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Validation of internal control genes for gene expression analysis in diabetic glomerulosclerosis

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Validation of internal control genes for gene expression analysis in diabetic glomerulosclerosis.

Background. Gene expression analysis is an invaluable tool in the study of diabetic glomerulosclerosis. The necessary denominator for the quantitative expression of a specific gene is the expression level of a second gene that is presumed to remain unchanged. Thus, it is critical that the stability of this housekeeping gene in diabetic glomeruli or in cultured glomerular cells is not altered by the disease or a high glucose environment, respectively. Although gene expression quantification, achieved by Northern blot analysis or real-time reverse transcription-polymerase chain reaction (RT-PCR) has been extensively applied in diabetic renal tissue in vivo and in vitro, there are no studies validating the use of any specific endogenous control gene in these measurements.

Methods. We performed real-time RT-PCR using RNA from microdissected diabetic glomeruli and from mesangial cells cultured in high glucose concentration to investigate gene expression stability of β-*actin*, *glyceraldehyde-3-phosphate dehydrogenase* (*GADPH*), *phospholipase* A_2, β_2-*microglobulin*, *acidic ribosomal protein 36B4*, and *cyclophilin A*.

Results. Using an analysis method which is independent of gene abundance and compares the pair-wise variation of a given housekeeping gene with all other control genes, β-*actin* and *phospholipase* A_2, were found to be the most stable genes in diabetic glomeruli and in primary mesangial cells exposed to 20 mmol/L glucose.

Conclusion. It is proposed that the expression level of these genes is the best reference to evaluate relative changes in gene activity in diabetic/high glucose exposed glomerular tissues.

Gene expression analysis is integral to unraveling the pathophysiology of diabetic nephropathy and particularly diabetic glomerulosclerosis. Common techniques include Northern blot analysis and real-time, reverse transcription-polymerase chain reaction (RT-PCR) which has become popular due to its ability to quantify genes in small quantities of tissue. Quantification is usually achieved by expressing the abundance of the gene of interest relative to that of an internal control gene, known as a housekeeping gene, which by necessity should not change under the experimental conditions (e.g., diabetes). Commonly used housekeeping genes in gene expression analyses of diabetic glomerulosclerosis have included *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and β-*actin* [1–11]. Surprisingly, there are few studies validating the stability of genes used as endogenous controls in kidney diseases and none that address this critical issue in diabetic glomerulopathy [12, 13]. We performed real-time, RT-PCR on RNA extracted from glomeruli microdissected from control and diabetic Munich-Wistar rats and from primary mesangial cells chronically exposed to 5 mmol/L and 20 mmol/L glucose to quantify the expression of β-*actin*, *GAPDH*, *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide,* also known as *phospholipase* A_2 (*YWHAZ*), β_2-*microglobulin* (β_2M), *acidic ribosomal protein 36B4* (*36B4*), and *cyclophilin A* (*CycA*). Each of these genes has been used as an endogenous control previously and many have been studied as potential references in nonrenal tissue [12–18]. The amount of total RNA used as template was also included as a possible reference of gene expression. The most stable internal control genes were identified using the GeNorm software, which applies a measure of gene-stability that is unaffected by gene abundance and does not require normalization [13].

Key words: diabetic nephropathy, housekeeping genes, internal control genes, RT-PCR.

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METHODS

Materials

Streptozotocin, dextran T-40, ribonucleoside vanadyl complex, and the total glycated hemoglobin kit (TGHb kit #442) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Isofluorane (Forane®) was obtained from Baxter (Deerfield, IL, USA). Ambion (Austin, TX, USA) supplied the RNase inhibitor SUPERaseIN. Qiagen Inc. (Valencia, CA, USA) provided the following kits for mRNA purification and quantification: RNeasy Mini Kit, RNase-free DNase Set, and Quantitect Probe

RT-PCR Kit. Primers and probes were purchased from TIB-Molbiol LLC (Adelphia, NJ, USA). Tissue culture flasks (75 cm^2) were obtained from Corning Inc. (Corning, NY, USA). RPMI 1640 culture medium and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA).

