

Tissue-specific selection of stable reference genes for real-time PCR normalization in an obese rat model

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Abstract

Obesity is a complex pathology with interacting and confounding causes due to the environment, hormonal signaling patterns, and genetic predisposition. At present, the Zucker rat is an eligible genetic model for research on obesity and metabolic syndrome, allowing scrutiny of gene expression profiles. Real-time PCR is the benchmark method for measuring mRNA expressions, but the accuracy and reproducibility of its data greatly depend on appropriate normalization strategies. In the Zucker rat model, no specific reference genes have been identified in myocardium, kidney, and lung, the main organs involved in this syndrome. The aim of this study was to select among ten candidates (*Actb*, *Gapdh*, *Polr2a*, *Ywhag*, *Rpl13a*, *Sdha*, *Ppia*, *Tbp*, *Hprt1* and *Tfrc*) a set of reference genes that can be used for the normalization of mRNA expression data obtained by real-time PCR in obese and lean Zucker rats both at fasting and during acute hyperglycemia. The most stable genes in the heart were *Sdha*, *Tbp*, and *Hprt1*; in kidney, *Tbp*, *Actb*, and *Gapdh* were chosen, while *Actb*, *Ywhag*, and *Sdha* were selected as the most stably expressed set for pulmonary tissue. The normalization strategy was used to analyze mRNA expression of tumor necrosis factor α , the main inflammatory mediator in obesity, whose variations were more significant when normalized with the appropriately selected reference genes. The findings obtained in this study underline the importance of having three stably expressed reference gene sets for use in the cardiac, renal, and pulmonary tissues of an experimental model of obese and hyperglycemic Zucker rats.

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Introduction

Obesity is a chronic, multifactorial, and complex disease resulting from environmental and genetic factors. The progression of this pathology has been associated with an increased predisposition for developing additional morbid conditions, including heart disease, gastrointestinal, pulmonary, renal, hematological and skeletal complications, vascular disease, arthritic disorders, and several types of malignancies (Kopelman 2000, Martin *et al.* 2000, Antic *et al.* 2003, Rubenstein 2005, Kuchta 2005, Poirier *et al.* 2006, Singer & Granger 2007).

Recent studies have highlighted that the prevalence of obesity in both adults and children has increased worldwide over the past two decades, probably due to changes in dietary habits and lifestyle modifications (French *et al.* 2001). In fact, the childhood obesity epidemic has begun to compromise the health of the pediatric population by promoting the premature development of atherosclerosis and metabolic syndrome, both of which significantly increase the risk of cardiovascular disease early in life (Lavrador *et al.* 2011).

At present, Zucker rats are bred to be an eligible genetic model for research on obesity and metabolic

syndrome due to their incorporation of transgenic and knockout technology (Cozzi *et al.* 2009, Geurts *et al.* 2009). The obese Zucker rat (OZR) bears a mutation in the leptin receptor gene, an autosomal trait (*fa/fa*) located on chromosome 5 (Bray 1977, Sone & Osamura 2001). Owing to the impairment of the leptin receptor, OZR manifests an impaired satiety reflex, resulting in a consistent hyperphagia that leads to obesity. With both hypertrophy and hyperplasia of adipocytes, OZR begins to manifest many of the characteristics of human obesity: moderate hypertension, hypertriglycemia, insulin resistance, and the evolution of a pro-inflammatory state (Bray 1977, Vandesompele *et al.* 2002, Frisbee 2005, Vaziri *et al.* 2005, Johnson *et al.* 2006).

To measure the gene expression profile of the main biomarkers involved in obesity, real-time PCR is the benchmark method for detecting and quantifying mRNA expression because it allows sensitive, specific, and reliable results. An increasing number of reports have shown how the accuracy and reproducibility of real-time PCR data are closely dependent on appropriate normalization strategies to reduce the noise of the method (Bustin 2000, Vandesompele *et al.* 2002, Huggett *et al.* 2005, Hendriks-Balk *et al.* 2007, Martino *et al.* 2011). Until now, housekeeping genes

were adopted from the literature as reference genes, i.e. transcripts stably expressed among different samples irrespective of their specific tissue-dependent behavior; however, recent studies have shown that the expression levels of traditional housekeeping genes can vary markedly across cells, tissues, metabolic conditions, and between experimental treatments, emphasizing the need to adopt alternative reference genes or appropriate strategies for their selection (Schmittgen & Zakrajsek 2000, Deindl *et al.* 2002, Dheda *et al.* 2005, Brattelid *et al.* 2007, de Jonge *et al.* 2007). The use of at least three reference genes for the correct normalization of real-time PCR data has been proposed by Vandesompele *et al.* (2002), and, to date, it is considered the best approach for normalizing real-time PCR data. A reference gene should fulfill several criteria in order to be suitable for normalizing; for example, it should exhibit constitutive, nonregulated, and stable expression profiles in the sample and conditions analyzed. The reference gene and the target selected should display a similar mRNA expression level in the same sample; moreover, it is important to avoid amplification of genomic DNA using DNase treatment or stringent design of intron-spanning primers as well as to promote RNA-specific amplification.

