Global Renal Gene Expression Profiling Analysis in B$_2$-Kinin Receptor Null Mice: Impact of Diabetes

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Abstract

Diabetic nephropathy (DN), the leading cause of end-stage renal failure, is clinically manifested by albuminuria and a progressive decline in glomerular filtration rate. The risk factors and mechanisms that contribute to the development and progression of DN are still incompletely defined. To address the involvement of bradykinin B$_2$-receptors (B$_2$R) in DN, we used a genome wide approach to study the effects of diabetes on differential renal gene expression profile in wild type and B$_2$R knockout (B$_2$R$^{-/-}$) mice. Diabetes was induced with streptozotocin and plasma glucose levels and albumin excretion rate (AER) were measured at predetermined times throughout the 23 week study period. Longitudinal analysis of AER indicated that diabetic B$_2$R$^{-/-}$D null mice had a significantly decreased AER levels compared to wild type B$_2$R$^{+/+}$D mice ($P = 0.0005$). Results from the global microarray study comparing gene expression profiles among four groups of mice respectively: (B$_2$R$^{-/-}$C, B$_2$R$^{+/+}$D, B$_2$R$^{+/-}$C and B$_2$R$^{-/-}$D) highlighted the role of several altered pathological pathways in response to disruption of B$_2$R and to the diabetic state that included: endothelial injury, oxidative stress, insulin and lipid metabolism and inflammatory process with a marked alteration in the pro-apoptotic genes. The findings of the present study provide a global genomics view of biomarkers that highlight the mechanisms and putative pathways involved in DN.

Introduction

Diabetic nephropathy (DN) is a major health epidemic and is the main cause of morbidity and mortality in diabetes. It is the single most common cause of end-stage renal failure [1,2]. A very characteristic and initial event of the development of DN is glomerulosclerosis, which is featured by increased thickness of the glomerular basement membrane, and widening of the mesangium with accumulation of extracellular matrix (ECM). Furthermore, the degree of mesangial expansion is strongly related to the clinical manifestations of diabetic nephropathy, such as albuminuria and decreased glomerular filtration rate [3,4]. Even though inherent susceptibility seems to influence the rate at which glomerular injury develops, hyperglycemia seems to be the primary driving force for cellular damage [5]. In this regard, intensive control of glycemia in type I diabetic patients was associated with a significant reduction in the development and progression of nephropathy [6].

Although, the underlying biochemical and cellular mechanisms that promote renal injury in diabetes are still undefined, accumulating evidence supports a relationship between the activity of the kallikrein-kinin system (KKS) and renal impairment. It has been shown that type I diabetic patients with hyperfiltration as well as diabetic rats with increased glomerular filtration rate (GFR) and renal plasma flow (RPF) are associated with increased active kallikrein excretion rate [7,8]. In addition, treatment of hyperfiltering diabetic rats with aprotinin, a kallikrein inhibitor, or with a B$_2$-kinin receptor (B$_2$R) antagonist, increases the renal vascular resistance and reduces GFR and RPF [9]. Furthermore, previous findings from our lab have shown that increased plasma prekallikrein activity is associated with increased albumin excretion rate; these data have been demonstrated in DCCT/EDIC-cohort of type 1 diabetic patients [10].

While most of the physiological actions of the KKS are attributed to the generation of BK and activation of B$_2$R, the intracellular signaling pathways initiated upon activation of B$_2$R leading to expression of pro sclerotic factors that ultimately result in glomerular injury are just beginning to be defined. Activation of B$_2$R by BK results in marked induction of connective tissue growth factor (CTGF), collagen I and transforming growth factor-$eta$ type II receptor (TGF-ßRII) in mesangial cells. Inhibition of B$_2$R by Icatibant significantly reduced the increase in collagen I and CTGF mRNA levels in response to BK challenge [11]. Of interest, it has been shown that the glomerular expression of B$_2$R is increased in diabetes and a targeted deletion B$_2$R protects against the development of DN [12,13]. Furthermore, diabetic B$_2$R$^{-/-}$null mice display reduced albumin excretion rate (AER), as well as reduced glomerular and tubular injury compared to diabetic B$_2$R$^{+/+}$ mice [13].
In this study, we employed a global microarray analysis coupled with systems biology study to investigate the differential gene expression in wild type control (B2R/+/+) and diabetic (B2R-/-/-) mice as well as in B2R knockout-control (B2R-/-/+) mice and in B2R knockout-diabetic (B2R-/-/-) mice in order to identify candidate genes that may be involved in the development of diabetic nephropathy. The objective of our study was to determine 1) whether deletion of B2 receptors will result in alteration in specific gene expression profiles whose specific functions can shed light on the role(s) of B2 receptors, and 2) whether diabetes will result in differences in the patterns of gene expression and pathways between B2R+/+ and B2R-/-/- mice that can be linked to the pathological manifestation observed after the induction of DN.

