTS

To determine the expression stability of four potential reference genes, BA, B2M, HPRT1 and TBP, mRNA levels were measured in 12 visceral adipose tissue samples from female rats (6 obtained from fructose-fed rats and 6 obtained from animals on standard laboratory diet) and cross-validated using two popular algorithms: geNorm 3.5 (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004). Genes were chosen according to previously published studies on these genes as reference controls.

Amplification Efficiency

To evaluate the qPCR efficiency of GR and the four tested reference genes, serial five-fold dilutions of one randomly chosen cDNA sample were amplified in each of the five assays. The calculated corresponding amplification efficiency for all assays were satisfactory and ranged from 94.54 to 102.69% (-3.46 > slope > -3.26) (Table 2). All five R^2 values were >0.998 showing the reaction's high linearity and stability for the quantification purposes. In the no-template controls, no amplification was detected.

Range of expression of candidate reference genes

In a qPCR assay, a positive reaction is detected by the accumulation of a fluorescent signal. The Ct is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. to exceed the background level), and thus is inversely proportional to the amount of target nucleic acid in the sample. Fig. 1 shows the expression profiles of the four evaluated endogenous control genes in visceral adipose tissues of female rats on regular and fructose-enriched diet. The relative amount of the four tested reference genes differed in the same manner in all samples: B2M was the most abundantly expressed gene, having the lowest mean Ct value. It was followed by BA, HPRT1 and finally TBP, which was the least expressed gene with an average Ct value around 32. The observed Ct values for the examined candidate genes in the control and fructose-fed rats are given in Table 3. In addition, interindividual variations were observed for each gene. B2M exerted the greatest variations in the expression level with the widest Ct range, while BA showed a more stable expression with the narrowest Ct range (Table 3).

Expression stability testing – identification of optimal reference genes

To evaluate further the candidate genes for their use as references in qPCR data normalization in the visceral adipose tissue of female rats fed with a fructose-enriched diet, expression stability was tested using the NormFinder and the geNorm algorithms.

When the statistical algorithm geNorm was applied, genes were ranked from the least to the most stable on the basis of their expression stability (M) – the lowest M value indicating the highest stability (Fig. 2). The authors of the algorithm recommend the M value of 1.5 as a cut-off for suitability as a ref-