This strategy of selecting reference genes has been applied in rat models for several different pathologies (Silver *et al.* 2008, Cook *et al.* 2010, Nelissen *et al.* 2010, Martínez-Beamonte *et al.* 2011), while in the OZR model, only one study on housekeeping gene expression has been published (Martínez-Beamonte *et al.* 2011) and, to the best of our knowledge, no specific reference genes have been identified in OZR myocardium, kidney, and lung, the main organs involved in metabolic syndrome.

The aim of this study was to select among ten candidates (*Actb*, *Gapdh*, *Polr2a*, *Ywhag*, *Rpl13a*, *Sdha*,

Ppia, *Tbp*, *Hprt1*, and *Tfrc*, Table 1) a set of reference genes that can be used for normalizing mRNA expression data obtained by real-time PCR in an OZR model. Although the search for appropriate reference genes is difficult and expensive, it is important to evaluate the suitability of different reference genes by assessing their stability simultaneously in control and obese rats both during fasting and during induction of acute hyperglycemia.

The normalization strategy used was tested by analyzing mRNA expression of tumor necrosis factor α (TNF α), the main inflammatory mediator in obesity, where its expression is correlated with the degree of adiposity and associated with insulin resistance (Ji *et al.* 2011).

Materials and methods

Ethics statement

National guidelines for the care and use of research animals (D.L. 116/92, implementation of EEC directive (609/86)) were followed.

Experimental animal model

The study included 20 male Zucker rats (Charles River Laboratories International, Inc., Wilmington, MA, USA) 9–11 weeks of age subdivided into two groups: obese rats (O, $n=10$, body weight = 351 ± 9.6 g) and age-matched lean rats (CO, $n=10$, body weight = 287.7 ± 24.3 g) as control. Rats were fasted for 12 h with unrestricted access to water. Some of the rats of each group were studied during fasting conditions (CO_{fb}, $n=5$, glycemia = 118 ± 14.4 mg/dl; O_{fb}, $n=5$, glycemia = 379 ± 42.7 mg/dl) and the remaining rats during the induction of acute hyperglycemia (CO_{AH}, $n=5$, glycemia = 351 ± 55 mg/dl;

Table 1 Full reference gene names

Genes	Full gene name	Gene functions
<i>Actb</i>	β -Actin	Component cytoskeletal structures involved in cellular process
<i>Gapdh</i>	Glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme involved in glycolysis and gluconeogenesis
<i>Hprt1</i>	Hypoxanthine phosphoribosyltransferase 1	Transferase played a central role in the generation of purine nucleotides
<i>Polr2a</i>	Polymerase (RNA) II (DNA directed) polypeptide A	DNA-dependent RNA polymerase
<i>Rpl13</i>	Ribosomal protein L13A	Component of the large 60S ribosomal subunit
<i>Sdha</i>	Succinate dehydrogenase complex, subunit A, flavoprotein	Protein complex catalyzed the oxidation of succinate
<i>Tbp</i>	TATA binding protein	Transcription factor played a central role in the initiation of eukaryotic mRNA synthesis by binding TATA box
<i>Tfrc</i>	Transferrin receptor	Plasma membrane transport glycoprotein involved in iron homeostasis
<i>Ywhag</i>	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	Involved in signal transduction pathways
<i>Ppia</i>	Peptidylprolyl isomerase A (cyclophilin A)	Accelerate protein folding

O_{AH} , $n=5$, glycemia = 448.5 ± 64.5 mg/dl). Hyperglycemia was induced by injecting a bolus of 0.5 ml of a 50% glucose solution.

At the time of killing, anesthesia was induced by inhalation of 2% isoflurane and maintained by i.p. administration of Zoletil (Virbac S.r.l., Milan, Italy; tiletamine and zolazepam 40 mg/kg) and xylazine (5 mg/kg; Guiducci *et al.* 2011). Tissue was collected from the left ventricle ($n=20$), kidney ($n=20$), and lung ($n=20$), immediately placed in ice-cold RNeasy lysis reagent (Qiagen S.p.A), and stored at -80°C for later use.

Tissue handling, RNA extraction and quality, and cDNA synthesis

The cardiac, renal, and pulmonary tissues were homogenized with an automated tissue lyser through high-speed shaking in plastic tubes with stainless steel beads (Qiagen S.p.A).

Total RNA was extracted using the acid guanidinium thiocyanate–phenol–chloroform method from tissue samples obtained from rats using an RNeasy Midi kit (Qiagen S.p.A) following the manufacturer's instructions. The RNA concentration and purity were determined spectrophotometrically (BioPhotometer; Eppendorf Italia, Milan, Italy) measuring spectral absorption at 260 nm. The reading ratio at 260 and 280 nm (A_{260}/A_{280}) provides an estimate of RNA purity with respect to contaminants absorbing in the u.v. spectrum, such as protein. The integrity and purity of total RNA was also detected by electrophoresis of samples on Gel Star Stain (Lonza, Rockland Inc., ME, USA) agarose gels. Samples showing clear and distinct 28S and 18S rRNA bands and having spectrophotometric OD 260:280 ratios of 1.9–2.1 were used. A known amount of total RNA (Ambion, Inc., Austin, TX, USA) was used as marker. The RNA samples were stored at -80°C for use in gene expression studies.