Animals

Simonsen Laboratories (Gilroy, CA, USA) supplied the animals. Studies were carried out according to protocols approved by the Institutional Animal Care and Use Committee of Henry Ford Health Sciences Center. Insulin-deficient diabetes was induced in 14 male Munich-Wistar rats at age 4 to 5 weeks old by a single intravenous dose of 55 mg/kg streptozotocin, dissolved in sodium citrate buffer-acidified normal saline, via the dorsal penile vein. Fourteen control animals received the same volume of solution vehicle. Postprandial (morning) blood glucose measurements were obtained twice weekly for 2 weeks then once every 3 to 4 weeks during the rest of the study. A blood sample was obtained for determination of glycosylated hemoglobin at the completion of the observation period.

Glomerular microdissection

After 22 weeks following streptozotocin injection, glomeruli were microdissected from the left renal cortex for isolation of total RNA, according to a modification of a previously described method [19]. Following isofluorane/oxygen anesthesia, retrograde perfusion of the left kidneys with 50 mL of ice-cold Hank's balanced salt solution (HBSS) containing 3.2% dextran T40 was carried out via a needle placed in the abdominal aorta below the renal arteries, while the organ was externally cooled with ice-cold 0.15 mol/L NaCl. Sagittal slices from the left kidney were made and placed in 0.8 μm pore filtered ice-cold HBSS, containing 5% ribonucleoside vanadyl complex. A total of 160 glomeruli were microdissected in less than 25 minutes and transferred into 50 μL of sterile phosphate-buffered saline (PBS) containing 2 U/μL of RNase inhibitor. Following the addition of 350 μL of lysis buffer (buffer RLT) (RNeasy Mini Kit) samples were rapidly homogenized with two 15 second bursts using a rotor-strator homogenizer fitted with a 5 mm diameter probe (Omni μH Micro Homogenizer) (Omni International Inc., Warrenton, VA, USA) and stored at −70°C. Total glomerular RNA was isolated from glomerular homogenates using the Qiagen's RNeasy Mini Kit, according to the manufacturer's instructions. This isolation included an on-column DNase digestion step. Five microliters of isolated RNA from each of the 14 individual glomerular preparations in each group was pooled. RNA was stored at −70°C until RT-PCR was performed.

Cell culture

Primary cultures of mesangial cells were obtained from outgrowths of isolated glomeruli from Munich-Wistar rats as previously described [20]. Cells were grown in 75 cm^2 culture flasks in RPMI 1640 medium, pH 7.4 containing 5 Mmol/L glucose, 2 mmol/L L-glutamine, 23.8 mmol/L $NaHCO_3$, 15% fetal bovine serum (FBS), 5 U/mL penicillin G, and 5 U/mL streptomycin. Culture medium was changed at 2- or 3-day intervals. When reaching confluency at 10 days of growth, cells were reseeded at 150×10^3 per flask. Eight weeks before the termination of the experiments the medium in the experimental group was changed to a similar medium containing 20 mmol/L glucose, while the control group was continuously grown in 5 mmol/L glucose. At the 12th passage, cultures were harvested and total RNA isolated when 90% to 100% confluent using Qiagen's RNeasy Mini Kit. This included the addition of 700 μL of lysis buffer (buffer RLT) (RNeasy Mini Kit) to each flask followed by homogenization as above. An on-column DNase digestion step was again included and RNA stored at −70°C until RT-PCR was performed.

Total RNA quantification

Total RNA was quantified using the RiboGreen® RNA Quantitation Kit (Molecular Probes Inc., Eugene, OR, USA) which uses 16S and 23S ribosomal RNA as its RNA standard. Samples were analyzed in triplicate using the Fluoroskan Ascent FL (Thermo Electron Co., Milford, MA, USA) microplate fluorimeter.