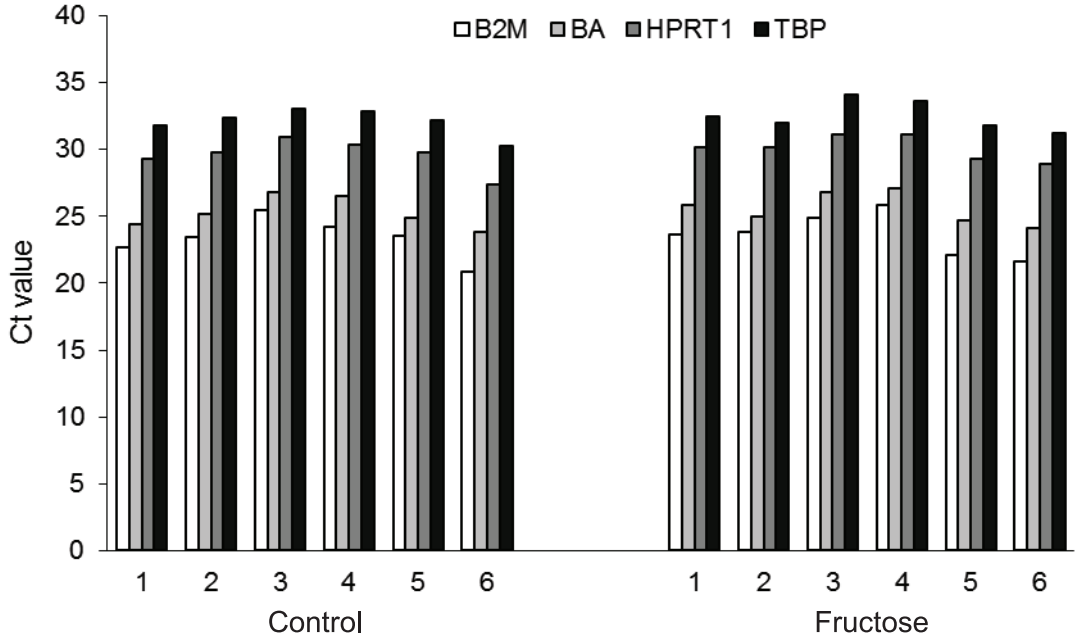

Figure 1. The expression profiles of four tested endogenous controls. Ct values of each gene and each animal are presented.

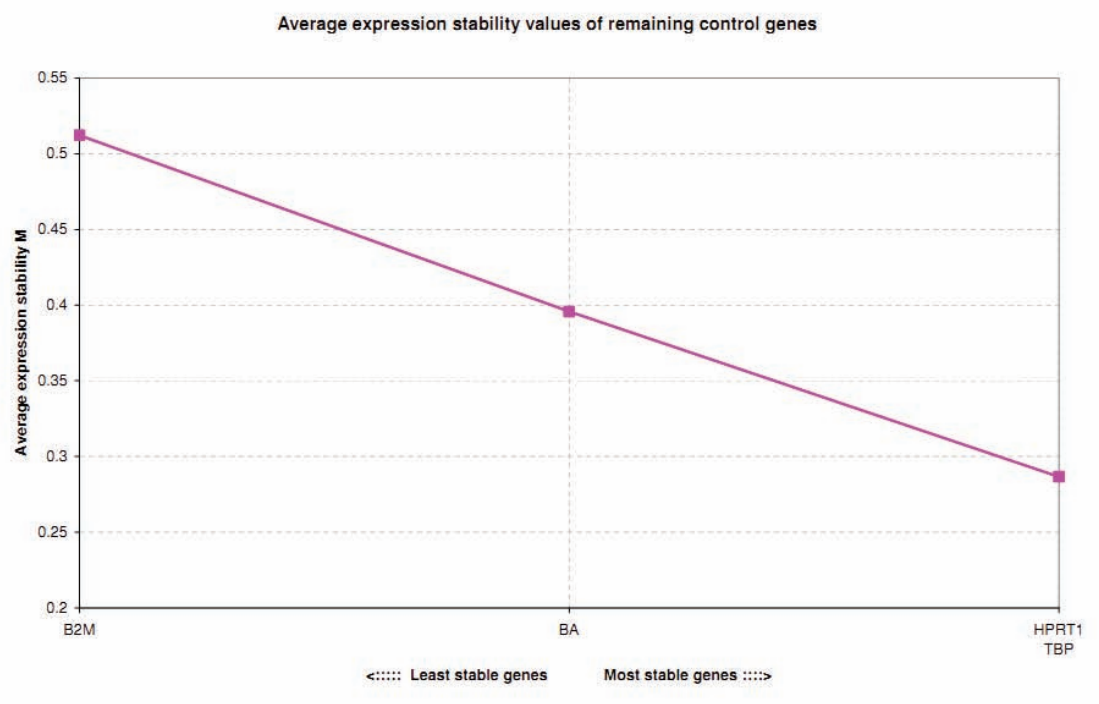

Figure 2. GeNorm output. Average expression stability values (M) of candidate reference genes in visceral adipose tissue. The highest M values characterize genes with the least stable expression. Stepwise elimination of the least stable gene generates a ranking of reference genes according to increasing expression stability and results in the identification of the most stable pair which cannot be ranked further. The x-axis indicates the ranking of reference genes based on expression stability starting from the least stable gene at the left. The M value threshold for stability of a gene according to GeNorm is 1.5.