Following DNase treatment (RNase-Free DNase Set, Qiagen S.p.A), first-strand cDNA was synthesized with iScript cDNA Synthesis kit (Bio-Rad) using about 1 μg total RNA as template. Reverse transcriptase reaction sequence consisted of incubation at 25°C for 5 min, followed by three different cycles at 42°C for 30 min and 45 – 48°C for 10 min, in order to better separate the strands. The reverse transcriptase enzyme was inactivated by heating to 85°C for 5 min. The cDNA samples obtained were placed on ice and stored at 4°C until further use.

Real-time PCR

Real-time PCR reactions were performed in duplicate in the Bio-Rad C1000 thermal cycler (CFX-96 real-time PCR detection systems; Bio-Rad). For monitoring cDNA

amplification, a third-generation fluorophore, EvaGreen, was used (SsoFAST EvaGreen Supermix; Bio-Rad). PCR was performed in a volume of 20 μl /reaction; to minimize the influence of PCR inhibitors in real-time applications, all cDNA samples were diluted 1:5. Reaction mixture included 2 μl template cDNA (100 ng/ μl), 0.2 μM of each primer (Sigma–Aldrich), 1 \times SsoFAST EvaGreen SuperMix (Bio-Rad), and sterile H_2O . Amplification protocol started with 98°C for 30 s followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. To assess product specificity, amplicons were systematically checked by melting curve analysis. Melting curves were generated from 65 to 95°C with increments of 0.5°C /cycle. Multiple inter-run calibrators were always used to allow comparison of C_t values obtained in different runs.

Reference gene selection

Ten candidate reference genes, among the most commonly used references in the literature (Table 1), and TNF α primer pairs, designed with Primer Express Version 2.0 (Applied Biosystems), were selected as listed in Table 2. Whenever possible, intron-spanning primers were selected to avoid amplification of genomic DNA. Reaction conditions of all primer pairs used were optimized. In particular, a gradient PCR was conducted to assess the optimal annealing temperature while a standard curve obtained by scalar dilution of a cDNA pool (1:5, 1:25, 1:125, and 1:625) was always generated to verify PCR efficiency (Table 2).

MIQE guidelines

In an effort to provide greater transparency of our results between research laboratories, this study was carried out to conform to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE; Bustin *et al.* 2009). A MIQE checklist is listed in Table 2.

Data analysis

Analysis of reference genes using CFX96 software and qbase^{PLUS} software

The mRNA expression of ten candidate reference genes was measured by real-time PCR in the left ventricle, kidney, and lung of obese and lean Zucker rats both at fasting and during acute hyperglycemia.

The combination of qbase^{PLUS} Software (Biogazelle NV, Belgium) with Bio-Rad's CFX96 manager software is based on GeNorm and qBase technology, and it provided an accurate gene expression analysis of the real-time PCR data. This analysis is carried out using

Table 2 Details of specific primers used in real-time PCR experiments

Gene	Primer sequence	GenBank accession no.	Junction	Amplicon length (bp)	Temperature (°C)	Efficiency (%)	R ²
<i>Actb</i>	F: GTCGTACCACTGGCATTGTG R: CTCTCAGCTGTGGTGGTGAA	NM_031144	Exon 4	181	60	104.3	0.998
<i>Gapdh</i>	F: CTACCCACGGCAAGTTCAAC R: CCAGTAGACTCCACGACATAC	NM_01708	Exon 4/6	98	60	98.4	0.999
<i>Hprt1</i>	F: CCCAGCGTCGTGATTAGTGATG R: TTCAGTCTGTCCATAATCAGTC	NM_012583	Exon 1/3	125	60	103	0.998
<i>Polr2a</i>	F: CGTATCCGCATCATGAACAGTGA R: TCATCCATCTTATCCACCACCTCTT	XM_343922	Exon 1	70	60	105	0.998
<i>Rpl13</i>	F: GGATCCCTCCACCCTATGACA R: CTGGTACTTCCACCCGACCTC	NM_173340	Exon 8	130	60	101	0.999
<i>Sdha</i>	F: CTCTTTTGGACCTTGTCTCTTT R: TCTCCAGCAATTTGCCTTAATCGG	NM_130428	Exon 1	102	60	104.7	0.999
<i>Tbp</i>	F: CACCGTGAATCTTGGCTGTAAAC R: CGCAGTTGTTCGTGGCTCTC	NM_001004198	Exon 4/6	123	60	105	0.998
<i>Tfrc</i>	F: ATCATCAAGCAGCTGAGCCAG R: CTCGCCAGACTTTGCTGAATTT	NM_022712	Exon 4/5	123	60	93.1	0.998
<i>Ywhag</i>	F: TTCCTAAAGCCCTTCAAGGCA R: GGCTTTCTGCACTAGTTGCTCG	NM_019376.2	Exon 1	100	60	95.3	0.997
<i>Ppia</i>	F: CCAACACAAATGGTCCCAGT R: ATTCCTGGACCCAAAACGCT	NM_017101	Exon 3/4	135	60	98.4	0.998
<i>TNFα</i>	F: GCCCAGACCCTCACACTC R: CCACTCCAGCTGCTCTCT	NM_012675	Exon 4	98	60	105	0.997