Quantitative real-time RT-PCR

Rapid cycle, real-time quantitative RT-PCR was carried out using the Roche LightCycler (Roche Molecular Biochemicals, Indianapolis, IN, USA). In this method, a one-step protocol was performed with the RT and PCR reactions carried out sequentially in the same reaction capillary. A commercially available reaction mix was used (QuantiTect™ Probe RT-PCR) and RT-PCR was performed according to the manufacturer's instructions (Qiagen) using 2 μL of sample RNA, 1 μmol/L primer, and 0.3 μmol/L probe. The housekeeping gene of interest was amplified, following the RT reaction, with specific primers and the quantity of product formation detected by fluorescence resonance energy transfer (FRET) reactions using sequence-specific pairs of hybridization probes labeled with fluorescein and an acceptor dye (LC-Red 705). Conditions of RT-PCR included 30 minutes reverse transcription at 50°C, 15 minutes polymerase activation at 95°C, and 35 cycles at 95°C for 0 seconds, annealing at optimal temperature for 40 seconds, and extension at 72°C for 40 seconds. Primers, probes, and the experimentally determined annealing temperature for each gene are listed in Table 1. All primers were specif-

Table 1. Primers, probes, and experimentally determined optimal annealing temperature (T_A) used to determine housekeeping gene expression

	Forward Primer Reverse Primer Donor Probe Acceptor Probe	T_A
β-*actin*	ACCCACACTgTgCCCATCTA ggCCATCTCTTgCTCgAA gCCACgCTCggTCAggATCTTCAT X LC705 AggTAgTCTgTCAggTCCCggCCA p	58°C
Cyclophilin A (*CycA*)	TACCTACATTCCAAgCCTgC CACCAgTgAgCTTCCTgC TgCCgTCTTTCACCTTCCCTAAg X LC705 CCACAACATgAAAgCCATCTgACC p	60°C
Phospholipase A_2 (*YWHAZ*)	TggTgATgACAAgAAAgggA TgTATCAAgTTCAgCAATggC AACCAACACATCCTATCAgACTggg X LC705 CTggCCCTTAACTTCTCTgTgTTCTA p	50°C
Acidic ribosomal phosphoprotein 36B4	ggCATCACCACTAAAATCTCCA CCAgTgggAAggTgTAgTCAg TCCCACCTTgTCTCCAgTCTTTATCAgC X LC705 gCACATCgCTCAggATTTCAATggT p	58°C
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	CTCCCTCAAgATTgTCAgCAA GTCAgATCCACAACggATACATT ACAgTCTTCTgAgTggCAgTgATggCA X LC705 ACTgTggTCATgAgCCCTTCCACgAT p	58°C
β_2-*microglobulin* ($\beta_2 M$)	TgCCATTCAgAAACTCCC TTCAgTgTgAgCCAggATgT CTATCTgAggTgggTggAACTgAgAC X LC705 CgTAgCAgTTgAggAAgTTgggCTT p	60°C

ically designed for intron-exon splice regions thereby preventing any possible genomic DNA amplification. After optimal RT-PCR conditions for each gene were determined, the resultant reaction products were separated electropheretically using a 1.5% agarose gel for verification by size with an appropriate DNA ladder. As controls, a sample with all reagents except RNA and a sample, including all reagents except reverse transcriptase, were included. Values were calculated as arbitrary units according to results from standard curves generated with total renal cortex RNA. Individual template concentrations of five-point standard curves were measured in triplicate with each real-time RT-PCR assay. Equal amounts of RNA, obtained from glomerular isolates in 14 individual control or diabetic animals, were pooled and measured in quintuplet. Their mean values were used in the GeNorm analysis. Similarly, equal amounts of total RNA, isolated from two 7×10^6 primary mesangial cell cultures grown in 5 mmol/L or 20 mmol/L glucose, were pooled. These pools were again measured in quintuplet and their mean values used in the GeNorm analysis. As for the isolated glomeruli, a full standard curve was also analyzed with each mesangial cell real-time RT-PCR assay.