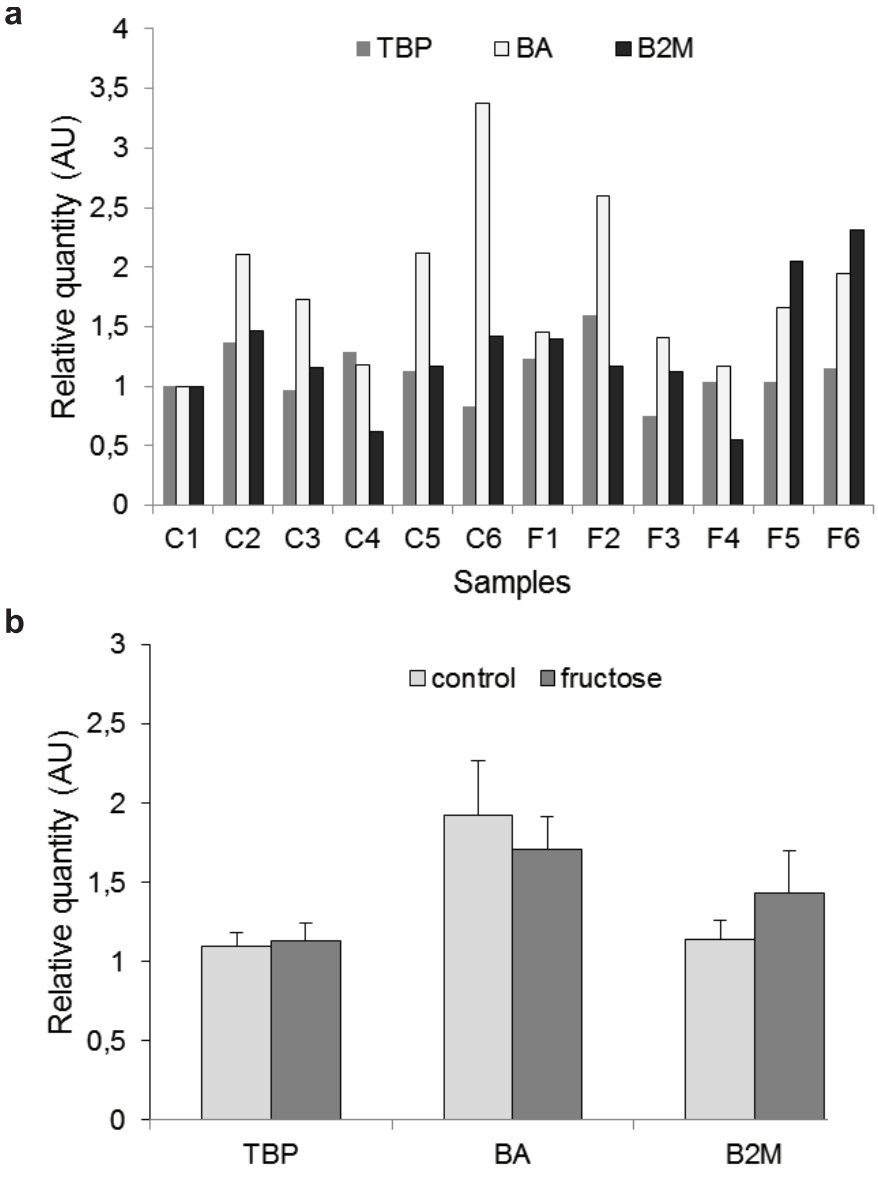

Figure 3. The relative quantities of TBP, BA and B2M after normalization to HPRT1. The expression of TBP, BA, and B2M in visceral adipose tissue of female rats on a standard (C) and fructose-enriched diet (F) is normalized to HPRT1 as a reference and to one control sample as a calibrator taken as 1. The data are presented as individual values for each sample (**a**) and as the means ± S.E.M. (**b**).

erence gene. All genes tested in this study were below this value. The respective individual M values are presented in Table 4. The least stable gene was B2M. After successive elimination of the least stable genes based on the highest M values and subsequent recalculation of the average M values, geNorm identified HPRT1 and TBP (M <0,287) as the two most stably expressed genes.