proven solutions for normalization analysis, data quality control including the elimination of erroneous data, normalization for removing sample-specific nonbiological variation, and inter-run calibration, which can remove the technical variation between samples analyzed in different runs. The GeNorm normalization strategy was used to assess the expression stability of each candidate reference gene to determine the ideal number of genes required for normalization and to calculate individual normalization factors for each sample based on the expression levels of the best reference genes. The average C_t values obtained from each duplicate was converted to a relative quantity ($\Delta C_{(t)}$) and analyzed with GeNorm algorithm, which is based on both the geometric mean of a user-defined number of reference genes and, consequently, the principle that the expression ratio of two ideal reference genes should be identical in all samples. The relative quantity and the normalization factor were automatically calculated as follows:

$$\text{Relative quantity}_{\text{sample(Refgen)}} = E_{\text{Refgen}}(C_{t(\min)} - C_{t(\text{sample})})$$

$$\text{Normalization factor}_{\text{sample(target)}} = (\text{RQ}_{\text{sample(Refgen1)}} \times \text{RQ}_{\text{sample(Refgen2)}} \times \dots \times \text{RQ}_{\text{sample(Refgen}_n\text{)}})^{1/n}$$

where E , efficiency of primers; $C_{t(\min)}$, average C_t for the sample with the lowest average C_t for the target studied; C_t , average C_t for any sample; RQ, relative

quantity; n , number of reference genes; Refgen, reference gene.

Whereas GeNorm Excel could not distinguish the two most stably expressed genes, the modified algorithm in GeNorm^{PLUS} allows ranking of candidate reference genes up to the single most stable gene. The reference gene stability is expressed by the M value, which is calculated as the average pairwise variation between one of the genes and all the others analyzed. Genes are ranked for their M value, and at each step of the analysis the least stable gene (highest M value) is excluded and M is recalculated. The most stable pair of genes is identified through the reiteration of this procedure, in a step-wise manner. The optimal number of genes for normalization is determined starting from the most stable pair and sequentially adding the other genes (from the most to the least stable) until the pairwise variation reaches a set threshold (usually, M value < 0.5 for homogeneous group and M value < 1 for heterogeneous group and pairwise variation < 0.15).

Statistical analysis

The geometric mean of the three most stably expressed genes in the specific tissue (heart, kidney, and lung respectively) was used for normalization of *TNF α* mRNA expression. Relative quantification of *TNF α* was calculated by $\Delta\Delta C_t$ method.

Normalization factor_{sample(target)}

$$= \frac{RQ_{\text{sample(Refgen 1)}}}{\left(RQ_{\text{sample(Refgen 1)}} \times RQ_{\text{sample(Refgen 2)}} \times \dots \times RQ_{\text{sample(Refgen } n)} \right)^{1/n}}$$

Differences between more than two independent groups were analyzed by Fisher's test after ANOVA using Statview 5.0.1 Software released for Windows Statistical (SAS Institute, Inc., Cary, NC, USA). The results are expressed as mean \pm s.e.m. and P value < 0.05 was considered significant.

Results

Assessment of real-time PCR condition

The concentration and purity of RNA were evaluated by absorption wavelengths of 260 and 280 nm and the absorption ratio (A_{260}/A_{280}) of all preparations was between 1.9 and 2.1. Moreover, to check the integrity of 18S and 28S rRNAs, all samples were subjected to gel electrophoresis. To optimize the thermocycle profile of each reference gene, the optimal annealing temperature and RNA concentration were assessed for each designed PCR primer.

An explanatory example relative to the assessment of reaction condition for *Hprt1* in the left ventricle is shown in Fig. 1. In particular, threshold cycles and standard curve obtained by serial dilution (1:5) of a pool template and the relative melting analysis are reported (Fig. 1a, b, c and d). Dilution series were run for all candidate reference genes to quantify real-time PCR efficiency (Table 2) that resulted in the range of 95–105% and a linear standard curve, $R^2 > |0.990|$.

Reference gene selection

In Table 3, mean C_t , s.d. and the coefficient of variation of the ten reference genes for each tissue are listed separately. All the selected genes were detectable in myocardium, kidney, and lung of both control and OZR, although the threshold cycle range was different among genes tested in a tissue-specific manner (Fig. 2). Thus, the stability of mRNA expression was evaluated in the entire system (control lean rats + OZR at fasting and during acute hyperglycemia) for selecting the best reference genes for relative normalization in the three tissues separately. This strategy is also important in order to avoid the confounding effect of hyperglycemia induction.