Methods

Study Design

To address the contribution of B2R to the development of diabetic nephropathy, we studied B2R knockout mice (B2R-/-/-) and their wild type littermates (B2R+/+). Male B2R-/-/- mice (strain # B6 129S1-BdKrb2, Jackson Laboratories, Bar Harbor, ME) and B2R+/+ mice (strain # B6 129S2/SJ, Jackson Laboratories, Bar Harbor, ME) weighing 20–30 g were used in our studies. Mice were housed three per cage in a light and temperature controlled room and had free access to food and water. Diabetes was induced by daily intraperitoneal injection of streptozotocin (50mg/kg body weight) for 3–5 days. Diabetes was confirmed in STZ-treated mice with systems biology study to investigate the differential gene expression in wild type control (B2R+/+), diabetic (B2R+/+/D); group 3, B2R knockout-control (B2R-/-/-), and group 4, B2R knockout-diabetic (B2R-/-/-). Glucose levels and body weights were measured at predetermined intervals to characterize the diabetic state and to ensure adequate metabolic control. Each week mice were placed in metabolic cages (Nalgene) for 24 h to acclimate, and then 24h urine collections were obtained from all mice to measure albumin excretion rate. The mice were sacrificed 6 months after the induction of diabetes. The studies were done in line with the Guide for the Care and Use of laboratory Animals published by the National Institutes of Health (NIH Publication No 83–23, revised 1996). The study was approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

RNA Extraction

Kidneys from control and diabetic mice (B2R+/+/-C, B2R+/+/-D, B2R-/-/-/C and B2R-/-/-/-D mice, n = 3 per group) were removed under anesthesia and cortices were cut off to extract RNA. For RNA extraction and purification, a method combined Trizol (Cat. No.15596-018, Invitrogen Life Technologies) and RNeasy Midi Kit (Cat. No.75144, QIAGEN) for total RNA isolation from animal tissue was used. Briefly, the cortices were homogenized using an appropriate volume of Trizol (1ml of Trizol/100 mg tissue). Then chloroform (0.2 ml/ml Trizol used) was added to separate the aqueous phase from protein phase. Total RNA was dissolved in the aqueous phase. RNA purification is followed the protocol of RNeasy kit handbook. The RNA concentration was determined in a spectrophotometer (ultraspec III, Pharmacia) by absorbance at 260 nm. The ratio of A260 to A280 was calculated to check the purity of RNA, and the rRNA ratio of 28S/18S using 2100 Bioanalyzer (Agilent) was measured to check the quality of RNA.

Synthesis of Double-stranded cDNA from Total RNA

Total RNA (10 µg) from each sample was used to synthesize ds-cDNA. In primer hybridization, 10 µg of RNA, T7-(dT)24 primer (100 pmol/µl, HPLC purified) and DEPC-H2O were added to the tube and incubated at 70°C for 10 min. Next, 5× first strand cDNA buffer 4 µl, DTT (0.1 M) 2 µl dNTP (10 mM) were added to each tube, incubated at 42°C for 2 min, and followed by addition of SuperScript II RT (200 U/µl) 2 µl and incubated at 42°C for 1 hour to synthesize the first strand of cDNA. The final volume for the first strand cDNA synthesis was 20 µl. In order to synthesis the second strand, the following reagents were added to the first strand synthesis tube: DEPC-treated water 91 µl, 5× second strand cDNA reaction buffer 30 µl, 10 mM dNTP mix 5 µl, 10 U/µl E.coli DNA ligase, 10 U/µl E.coli DNA polymerase 1 µl and 2 U/µl E.coli RNase H. The final volume of the second strand reaction was 150 µl. The reaction tubes were incubated at 16°C for 2 hours in a cooling water bath. After the incubation, 2 µl of [10 U] T4 DNA polymerase was added to the reaction tube, incubated at 16°C for 5 min, followed by addition of 10 µl of 0.5 M EDTA to complete synthesis of the second cDNA strand.