In addition, geNorm provides information on the optimal number of reference genes to be used in the analysis of gene expression. This number is determined by the level of variation in average reference gene stability, a measure known as pair-wise variation (V). Calculating the V value between two sequential normalization factors for all genes determines the number of reference genes sufficient for

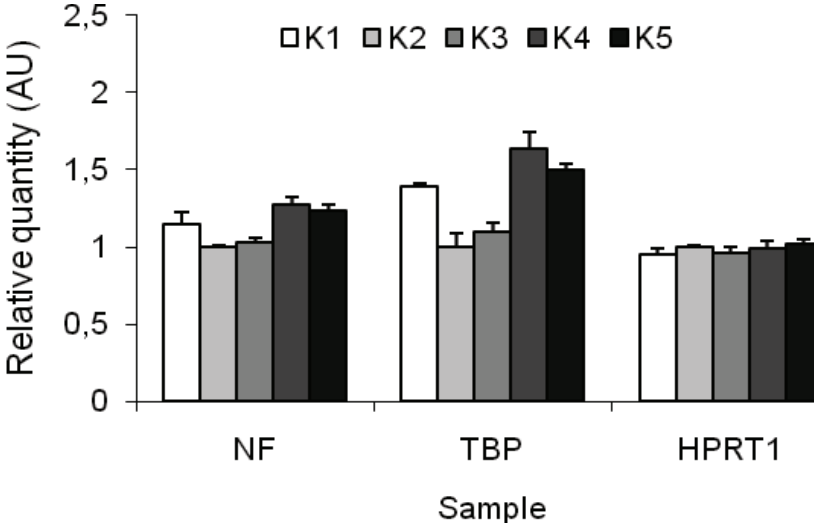

Figure 4. The relative quantity of GR in visceral adipose tissue of female rats fed on a standard diet. The relative quantity of GR mRNA in five control samples was normalized to the normalization factor (NF; geometric average of HPRT1 and TBP), and to HPRT1 and TBP individually.

accurate normalization. The geNorm analysis of our data indicated that the V value for V2/3 was 0.143, which is below the proposed cut-off value of 0.15. Therefore, the subsequent addition of the next most stable gene does not significantly affect the normalization factor.

NormFinder is another freely available application that identifies the best overall endogenous control gene, as well as the best combination of two endogenous controls. Its statistical framework provides information on both intra- and intergroup variability. More stable gene expression is indicated by lower average expression stability values.

As presented in the Table 4, the ranking data for the four tested genes obtained by NormFinder are similar to those obtained by geNorm. The most stably expressed gene identified by NormFinder was HPRT1 (Table 4), which was also one of the two most stable genes identified by geNorm analysis. The best combination of genes according to Normfinder was HPRT1 and TBP with an indicated stability value of 0.052, while the expression of B2M was indicated as unstable in both experimental groups.

To examine whether the use of HPRT1 alone as the reference gene would affect the results, we calculated the relative quantity of the other examined candidate reference genes by normalization to HPRT1 using one of the control samples as calibrator. The results are presented in Fig. 3. TBP showed the least variation in expression when compared to BA and B2M (Fig. 3, Panel a), which is in line with geNorm and Norm Finder results pointing to TBP as the second best reference gene. Furthermore, TBP was equally expressed in both study groups, while the expression of BA was decreased by 10% and the expression of B2M was increased by 26% after the treatment; however, none of these changes were statistically significant (Fig. 3, Panel b).

The effect of the best candidate reference genes on relative GR gene expression

To determine if HPRT1 solely could be used as the reference gene in our experimental design, the expression of GR as a target gene was normalized to HPRT1 and TBP separately, as well as to normalization factor (NF), calculated as the geometric average of these two genes within five control samples (Fig.4). The results showed higher interindividual

variations in the GR expression after normalization to TBP alone or to NF as compared to normalization to HPRT1 alone.

DISCUSSION

This study was designed to evaluate expression variability among different commonly used endogenous control genes and to identify the most reliable reference for the gene expression analysis in the adipose tissue of female rats on a fructose-enriched diet, an animal model that displays numerous features of the metabolic syndrome (Tran et al., 2009). Four housekeeping genes, BA, B2M, HPRT1 and TBP, were analyzed by direct comparison of Ct values and by geNorm and NormFinder software. HPRT1 and TBP were identified as the most stable combination of reference genes in this experimental paradigm. Moreover, HPRT1 alone was shown to be stable enough as the reference gene under these experimental conditions. All analytical methods used in this study identified B2M as the least stable gene in both experimental groups.