As the first point, we analyzed the stability of reference gene expression considering each tissue separately but always considering control and OZR samples

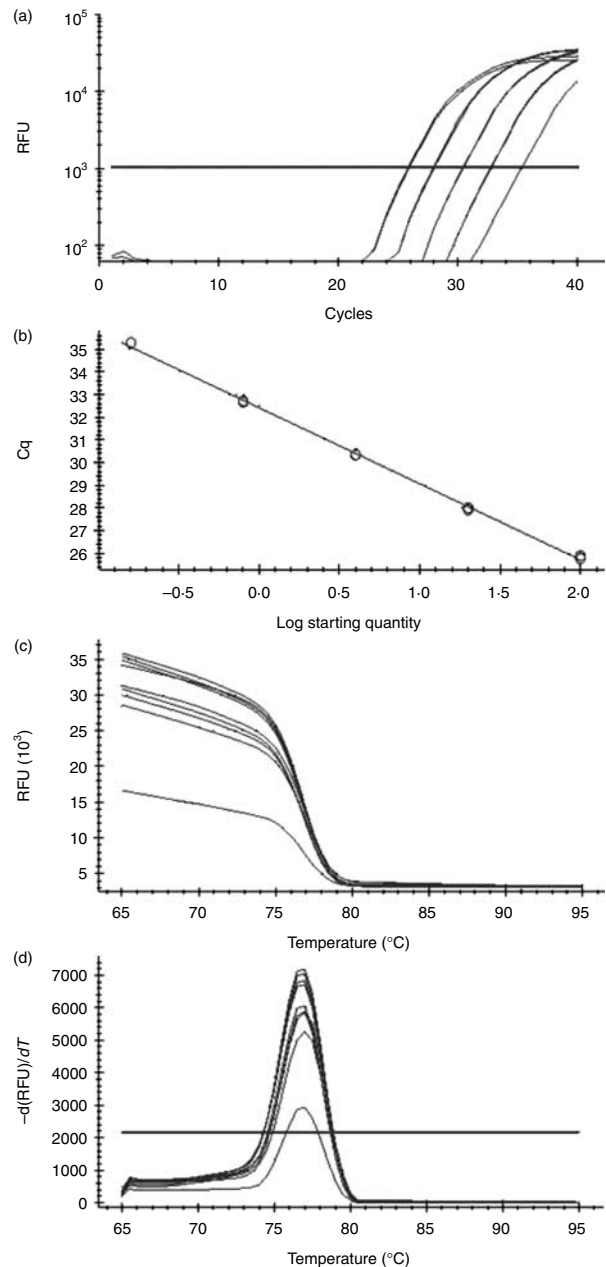


Figure 1 Explicative example of real-time PCR results obtained for *Hprt1* in rat cardiac tissue (left ventricle). (a) Threshold cycles (C_t) obtained by a standard curve (serial dilution, 1:5, of a pool template); (b) plot of the log of starting cDNA template quantity vs C_t : slope -2.32 ($\log_2(5) = 2.32$); efficiency: 99%; R^2 : 0.998; (c) melting curve; (d) rate of change of the relative fluorescence units (RFU) with time (T) ($-d(\text{RFU})/dT$) on the y-axis vs the temperature on the x-axis (peak at the melting temperature).

together. This analysis provided the gene expression stability measure (M) for each reference gene, which allowed ranking of them from the least stable (higher M value) to the most stably expressed (lowest M value).

Table 3 Mean C_t , s.d. and coefficient of variation (CV) of reference genes in cardiac, renal, and pulmonary tissues of control lean Zucker rats and obese Zucker rats during both fasting and acute hyperglycemia conditions

Gene	Cardiac tissue			Renal tissue			Pulmonary tissue		
	Mean C_t	s.d.	CV%	Mean C_t	s.d.	CV%	Mean C_t	s.d.	CV%
<i>Tfrc</i>	32	2.7	8.4	30	2.9	9.7	31	2	6.4
<i>Ppia</i>	28	1.7	6.1	27	2.2	8	22	1.8	8.2
<i>Rpl13</i>	25	1.5	6	22	1.4	6.4	20	1.5	7.5
<i>Ywhag</i>	32	1.7	5.3	31	2.4	7.7	29	2.2	7.6
<i>Polr2a</i>	34	1.8	5.3	30	3.1	10	28	2.3	8.2
<i>Actb</i>	29	1.7	5.9	25	2	8	25	2.3	9.2
<i>Gapdh</i>	25	1.5	6	24	2	8.3	25	1.5	6
<i>Sdha</i>	31	1.8	5.8	28	2	7.1	26	2.2	8.4
<i>Tbp</i>	34	1.7	5	32	1.7	5.3	32	1.8	5.6
<i>Hprt1</i>	30	1.5	5	29	2	7	27	1.8	6.7

Thus, ordering genes according to M values ($M < 1$ for heterogeneous groups) in the analyzed conditions, the rank was different depending on the tissue considered. In the left ventricle, the most stably expressed genes were *Hprt1*, *Tbp*, and *Sdha*; while in kidney, they were *Actb*, *Gapdh*, and *Tbp*, and in pulmonary tissue, the most stable were *Hprt1*, *Ywhag*, and *Sdha* (Fig. 3).