Synthesis of Biotin-labeled cRNA

Before Synthesis of biotin-labeled cRNA, double-strand cDNA was cleaned according to the GeneChip Sample Cleanup Module. The following reagents were used in the final reaction volume (40 µl): 4 µl of 10×HY reaction buffer, 4 µl of 10×Biotin-labeled ribonucleotides, 4 µl of 10×DTT, 4 µl 10×RNase inhibitor mix, 2 µl 20×T7 RNA polymerase and distilled water. All of the reagents were mixed and incubated at 37°C for 5 hours, with gentle mixing of the tube every 30 min. The biotin-labeled cRNA was cleaned according to the GeneChip Sample Cleanup Module before quantification.

cRNA Fragmentation and Microarray Procedure

To reach a final concentration of 1 µg/µl, 20 µg cRNA and 8 µl of 5×fragmentation buffer were incubated at 94°C for 35 min. A total of 15 µl of each sample (1.0 µg/µl) was used for preparation of hybridization cocktail that was loaded onto the GeneChips [Mouse Expression Array 430 A, Affymetrix] and hybridized for 16 h at 45°C in the Affymetrix GeneChips oven 640. Following this, the chips were loaded into the Affymetrix GeneChip Fluidics Station 400 with double stain antibody amplification solution for washing and staining. Finally, the GeneChips were scanned using the Hewlett Packard GeneArray Scanner 2500.

Expression values were derived using RMA (for normalization and background subtraction) as executed by the software MAStress (University of California, Berkeley). Expression genes were determined according to the following criteria: any gene for which a sample had an average detection p-value (MASS >0.04 (standard threshold for MASS “presence” call); all other genes were excluded from further consideration. RAW expression values were converted from log-base 2 and imported into dChip. Dchip was used to perform comparisons for all desired group comparisons. Criteria for comparison were: Fold change of 1.8; 90% confidence bound of fold change was used; T-test with p-value <0.05; false discovery rate was calculated as the median number genes discovered in 50 iterations of permuted samples.

Real-Time PCR

Total RNA (2 µg) was converted to cDNA using MLV Reverse Transcriptase (Promega, Madison, WI) according to the manufacturer’s protocol at 37°C for 1 hr. To determine the validity of
Figure 1. Plasma glucose levels (A) and body weights (B) in diabetic (B2R\(^{+/−}\)D and B2R\(^{−/−}\)D) and control (B2R\(^{+/+}\)C and B2R\(^{−/−}\)C) mice. (A) Plasma glucose levels were significantly increased two weeks after STZ injection in both diabetic groups (B2R\(^{+/−}\)D and B2R\(^{−/−}\)D) compared to B2R\(^{+/+}\)C and B2R\(^{−/−}\)C (\(P<0.001\)) and remained significantly elevated for the duration of the study. (B) Initial body weights were not significantly different between diabetic and control mice. However, B2R\(^{−/−}\)D mice had significantly reduced body weight after 14 weeks and B2R\(^{+/−}\)D after 20 weeks compared with B2R\(^{+/+}\)C and B2R\(^{−/−}\)C mice and this reduction in body weight was maintained for the duration of the study (\(P<0.001\) vs. B2R\(^{+/+}\)C and B2R\(^{−/−}\)C).

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Figure 2. Albumin excretion rate (AER) in diabetic (B2R\(^{+/−}\)D and B2R\(^{−/−}\)D) and control (B2R\(^{+/+}\)C and B2R\(^{−/−}\)C) mice. AER was significantly higher in B2R\(^{+/−}\)D mice compared to B2R\(^{−/−}\)D (\(\dagger P<0.05\)) and to B2R\(^{+/+}\)C and or B2R\(^{−/−}\)C (*\(P<0.001\)), as early as two weeks after induction of diabetes and remained elevated for the duration of the study period.

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Figure 3. Hierarchical clustering of gene expression in the kidney among four groups of mice: $B_2R^{+/+}$ C, $B_2R^{+/+}$ D, $B_2R^{+/2}$ C and $B_2R^{+/2}$ D. Each column represents one sample, and the color bars represent the median value of three array experiments for an individual mouse for that gene.

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primers and appropriate Tm for Real Time PCR, the primers were first amplified in a PCR reaction to ensure that only one band is amplified. The following primers were designed so that all of the PCR products are within 75–150 bp (Integrated DNA Technologies Inc).