Quantitative PCR, as an extensively used method for quantifying gene expression, relies on the accurate normalization of gene expression data which is essential for overcoming sample-to-sample and non-biological variations (Bustin, 2000). The most commonly used method of data normalization is normalization to the reference gene. However, gene expression analysis is susceptible to misinterpretation if the gene of interest is compared to an inappropriate reference gene. That is, the under- or overestimations of the relative gene expression levels can be interpreted as pseudo-variation or can mask the real biological variation (Dheda et al., 2005). The high sensitivity of qPCR revealed that many of the traditionally used housekeeping genes show considerable variations in expression level in different experimental treatments, biological processes, tissues and even genders (Bustin, 2002; Verma and Shapiro, 2006; Derks et al., 2008), presumably because they are basically multifunctional proteins. Therefore, several statistical models and software packages have been designed for the identification of the most stable reference gene. As regards gender differences in the prevalence of metabolic syndrome, limited information is available on the suitable reference genes to be used in gene expression studies involving the visceral adipose tissue of female rats.

Herein, we used two popular approaches, geNorm and NormFinder, to detect the most suitable reference gene for analyzing fructose-induced gene-expression alterations in the visceral adipose tissue of female rats. HPRT1 and TBP were identified by both programs as the most stable combination of reference genes and NormFinder also pointed to HPRT1 as a single best reference gene. Similar results were obtained in our study involving fructose-fed male rats (Djordjevic et al., 2012). In view of the gender-related differences in susceptibility to metabolic syndrome, these observations indicate the reliability of a combination of HPRT1 and TBP genes as normalization factors for both genders.

Notably, in the present study, we have shown by evaluating GR gene transcript quantity that HPRT1 was reliable enough as the reference gene in our experimental paradigm. The results show higher interindividual variations in GR expression after normalization to TBP or to NF when compared with normalization to HPRT1, thereby supporting the view that HPRT1 alone could be used as a reference gene in our experimental design. Although the use of two or more reference genes is strongly recommended (Vandesompele et al., 2002; Brkljacic et al., 2010), it is not always a practical solution since it increases both the expense and the amount of samples used. Previously, HPRT1 was utilized in several animal studies as a reference gene in the adipose tissue of male rats and mice, either alone (Gosmain et al., 2005; Tharp et al., 2008) or in combination with ribosomal protein S15 (van Schothorst et al., 2009). TBP was used as an endogenous control in male mice on a high-fat diet (Scheja et al., 2008), in mature adipocytes isolated from rats (Du et al., 2011), but also in human studies on the visceral adipose tissue of women (Ellero et al., 2010).