Relevance of selecting specific reference genes for the evaluation of *Tnf α* expression

The expression of *TNF α* in the different tissues, in terms of C_t , was similar in the heart (mean \pm s.d., 35.0 ± 1.10) and in the kidney (mean \pm s.d., 35.5 ± 1.6) but was significantly lower in the lung (mean \pm s.d., 33.4 ± 1.7 ; $P=0.007$ vs heart, $P=0.0006$ vs kidney).

To assess whether the normalization strategy used was effective, *Tnf α* mRNA levels were analyzed using different normalization settings. In particular, *Tnf α* mRNA gene expression was normalized separately for each tissue, with the subset of the three most stably expressed genes selected in our study, and against *Gapdh*, one of the most frequently used reference genes in the literature.

As shown in Fig. 4, when *Tnf α* was normalized with the three best genes in the left ventricle (*Sdha*, *Tbp*, and *Hprt1*), its mRNA levels showed an increase in fasting OZR (O_{fc}) with respect to controls (CO_{fc}), although not significantly, while in hyperglycemic OZR (O_{AH}) and respective controls (CO_{AH}), a similar variation was not observed. A statistical significance was obtained between fasting and hyperglycemic OZR ($P=0.049$). On the contrary, after normalization against *GAPDH*, *TNF α* relative expression differences were blunted and statistical significance was suppressed.

This normalization strategy comparison was also applied in renal and pulmonary tissues and the results obtained are shown in Fig. 4.

When controls and OZR were considered without splitting in fasting and hyperglycemic, no significant difference was observed even if the variation of *Tnf α* mRNA expression was more emphasized after

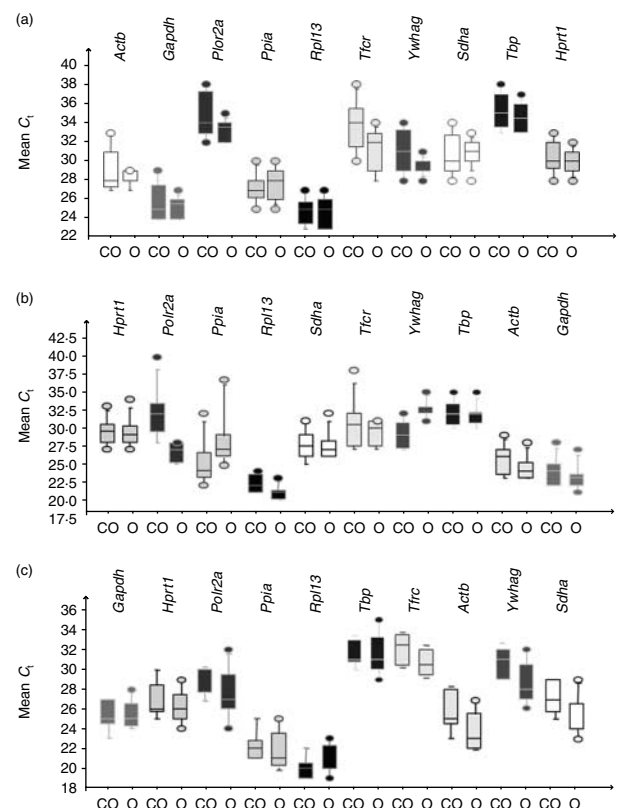


Figure 2 C_t values in control lean Zucker rats and obese Zucker rats (during fasting and acute hyperglycemia): each box consists of five horizontal lines displaying the 10th, 25th, 50th (median), 75th, and 90th percentiles of the variable. All values above the 90th percentile and below the 10th percentile are plotted separately. (a) C_t value ranges in the left ventricle; (b) C_t value ranges in the kidney; (c) C_t value ranges in the lung of control lean Zucker rats ($CO_{fc}+CO_{AH}$) and obese Zucker rats ($O_{fc}+O_{AH}$).