- **β-actin**: 5’- actgcccgtctctc-3’; 5’- ccgctgcaatagta-3’; **Growth hormone receptor**: 5’- ttcctgggaagcctcgattcaca-3’, 5’- cagctgttcgcttgccttt-3’; **Insulin growth factor binding protein-1 (IGFBP1)** 5’- agatcgccgacctgaagaatgga-3’, 5’- tgttgggctgcagctaatctct-3’; **IGFBP4**: 5’- tcggaaatcgaagccatccaggaa-3’, 5’- tgaagctgttgttggatgttcg-3’; **Extracellular superoxide dismutase (EC-SOD)** 5’- tgcattgcatactgacggtac-3’, 5’- aagagaaccacaagcgttgtgtt-3’, 5’- atgggaatactgttcggaac-3’; Glutathione-S-transferase a-2 (GSTa-2) 5’- atgcaagagctaattgggca-3’, 5’- ggtggcaacagtctcaacat-3’. For each target gene, a standard curve was established. This was achieved by performing a series of 3-fold dilutions of the gene of interest. Negative control was made using the same volume of RNase-free water instead of sample. The master mix was prepared as follows: 2× SYBR Green Supermix (cat. No. 170–8880, BIO-RAD) 12.5 µl, forward and reverse primer 0.25 µl respectively and ddH2O 12 µl. For each well, 22 µl of master mix was loaded first, followed by 3 µl of sample, mixed well to get total reaction volume of 25 µl. For plate setup, SYBR-490 was chosen as fluorophore. The plate was covered with a sheet of optical sealing film. PCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s.

**Figure 4. Biological processes depicting genes that are altered in response to B2R disruption are shown in pie chart.** Data compares genes altered in B2R−/− C vs. B2R+/+ C (A) upregulated genes and (B) downregulated genes. doi:10.1371/journal.pone.0044714.g004
global gene profiling in diabetic B2R-null mice

Table 1. Upregulated and Downregulated Genes in B2R−/−C vs. B2R+/+C.

| Accession ID | Gene                              | Gene ID | Fold Change | P value |
|--------------|-----------------------------------|---------|-------------|---------|
| NM_018731    | Atp4a ATPase, H+/K+ exchanging, gastric, alpha polypeptide | 11944   | 2.77        | 0.001678 |
| AK007618     | Ak3 adenylate kinase 3            | 56248   | 1.85        | 0.017419 |
| NM_001164745 | Ptp4a2 protein tyrosine phosphatase 4a2 | 19244   | 2.97        | 0.028458 |
| NM_012032    | Serinc3 serine incorporator 3     | 26943   | 2.14        | 0.013514 |
| NM_013467    | Aldh1a1 aldehyde dehydrogenase family 1, subfamily A1 | 11668   | −4.13       | 0.003764 |
| BC027434     | Hbb-b2 hemoglobin, beta adult minor chain | 15130   | −2.42       | 0.002817 |
| NM_006218    | Hba-a1 hemoglobin alpha, adult chain 1 | 15122   | −2.54       | 0.00193  |
| NM_011921    | Aldh1a7 aldehyde dehydrogenase family 1, subfamily A7 | 26358   | −2.35       | 0.005015 |
| BC005569     | Rnase4 ribonuclease, RNase A family 4 | 58809   | −4.28       | 0.00286  |
| AF031467     | Bcat2 branched chain aminotransferase 2, mitochondrial | 12036   | −2.49       | 0.047991 |
| NM_011844    | Mgll monoglyceride lipase         | 23945   | −3.01       | 0.028657 |
| BC027279     | Bhbr biliverdin reductase B (flavin reductase (NADPH)) | 233016  | −1.94       | 0.016502 |
| NM_001003953 | Kdm2b lysine (K)-specific demethylase 2B | 30841   | −1.81       | 0.001347 |

10 sec, 58°C for 1 min for β-actin and for all the other genes 60°C for 1 min, then 95°C for 1 min, 55°C for 1 min and 100 cycles of 55°C for 10 sec. All of the reactions were done in duplicate. The correlation coefficient is between 0.98-1, PCR efficiency is between 75–130%. The mRNA levels were expressed relative to β-actin mRNA. Realtime PCR using iCycleTM iQ optical system software (version 3.0a) was used in our studies.

Urinary Albumin Excretion Rate

The urinary albumin excretion rate was measured with a murine microalbuminuria ELISA kit (Exocell Inc., PA) according to the manufacturer’s suggestions.

Systems Biology Analysis

The microarray differential expression of the wild type B2R vs. knockout (B2R−/−) in control and diabetic phenotypes was further analyzed using a systems biology approach to assess the altered pathway(s) relevant to differential B2R knockout (B2R−/−) phenotype mice and its contribution to the development of Diabetes. PathwayStudio software (v 9.0; Ariadne Genomics, Rockville, MD, USA) was applied for the systems biology analysis. This software helps to interpret biological meaning from differential gene expression, build and analyze pathways, and identify altered cellular processes and molecular functions involved. PathwayStudio comes with a built-in resource named ResNet, which is a database of molecular interactions based on natural language processing of scientific abstracts in PubMed.