Data analysis

Characteristics of control and diabetic rats were expressed as mean ± SE. Statistical significance of differences between means was determined by unpaired *t* test and the distribution of *t* in a two-tailed test. *P* values less than 0.05 were considered significant.

To determine the stability of endogenous control genes, analysis was performed using the GeNorm 3.3 visual basic application for Microsoft Excel (*http://medgen31.ugent.be/jvdesomp/genorm/*) validated previously [13]. Briefly, this method is based on the principle that the expression ratio of two ideal control genes should be identical in all samples and unchanged by the experimental conditions. An increase in the ratio variability represents decreased expression stability of one, or both, of the genes being evaluated. The program calculates the standard deviation of log-transformed control gene ratios. These ratios do not require normalization or equality of statistical variance making them strongly representative of the variation between two control genes. The ratios are also independent of differences in gene abundance and are equally affected by outlying ratios. The GeNorm software calculates the gene-stability measure (M) by determining the average pair-wise variation between a particular gene and all other control genes. By stepwise exclusion of the least stable gene (highest M value) and recalculation of M values after each exclusion, the two most stable genes are identified.

RESULTS

Diabetic rats had elevated blood glucose (control 75 ± 2 mg/dL, diabetic 461 ± 22 mg/dL) ($P < 0.0001$) and

Table 2. Relative abundance of gene specific mRNA as quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR) for microdissected glomeruli and primary mesangial cells listed from most abundant to least abundant (top to bottom)

Gene	Control microdissected glomeruli *arbitrary units*[a]	Gene	Control primary mesangial cells *arbitrary units*[a]
β-*actin*	22.3	β-actin	20.7
Phospholipase A₂ (YWHAZ)	16.5	*Phospholipase A₂ (YWHAZ)*	18.6
β2-*microglobulin* (β_2M)	12.0	*Acidic ribosomal phosphoprotein 36B4*	11.7
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	6.4	*Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*	11.6
Acidic ribosomal phosphoprotein 36B4	5.1	β2-*microglobulin* (β_2M)	7.0
Cyclophilin A (CycA)	1.0	*Cyclophilin A (CycA)*	1.0

[a]Results are expressed relative to *cyclophilin A* (least abundant gene).

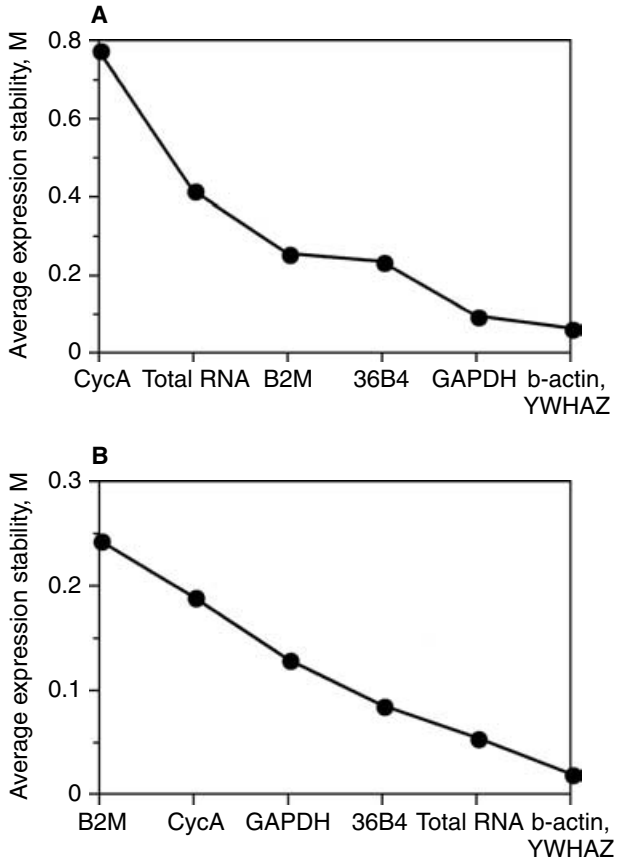

Fig. 1. Expression stability of internal control genes using the GeNorm expression analysis software, plotted from least stable to most stable (left to right). (*A*) Pooled samples of microdissected glomerular RNA from control and diabetic rats (160 glomeruli/animal). (*B*) RNA extracted from primary mesangial cells cultured for 8 weeks in 5 mmol/L or 20 mmol/L glucose.