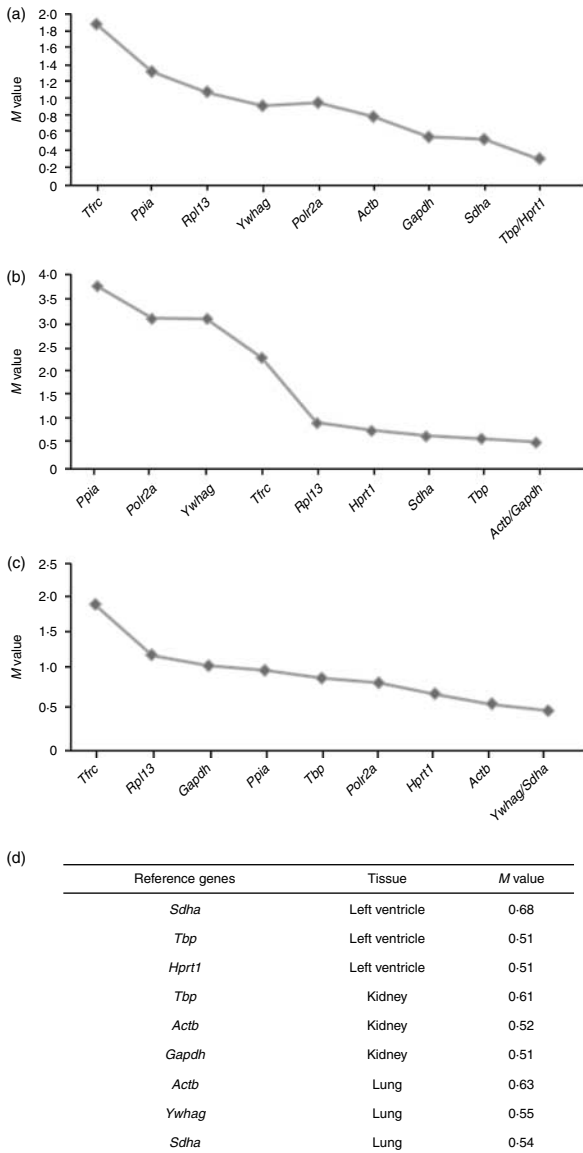


Figure 3 Evaluation of reference gene expression stability (M value) during stepwise exclusion of the least stable gene. Evaluation of reference gene expression stability (M value) during stepwise exclusion of the least stable gene in the left ventricle (a), kidney (b), and lung (c). Summary of M values of the most stable pair in cardiac, renal, and pulmonary tissues (d).

normalization with the most stably expressed reference gene (heart: $TNF\alpha$ mRNA expression normalized with *Gapdh*, $CO = 1.23 \pm 0.3$ vs $O = 1.42 \pm 0.3$; normalized with *Sdha-Tbp-Hprt1*, $CO = 2.03 \pm 0.4$ vs $O = 3.05 \pm 0.9$; kidney: $TNF\alpha$ mRNA expression normalized with *Gapdh*, $CO = 1.32 \pm 0.4$ vs $O = 1.19 \pm 0.3$; normalized with *Actb-Gapdh-Tbp*, $CO = 1.33 \pm 0.4$ vs $O = 1.02 \pm 0.2$; lung, $TNF\alpha$ mRNA expression normalized with *Gapdh*, $CO = 0.32 \pm 0.1$ vs $O = 1.74 \pm 0.6$; normalized with *Hprt1-Ywhag-Sdha*, $CO = 0.48 \pm 0.07$ vs $O = 1.38 \pm 0.4$).

Discussion

Very little is known about the expression of reference genes in cardiac, renal, and pulmonary tissues of the Zucker rat, the eligible genetic model of obesity (Bray 1977, Frisbee 2005, Johnson *et al.* 2006). To the best of our knowledge, this is the first report dealing with the selection of reference genes in three different types of tissues, such as heart, lung, and kidney, and the first one to explore the variability between control lean Zucker rats and OZR, both during fasting and during acute hyperglycemia, suggesting that specific subsets of reference genes should be used for each tissue analyzed.

As a matter of fact, the importance of an adequate normalization strategy of data provided by real-time PCR is still underestimated. As proposed by Vandesompele *et al.* (2002), the use of multiple reference genes, identified on the basis of their stability in experimental conditions allows a more accurate normalization of expression data, in particular for genes with slight expression difference.