For gene ontology analysis including differential molecular function and biological processes involved, PANTHER software (Protein ANalysis THrough Evolutionary Relationships; http://www.pantherdb.org/genes/batchIdSearch.jsp) was utilized to classify proteins into distinct categories of molecular functions and biological processes. Panther software uses published scientific experimental evidence and evolutionary relationships abstracted by curators with the goal of predicting function even in the absence of direct experimental evidence. Proteins are classified into families and subfamilies of shared function, which are then categorized using a highly controlled vocabulary (ontology terms) by biological process, molecular function and molecular pathway.

Statistical Methods

Power Analysis

Sample size calculation for our study was determined by using the formula by Hedeker D et al, for longitudinal data [14]. In this study we assumed 80% power, significance of 5%, repeated measure correlation of 0.5, 9 measurement time points, within subject variance of 4.2, and medium effect size of 0.3. This resulted in 2.3 mice per group, and accounting for possible attrition effect we inflated our sample size by 20% so the sample size in each group will be 2.76 mice.

Statistical Analysis

Results are expressed as mean ± standard error, unless stated otherwise. All data were analyzed using SAS (SAS Institute Inc., Version 8, Cary, NC). t-tests were used to analyze continuous outcomes versus each covariate separately. To compare means values across three or more groups, ANOVA was used. Generalized linear models and generalized estimating equations were used to compare albumin excretion rates, plasma glucose levels and body weights within mice and across groups over time. A longitudinal data analysis was conducted to assess the effect of group on the AER levels over time. A mixed model was fit and spatial data covariance structure was used to accommodate for the unequally- spaced measurement time points. In this context, a continuous-time model was employed using variance-covariance matrix with type = sp (pow) in SAS PROC MIXED. Bonferroni correction was used to adjust for inflated type I error when making multiple comparisons. Statistical significance was determined using a two-sided test and significance was assumed for P-values ≤0.05.

Results

Characteristics of the Diabetic State

Plasma glucose levels were markedly elevated 2 weeks after STZ injection in both B2R−/+D and B2R−/−D groups of mice compared to their non-diabetic controls, and remained elevated throughout the study period (Figure 1A). On average plasma glucose levels increased by 205 mg/dl in B2R−/−D null mice and by 251 mg/dl in B2R−/+D null mice compared to B2R−/+C mice, P<0.001. No significant difference in plasma glucose levels was
observed between B2R$^{+/+}$C mice and B2R$^{+/−}$C mice, $P = 0.276$. No significant time effect on plasma glucose level was observed, $P = 0.2647$. Also no significant effect of group by time interaction on plasma glucose levels was detected, $P = 0.28$. Hence, the observed difference in plasma glucose levels across groups was primarily due to group effect.

Initial body weights were not significantly different between diabetic and non-diabetic mice. However, B2R$^{+/−}$D mice had significantly reduced bodyweight after 14 weeks and B2R$^{+/*}$D after 20 weeks compared with B2R$^{+/+}$C and B2R$^{+/−}$C mice and this reduction in body weight was maintained for the duration of the study (Figure 1B). Body weight analyses revealed that there was no significant group effect on bodyweights over time, but there was
a significant effect of time on bodyweights, \( P = 0.0011 \). In addition, there was interaction between time and group effect on changes in body weights \( P = 0.0011 \). Thus, the decrease in bodyweights in \( B_2R^{\text{+/+}} / D \) null mice and \( B_2R^{\text{+/+}} / D \) mice compared to \( B_2R^{\text{+/+}} / C \) mice are a result of time effect.

**Albumin Excretion Rate**

The albumin excretion rate results are presented in Figure 2. Groups were defined as \( B_2R^{\text{+/+}} / C \), \( B_2R^{\text{+/+}} / D \), \( B_2R^{\text{+/}} / C \) and \( B_2R^{\text{+/}} / D \). AER was modeled with a time and group main effect and a time by group effect. Since AER in each mouse was measured up to 10 times over 23 weeks, a longitudinal data analysis was conducted to assess the effect of group on the AER levels over time. A mixed model fit was spatial and data covariance structure was used to accommodate for the unequally-spaced measurement time points. Our results showed that there was a significant overall group effect with \( P < 0.0001 \). In particular, when the wild type control group \( B_2R^{\text{+/+}} / C \) was considered as the reference group, we observed that \( B_2R^{\text{+/+}} / C \) had a significant increase in the AER by 13.5 mg/24 h, \( P = 0.001 \). Overall, a significant increase by about 28.5 mg/24 h in AER was also observed for \( B_2R^{\text{+/+}} / D \) mice compared to \( B_2R^{\text{+/+}} / C \) mice \( P < 0.0001 \). No significant differences in AER was observed between \( B_2R^{\text{+/+}} / C \) and \( B_2R^{\text{+/}} / D \). Each column represents one sample, and the color bars represent the median value of three array experiments for an individual mouse for that gene (Figure 3).