increased glycated hemoglobin (control 5.4 ± 0.4%, diabetic 11.8 ± 0.4%) ($P < 0.0001$) as compared to controls. Diabetic animals had less weight gain than controls resulting in lower final weight (control 349 ± 6 g, diabetic 265 ± 10 g) ($P < 0.0001$).

RT-PCR results of RNA standards yielded linear time-cycle/log concentration regressions for all genes, with correlation coefficients of 0.98 or greater and standard errors below 0.12. The housekeeping genes selected demonstrated a wide variation in their expression levels (Table 2). The GeNorm analysis demonstated a greater overall stability of internal control gene expression in cultured mesangial cells than in microdissected glomeruli (Fig. 1). β-*actin* and *YWHAZ* offered the most stable expression in diabetic microdissected glomeruli as well as in primary mesangial cells cultured in 20 mmol/L glucose (Table 3). For the purpose of illustration, Tables 4 (isolated glomeruli) and 5 (cultured mesangial cells) contain the relative expression ratios, prior to log-transformation, of the two extremes of control gene stability as compared to the rest of the endogenous controls tested.

Table 3. Internal control genes ranked from most stable expression (top) to least stable expression (bottom) in RNA samples from control and diabetic microdissected glomeruli and from primary mesangial cells cultured in 5 mmol/L or 20 mmol/L glucose

Microdissected glomeruli	Primary mesangial cells
β-*actin, phospholipase A₂ (YWHAZ)*	β-*actin, phospholipase A₂ (YWHAZ)*
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	*Total RNA*
Acidic ribosomal phosphoprotein 36B4	*Acidic ribosomal phosphoprotein 36B4*
β2-*microglobulin* (β_2M)	*Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*
Total RNA	*Cyclophilin A (CycA)*
Cyclophilin A (CycA)	β2-microglobulin (β_2M)

Total RNA concentration in the specimens was 6.3 ng/μL and 7.7 ng/μL in the pooled control and diabetic glomeruli, respectively. In the samples of primary mesangial cells cultured in 5 mmol/L and 20 mmol/L glucose, the corresponding concentrations of total RNA were 194 ng/μL and 208 ng/μL, respectively. In glomerular samples, total RNA template used in the reaction was a poor marker for endogenous control gene stability while in primary mesangial cells, total RNA correlated reasonably well with stability of housekeeping gene expression (Fig. 1).

DISCUSSION

Enhanced expression of multiple genes is expected in diabetes and after exposure to a high glucose

Table 4. Relative expression ratios for the best [β-*actin* and *phosholipase* A_2 (*YWHAZ*)] and worst [*cyclophilin A* (*CycA*)] internal control genes prior to log-transformation using total RNA isolated from control and diabetic microdissected glomeruli (the ratio between two ideal endogenous controls should be identical across treatment groups)