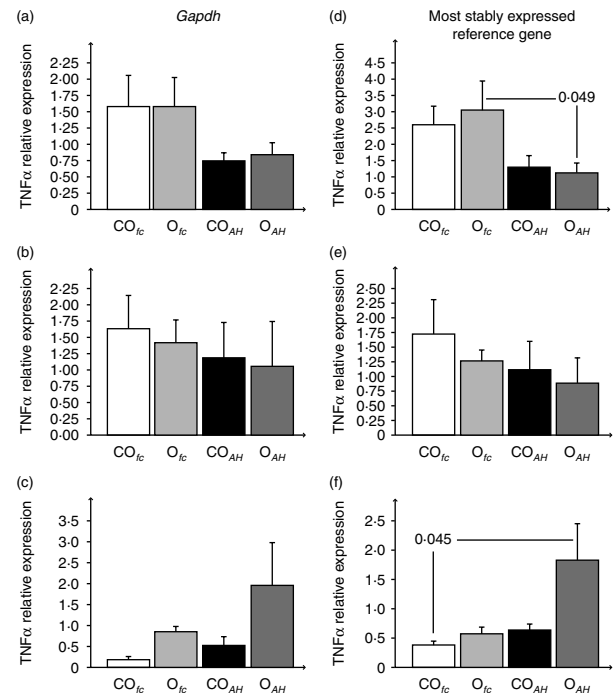


Figure 4 $Tnf\alpha$ mRNA expression in cardiac, renal, and pulmonary tissues. On the left side: $Tnf\alpha$ expression normalized for *Gapdh* relative levels in (a) left ventricle, (b) kidney, and (c) lung respectively. On the right side: $Tnf\alpha$ expression normalized with the most stable genes selected for (d) left ventricle (*Sdha*, *Hprt1*, and *Tbp*), (e) kidney (*Tbp*, *Actb*, and *Gapdh*), (f) lung (*Actb*, *Ywhag*, and *Sdha*) respectively. CO_{fc}, control lean Zucker rat during fasting condition; O_{fc}, obese Zucker rat during fasting condition; CO_{AH}, control lean Zucker rat during acute hyperglycemia; O_{AH}, obese Zucker rat during acute hyperglycemia.

Following this methodological approach, our study found that the number of potential reference genes used in the selection of the most stably expressed set has a relevant influence on the outcome of our final real-time PCR results. Thus, we evaluated ten reference genes among the most commonly used genes in different species and conditions in order to identify the most stable combination, specific for each tissue in the Zucker rat model: heart, kidney, and lung.

The integration of GeNorm in the qbase^{PLUS}/qPCR data analysis software (Bio-Rad's CFX96 real-time PCR systems) provided us with a fully automated calculation, handling of missing data, ranking of candidate reference genes up to the single most stable gene, and expert reporting for understanding of GeNorm study. Moreover, whereas GeNorm Excel could not distinguish the two most stably expressed genes, the modified algorithm in GeNorm^{PLUS} allowed us to rank of candidate reference genes up to the single most stable gene.

Using the GeNorm^{PLUS} algorithm, we were able to identify the set of three reference genes that were used for gene expression normalization in real-time PCR experiments on this rat model of obesity. Each gene was detectable in rat samples, although a certain degree of variability in its expression was shown. Interestingly, substantial differences in gene expression stability, derived from the *M* values, were observed between heart, kidney, and lung. We evaluated the impact of the addition of other reference genes to the best pair and we observed that the addition of a third gene has the greatest impact on reducing variability.

However, *Sdha*, *Tbp*, and *Hprt1* were the best candidates for use as reference genes in cardiac tissue; *Tbp*, *Actb*, and *Gapdh* were adopted as reference genes for kidney; and *Actb*, *Ywhag*, and *Sdha* were selected as the most stably expressed set for pulmonary tissue. The three sets of housekeeping genes selected for each tissue separately encoded for proteins with independent physiological functions, avoiding confounding effects in normalization analysis.

In the light of recent studies showing that the fold-change in expression of a target gene may be widely under/overestimated when using a single, less stable reference gene (Kosir *et al.* 2010, Piehler *et al.* 2010), we have shown the importance of this strategy by evaluating the relative expression of *Tnfa*. Our results showed differences in the levels of *Tnfa* mRNA normalized with a single commonly used reference gene (*Gapdh*) or with our set of selected reference genes. Citing cardiac tissue as an explicative example, *Tnfa* mRNA expression was significantly lower in hyperglycemic OZR (*O_{AH}*) with respect to the group of fasting OZR (*O_{fc}*) if normalized with the three most stably expressed genes (*Sdha*, *Tbp*, and *Hprt1*), while no significant difference was observed using only *Gapdh*.

Regarding the other tissues, in the kidney, no statistical difference between TNF α values normalizing either with *Gapdh* or with the stable set selected was found, while in the lung, a significant difference was observed between fasting lean Zucker rats with respect to hyperglycemic OZR by the selected reference genes (*Hprt1*, *Ywhag*, and *Sdha*).

The combination of inflammation and insulin resistance in this model of obesity forms a common risk platform for the development of a different organ-specific vulnerability; thus, the lack of statistical significance in kidney and the slight differences observed in lung compared with left ventricle might be due to different susceptibilities to developing disease. We can assume that the lung and kidney, organs that are more easily predisposed to cancer, developed pathologies more slowly than the heart, in which obesity can induce cardiac pathologies.

The significant alteration of TNF α observed in the left ventricle could be important for its clinical significance because glucose disposal is thought to be controlled by TNF α release to optimize insulin signaling (Hotamisligil *et al.* 1995, Del Aguila *et al.* 1999). Further studies are also necessary to better understand this complex mechanism of regulation mediated by TNF α .

As the use of unvalidated reference genes can generate biased results, it appears to be of crucial importance to provide a set of reference genes specifically selected for the experimental conditions chosen to perform real-time PCR studies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

As to the contribution of each author, S D R and M C designed the study, analyzed and interpreted the results, and drafted the manuscript; S R, C C, M A G, A D, and T P collected the biological samples, performed the experiments, and built the database; and D G contributed to data discussion and interpretation. All authors participated in the discussion and interpretation of the results and in the final approval of the manuscript submitted.

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