**Gene Regulation in Response to Disruption of \( B_2R \)**

Upon deletion of \( B_2R \), there were a total of 14 altered genes (4 upregulated and 9 downregulated shown in Table 1); these include genes that code for ATPase activity, hemoglobin and enzymes involved in protein metabolism. Among the altered genes, Monoglyceride lipase (MGLL; EC 3.1.1.23) and lysine (K)-specific demethylase 2B (KDM2B) were found to be downregulated due to \( B_2R \) deletion. KDM2B gene encodes a member of the F-box protein family lysine (K)-specific demethylase 2B which function in phosphorylation-dependent ubiquitination while MGLL gene functions together with hormone-sensitive lipase to

| Accession ID | Gene ID | Fold Change | P value |
|--------------|---------|-------------|---------|
| NM_019659    | Kcnj1   | 2.1         | 0.005281|
| NM_011819    | Gdf15   | 1.82        | 0.031049|
| AK007630     | Cdkn1a  | 4.61        | 0.000166|
| AK008108     | Slu2    | 1.91        | 0.027765|
| AK013376     | Aplp2   | 1.88        | 0.022803|
| AK007618     | Ak3     | 1.99        | 0.010866|
| NM_030558    | Car15   | 1.99        | 0.013746|
| AAC42082     | Ccnq1   | 1.91        | 0.007673|
| NM_012032    | Serinc3 | 2.02        | 0.018166|
| BC010197     | Cpe     | -2.91       | 0.001076|
| NM_008321    | Id3     | -1.97       | 0.007472|
| NM_013475    | Apor    | -2.55       | 0.005274|
| BC027434     | Hbb-b2  | -2.17       | 0.003365|
| NM_008218    | Hba-a1  | -2.1        | 0.001414|
| D89669       | Cyp2a1a | -2.34       | 0.038907|
| BC020534     | Ccak     | -2.28       | 0.032112|
| NM_007812    | Cyp2a5  | -1.85       | 0.038301|
| BC005569     | Rnase4  | -3.31       | 0.002108|
| NM_030888    | C1qtnf3 | -2.24       | 0.025602|
| BC013343     | Hpd     | -0.98      | 0.027661|
| S64539       | Odc1    | 1.86        | 0.045764|
| AK011116     | Hba-a1  | -1.98      | 0.004592|
| AAH23851     | Hmgscl  | -2.71       | 0.013067|
| NM_001004148 | S1c13a5 | -1.84     | 0.00636|
| EDL41048     | Id4     | -1.95        | 0.031173|

Table 2. Upregulated and Downregulated Genes in \( B_2R^{\text{+/+}} / D \) vs. \( B_2R^{\text{+/+}} / C \).
hydrolyze intracellular triglyceride stores in adipocytes and other cells to fatty acids and glycerol. The biological processes depicting genes that are altered in response to B2R disruption are shown in Figures 4 A and B.

Gene Regulation in Response to Diabetes

Upon Diabetes induction, a total of 9 genes were found to be upregulated and 16 genes downregulated compared to B2R+/+ C wild type mice. An enriched pathways analysis identified genes associated with potassium transport, cell cycles and lipid metabolism as shown in Table 2. The biological processes depicting genes that are altered in response to diabetes are shown in Figures 5A and B.