While HPRT1 and TBP were selected as the most stable genes in both tests, the ranking data obtained by NormFinder was slightly different than the one obtained by geNorm. The discrepancy in gene ranking calculated by the different programs has been shown previously (Seol et al., 2011). The disparities in the rankings of genes between the two algorithms derive from the different methodologies used to calculate the gene stabilities. Namely, the geNorm applet selects genes based on two basic parameters, average expression stability and pairwise variation. The pair showing least variation in expression ratio across samples represents genes that share an expression profile. In contrast, NormFinder estimates stability values for endogenous controls considering overall stability, as well as combined intra- and intergroup variation. This algorithm gives higher rank to the genes whose expression does not show grouping effect than to genes whose expression varies in a group-like manner. Consequently, geNorm ranked BA as third, while NormFinder ranked it as the second gene. Nevertheless, it was not selected as one of the two best genes in combination by NormFinder because it exerted a higher intergroup variation compared to TBP. In addition, when the relative quantity of TBP, BA and B2M was calculated by normalization to HPRT1, BA and B2M showed greater inter- and intragroup variations in expression compared to TBP. Interestingly, when Ct values were directly compared interindividual variations were noticed for all of the four tested genes, and BA showed the lowest Ct range. While this finding confirms that the sole use of Ct values can be misleading for gene expression analyses, in animal studies BA appears to be most commonly used reference gene in the adipose tissue. It was used as reference gene for the gene expression analysis in the adipose tissue of male rats fed with different sugars including fructose and sucrose (London et al., 2007; Alzamendi et al., 2009; London and Castonguay, 2011; Sivakumar and Anuradha, 2011), in the adipose tissue of rats on the caloric-restriction low-fat diet (Yamaguchi et al., 2012), as well as in different fat depots such as the kidney, subcutaneous and mesenterial (Schmid et al., 2012). This gene was also utilized as a reference gene in the adipose tissues of androgenized fructose-fed female rats (Alzamendi et al., 2010) and ovariectomized females with estrogen replacement (Paulsen et al., 2008). In contrast, there are opposing results regarding the use of BA as a reference gene in human studies. Mehta et al. (2010) validated reference genes for qPCR analyses of human visceral adipose tissue samples and identified BA and RNA polymerase II complex as the most appropriate reference genes. However, Catalán et al. (2007) have shown that the BA expression level in different fat depots was affected by obesity and type 2 diabetes mellitus, suggesting that it could be an inappropriate reference for gene expression studies in metabolic syndrome.

Finally, both statistical algorithms used in this study identified B2M as the gene with unstable expression in both experimental groups, indicating its ineffectiveness as a reference gene. B2M also showed the most variable Ct values in a direct comparison of Ct values, and considerable variation when its relative quantity was calculated by normalization to HPRT1. Nevertheless, this gene was used as a normalization factor in different adipose depots, including visceral, subcutaneous and retroperitoneal, in several human studies in which both men and women were examined (Madsen et al., 2003; van Beek et al., 2007; Costa Cdos et al., 2010).

Because the geNorm software uses pairwise comparison, it is recommended to consider as candidate genes, genes that are not co-regulated. The four candidate reference genes considered in the present analysis have different physiological/molecular functions: BA is a ubiquitous cytoskeleton protein; B2M is a component of the major histocompatibility complex class I molecule; HPRT1 is engaged in the metabolic purine salvage pathway, and TBP is a general transcription factor. Thus, it is tempting to assume that the genes analyzed in the present study are not subject to co-regulation.

The commonly used reference gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was not analyzed in this study because it plays a pivotal role in carbohydrate metabolism and energy production pathways. It is likely that fructose-in-

duced metabolic disturbances, especially those relating to insulin resistance and hyperinsulinemia, may affect the expression level of this gene, since it carries multiple insulin-responsive elements in its promoter (Nasrin et al., 1990) and its transcription is known to be stimulated by insulin (Rolland et al., 1995; Barroso et al., 1999). In addition, another common reference gene, 18S rRNA, was omitted in this evaluation study. As postulated by Vandesompele et al. (2002), this gene is considered inappropriate for the normalization of gene expression because it is significantly more abundant compared to many plausible target genes. Besides, Mehta et al. (2010) recognized it as one of the least stable genes in human visceral adipose tissue.

A better understanding of the gender-related differences in gene expression perturbations in adipose tissue may prevent the growing rate of diabetes, metabolic syndrome and obesity. Selection of an appropriate reference gene is the basis of an accurate estimation of fructose-induced alterations in the gene expression implicated in the development of metabolic disorders.

Overall, the results obtained by two different statistical applets, geNorm and NormFinder, were consistent, confirming the reliability of the validation for reference gene performed within the current study. Both algorithms identified the same pair of genes as the most reliable, despite differences in methodologies.

In summary, our research revealed HPRT1 as a reliable single reference gene for gene expression normalization in the study of adipose tissue of fructose-fed female rats. Since the use of more than one reference gene is recommended, HPRT1 and TBP were identified as the best combination of genes suitable for a normalization strategy. B2M should be avoided as a reference gene in these experimental conditions.

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