	β-*actin:* *YWHAZ*	β-*actin:glyceraldehyde-*3-phosphate *dehydrogenase* (*GAPDH*)	β-*actin:* Total RNA	β-*actin:acidic ribosomal phosphoprotein 36B4*	β-*actin:*β2-*microglobulin* (β_2M)	β-*actin:CycA*
Control	1.35	3.45	0.87	4.40	1.85	22.4
Diabetes	1.43	3.20	1.53	3.04	1.27	4.27
% difference	5.9%	8.0%	43.5%	44.9%	45.8%	423%
	*YWHAZ:*β-*actin*	*YWHAZ: GAPDH*	*YWHAZ:* Total RNA	*YWHAZ:36B4*	*YWHAZ:*β_2M	*YWHAZ:CycA*
Control	0.74	2.57	0.64	3.27	1.37	16.6
Diabetes	0.70	2.23	1.07	2.12	0.89	2.99
% difference	6.3%	14.8%	39.9%	53.9%	54.9%	456%
	CycA:β_2M	*CycA:36B4*	*CycA: GAPDH*	*CycA:*β-*actin*	*CycA:YWHAZ*	*CycA:*Total RNA
Control	0.08	0.20	0.15	0.04	0.06	0.04
Diabetes	0.30	0.71	0.75	0.23	0.33	0.35
% difference	72.1%	72.3%	79.3%	80.9%	82.0%	89.2%

Table 5. Relative expression ratios for the best [β-*actin* and *phospholipase* A_2 (*YWHAZ*)] and worst β_2-*microglobulin* (β_2M)] internal control genes prior to log-transformation using total RNA isolated from primary mesangial cells cultured in 5 mmol/L and 20 mmol/L glucose (the ratio between two ideal endogenous controls should be identical across treatment groups)

	β-*actin: YWHAZ*	β-*actin*: Total RNA	β-*actin*: *acidic ribosomal phosphoprotein 36B43*	β-*actin*: *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*)	β-*actin*: *cyclophilin A* (*CycA*)	β-*actin:*β_2M
5 mmol/L	1.11	0.063	1.77	1.78	0.21	2.97
20 mmol/L	1.13	0.062	1.95	1.49	0.27	4.43
% difference	1.9%	2.2%	9.5%	19.2%	24.3%	33.0%
	*YWHAZ:*β-*actin*	*YWHAZ:* Total RNA	*YWHAZ:36B4*	*YWHAZ:GAPDH*	*YWHAZ:CycA*	*YWHAZ:*β_2M
5 mmol/L	0.90	0.057	1.59	1.60	0.19	2.67
20 mmol/L	0.88	0.054	1.72	1.32	0.24	3.91
% difference	1.9%	4.1%	7.7%	21.5%	22.9%	31.7%
	β_2M*:CycA*	β_2M*:36B4*	β_2M*:YWHAZ*	β_2M:β-*actin*	β_2M*:* Total RNA	β_2M*:GAPDH*
5 mmol/L	0.070	0.60	0.37	0.34	0.021	0.60
20 mmol/L	0.062	0.44	0.26	0.23	0.014	0.34
% difference	12.9%	35.1%	46.4%	49.1%	52.4%	77.9%

environment as a result of glomerular cell hypertrophy [21–23]. The imperative question is if there is differential expression among some of these genes. Investigators have demonstrated changes in expression patterns among several genes believed to be involved in the development of diabetic glomerulopathy, including *transforming growth factor*-β (*TGF*-β), *Smad3*, *fibronectin*, *type IV collagen*, and *monocyte chemoattractant protein-1* (*MCP-1*) [1–11]. We present herein the first validation of endogenous control genes used to measure the relative expression of these pathogenetically relevant genes.

Vandesompele et al [13] analyzed several internal control genes including *GAPDH*, β_2M, β-*actin*, and *YWHAZ* by RT-PCR in various human tissues. Importantly, they developed and validated the GeNorm analysis software used herein to analyze the stability of housekeeping genes without prior knowledge of the amount of starting RNA by calculating the standard deviation of the log-transformed control gene ratios. This logarithmic transformation is necessary to control for possible outlying values and differences in gene abundance.

YWHAZ was found to be a robust endogenous control gene in human leukocyte samples but not as stable in other human tissues by Vandesompele et al [13]. Their analysis did not include kidney tissue or cultured renal cells. β-*actin* has been used as an internal control gene for gene expression analysis by investigators studying diabetic nephropathy [2, 6, 8]. Whereas β-*actin* was shown to have stable expression in human skeletal muscle, this stability could not be duplicated in adipose or colonic tissue, emphasizing the necessity to validate endogenous controls in different tissues [14–16].