Of great interest, in B2R−/− null mice, a total of 181 genes were regulated by diabetes including 91 upregulated genes and 90 downregulated genes, respectively (Table 3). A thorough systems biology analysis of specific enriched pathways, several genes were found to be associated with: endothelial cellular injury, insulin & lipid metabolism, oxidative stress, cardiac and kidney toxicity as illustrated in the biological processes (Figures 6A & B).
| Accession ID | Gene ID | Gene | Fold Change | P value |
|-------------|---------|------|-------------|---------|
| BC009155    | Mgst1   | microsomal glutathione S-transferase 1 | 56615  | 1.97    | 0.035109 |
| NM_019703   | Pfkp    | phosphofructokinase, platelet | 56421  | 1.99    | 0.045538 |
| EDL25631    | Mpi2l   | myelin protein zero-like 2 | 14012  | 1.86    | 0.035095 |
| NM_019423   | Elo2l   | elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 2 | 54326  | 2.03    | 0.003224 |
| NM_013467   | Aldha1   | aldehyde dehydrogenase family 1, subfamily A1 | 11668  | 8.21    | 0.015004 |
| NM_009994   | Cyp1b1   | cytochrome P450, family 1, subfamily b, polypeptide 1 | 13078  | 1.84    | 0.022511 |
| NM_009255   | Serpin2e | serine (or cysteine) peptidase inhibitor, clade E, member 2 | 20720  | 2.25    | 0.002624 |
| NM_021897   | Trp53inp1 | transformation related protein 53 inducible nuclear protein 1 | 60599  | 1.84    | 0.024129 |
| BC027434    | Hbb-b2   | hemoglobin, beta adult minor chain | 15130  | 2.69    | 0.013965 |
| NM_025284   | Tmsb10   | thymosin, beta 10 | 19240  | 1.9     | 0.024637 |
| NM_007631   | Ccnd1    | cyclin D1 | 12443  | 2.95    | 0.000665 |
| NM_007752   | Cp       | ceruloplasmin | 12870  | 2.49    | 0.007697 |
| NM_008218   | Hba-a1   | hemoglobin alpha, adult chain 1 | 15122  | 3.2     | 0.019897 |
| AF047838    | Clca1    | chloride channel calcium activated 1 | 12722  | 3.37    | 0.011734 |
| BC021776    | Apoc3    | apolipoprotein C-III | 11814  | 2.01    | 0.000641 |
| NM_009244   | Serpina1b | serine (or cysteine) peptidase inhibitor, clade A, member 1B | 20701  | 2.24    | 0.006778 |
| NM_008332   | Ift2    | interferon-induced protein with tetratricopeptide repeats 2 | 15958  | 2.05    | 0.022893 |
| NM_00119860 | H2-Q7   | histocompatibility 2, Q region locus 7 | 15018  | 1.84    | 0.013281 |
| NM_011921   | Aldha1a7 | aldehyde dehydrogenase family 1, subfamily A7 | 26358  | 3.96    | 0.030189 |
| NM_013492   | Clu      | clusterin | 12759  | 1.92    | 0.000802 |
| NM_009705   | Arg2     | arginine type II | 11847  | 1.98    | 0.00093 |
| D89869      | Cyp24a1  | cytochrome P450, family 24, subfamily a, polypeptide 1 | 13081  | 3.09    | 0.079614 |
| NM_008341   | Igfbp1   | insulin-like growth factor binding protein 1 | 16006  | 2.81    | 0.042115 |
| NM_031161   | Cck      | cholecystokinin | 12424  | 2.17    | 0.002063 |
| NM_010281   | Gglh     | gamma-glutamyl hydrolase | 14590  | 2       | 0.017566 |
| NM_019738   | Nupr1    | nuclear protein 1 | 56312  | 1.85    | 0.001812 |
| NM_008935   | Prom1    | prominin 1 | 19126  | 2.21    | 0.014173 |
| NM_031185   | Akap12   | A kinase (PRKA) anchor protein (gravin) 12 | 83397  | 1.86    | 0.00053 |
| NM_008831   | Ccng1    | cyclin G1 | 12450  | 2.15    | 0.007653 |
| NM_008182   | Gsta2    | glutathione S-transferase, alpha 2 (Yc2) | 14858  | 2.65    | 0.000003 |
| NM_011313   | S100a6   | S100 calcium binding protein A6 (calcyclin) | 20200  | 3.53    | 0.000785 |
| NM_011169   | Prlr     | prolactin receptor | 19116  | 3.04    | 0.020601 |
| NM_010145   | Ephx1    | epoxide hydrolase 1, microsomal | 13849  | 5.62    | 0.000013 |
| NM_013602   | Mt1      | metallothionein 1 | 17748  | 2.96    | 0.003938 |
| NM_009256   | Serpinb9 | serine (or cysteine) peptidase inhibitor, clade B, member 9 | 20723  | 1.88    | 0.031251 |