Here it is shown that, in primary mesangial cells exposed to a high glucose environment, while β-*actin* and *YWHAZ* were the best internal control genes, all of the genes studied appeared relatively stable as represented by their low M values, the marker of gene stability. In contrast, results in microdissected glomeruli showed variable expression stability of internal control genes. Our analysis found *YWHAZ* and β-*actin* had the most stable expression in diabetic glomeruli. To illustrate the significance of housekeeping gene expression variability, analysis of the

relative expression ratios of the two most and the least stable endogenous control genes was carried out prior to logarithmic transformation. As an example, in cultured primary mesangial cells (Table 5), the use of a robust housekeeper like *YWHAZ* or β-*actin* would result in a 2% error in the differential expression of a given gene by the high glucose environment. Use of a less stable gene like *GAPDH* would result in 19% variation whereas use of $\beta_2 M$ would yield a 33% error. Stated another way, using $\beta_2 M$ to normalize gene expression changes caused by high glucose would overestimate the target gene expression by one third. Similarly, use of *CycA* as the denominator for gene quantification in diabetic isolated glomeruli would underestimate the expression of the gene of interest by fourfold (Table 4). These results were determined using real-time RT-PCR and therefore extrapolation to other gene expression techniques (e.g., quantitative competitive PCR, semiquantitative PCR, or Northern blot analysis) may not be appropriate.

In our analysis, the most abundant genes generally displayed the least variability in gene expression induced by the high glucose state. This is not entirely surprising since a small quantitative change in gene expression induced by high glucose may have an apparent smaller overall effect on a gene already expressed in large quantities at baseline as compared to one expressed in small quantities.

Schmid et al [12] recently published an analysis of *CycA*, *GAPDH*, and 18S rRNA as endogenous controls for microdissected human renal biopsy tissue. They found a high degree of correlation between *CycA* and 18S rRNA in the tubulointerstitial compartment. However, *GAPDH* correlated poorly with 18S rRNA in both glomerular and tubulointerstitial compartments. In this study, we have demonstrated *CycA* to be a poor reference gene in microdissected diabetic glomeruli. Similar to the results of Schmid et al in other tissues, we also found *GAPDH* to be a poor reference in microdissected glomeruli. In contrast, *GAPDH* has been demonstrated to be a stable endogenous control gene in human leukocytes, bone marrow, and adipocytes [13, 16].

The acidic ribosomal protein 36B4 has been used as an internal control gene in the breast cancer cell line MCF-7 [17, 18]. However, Gorzelniak et al [16] demonstrated variable expression of this protein in adipocytes and preadipocytes. Similarly, our results show 36B4 to be a suboptimal housekeeping gene in diabetic glomeruli. In addition, we also found $\beta_2 M$ to be a poor internal control gene for gene expression analysis in glomeruli and mesangial cells. Studies in human skeletal muscle, adipocytes, pooled human tissue, and several human cell lines have similarly demonstrated variable behavior [13, 14, 16].

Finally, while total RNA was a stable parameter for reference in tissue culture it varied in glomeruli. Total RNA primarily consists or rRNA but it has been suggested to be unsuitable as an endogenous control because of imbalances between the rRNA and mRNA fractions [13]. Hence, using the amount of total RNA template added to the RT-PCR reaction as the denominator for normalization of samples is not reliable in diabetic glomeruli.

Taken together these results show that the housekeeping genes of choice for analyzing relative gene expression in glomeruli from diabetic rats or primary mesangial cells chronically exposed to high glucose are β-*actin* and *YWHAZ*. This choice appears much more important for glomeruli. As others have suggested, although not always feasible, the expression data become much more robust if expressed relative to two internal controls instead of one [13]. Furthermore, if bioactive substances are tested, it would be prudent to evaluate their effects on the stability of housekeeping gene-expression as part of the study of diabetic animals.

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