| NM_001166409 | Rbm3   | RNA binding motif protein 3 | 19652  | 2.57    | 0.034705 |
| NM_009162   | Sg5      | secretogranin V | 20394  | 2.08    | 0.031656 |
| NM_013590   | Lyc1    | lysozyme 1 | 17110  | 2.11    | 0.004781 |
| BC010291    | Ifitm3   | interferon induced transmembrane protein 3 | 66141  | 2.1     | 0.001773 |
| AK007630    | Cdk11a   | cyclin-dependent kinase inhibitor 1A (P21) | 12575  | 4.38    | 0.018945 |
| BC010747    | Cyp4a10  | cytochrome P450, family 4, subfamily a, polypeptide 10 | 13171  | 2.97    | 0.008145 |
| BC019601    | Wsb1    | WD repeat and SOCS box-containing 1 | 78889  | 2.08    | 0.017203 |
| AK011116    | Hba-a1   | hemoglobin alpha, adult chain 1 | 15122  | 2.87    | 0.004126 |
| NM_008630   | Mt2      | metallothionein 2 | 17750  | 2.3     | 0.000286 |
| AK002562    | Reep6    | receptor accessory protein 6 | 70335  | 1.83    | 0.006197 |
| AK019319    | Apoe     | apolipoprotein E | 11816  | 2.03    | 0.013394 |
| NM_009964   | Cryab    | crystallin, alpha B | 12955  | 1.98    | 0.008461 |
| NM_009700   | Aqp4    | aquaporin 4 | 11829  | 2.24    | 0.04758 |
| NM_011403   | Slc4a1  | solute carrier family 4 (anion exchanger), member 1 | 20533  | 1.88    | 0.005501 |
| Accession ID | Gene ID | Fold Change | P value |
|-------------|---------|-------------|---------|
| NM_028071   | Cotl1   | 2.52        | 0.045007|
| NM_033521   | Laptm4b  | 2.24        | 0.022123|
| NM_017372   | Lys2    | 3.11        | 0.0077  |
| NM_019989   | Sh3bg1   | 2.1         | 0.02642 |
| NM_01206367 | Gsn     | 1.82        | 0.004725|
| NM_01039392 | Tmsb10  | 2.41        | 0.009177|
| NM_010169   | F2r     | 2.66        | 0.008048|
| NM_007569   | Btg1    | 1.9         | 0.027652|
| NM_013492   | Clu     | 3.04        | 0.023634|
| NM_010664   | Krt18   | 2.33        | 0.032815|
| NM_009242   | Sparc   | 2.77        | 0.01031 |
| NM_021281   | Cts5    | 1.94        | 0.003757|
| NM_007631   | Ccnd1   | 2.36        | 0.01662 |
| NM_011579   | Tgp1    | 2.11        | 0.048236|
| NM_010501   | Ifit3   | 2.4         | 0.043235|
| NM_019975   | Hacl1   | 2.68        | 0.000059|
| NM_012006   | Aco1    | 2.11        | 0.001113|
| NM_009735   | B2m     | 2.8         | 0.025118|
| NM_009517   | Zmat3   | 1.93        | 0.005952|
| NM_054102   | Ivns1abp| 1.98        | 0.02154 |
| NM_009254   | Serpinb6a| 2.45       | 0.016523|
| NM_011844   | Mgll    | 2.2         | 0.021782|
| AF177041    | Akr1c12 | 2.06        | 0.010183|
| NM_016668   | Bhtm    | 3.04        | 0.006564|
| NM_010379   | H2-Ab1  | 1.86        | 0.01325 |
| NM_010169   | Coagulation factor II (thrombin) receptor | 2.02 | 0.003274 |
| AF263458    | Plac8   | 1.84        | 0.001426|
| BC008184    | Aldoc   | 2           | 0.008866|
| BC027340    | Lypal1  | 1.8         | 0.001368|
| BC012874    | Serpina1b| 2.34       | 0.024747|
| NM_009735   | B2m     | 1.88        | 0.015559|
| AK011116    | Hba-a1  | 2.52        | 0.003324|
| NM_013492   | Clu     | 2.45        | 0.011136|
| NM_001042611|        |             |         |
| NM_010362   | Gsto1   | 2.53        | 0.001403|
| NM_009369   | Tgfb1   | 1.82        | 0.021522|
| NM_011701   | Vim     | 1.93        | 0.022829|
| NM_008538   | Marcks  | 1.83        | 0.018183|
| NM_007620   | Cbr1    | 3.36        | 0.016972|
| AF108501    | Clca2   | 4.02        | 0.000586|
| NM_013470   | Anxa3   | 1.81        | 0.022226|
| NM_009156   | Sepv1   | 2.02        | 0.001731|
| NM_008509   | Lpl     | –3.3        | 0.000892|
| BC010197    | Cp      | –2.11       | 0.000013|
| AF145253    | Sec61a1 | –2.13       | 0.005158|
| NM_007823   | Cyp4b1  | –1.92       | 0.00051 |
| BC013477    | Adh1    | –1.92       | 0.007629|
| NM_013560   | Hspb1   | –2.56       | 0.004221|
| NM_013475   | Apoh    | –2.66       | 0.003569|
| Accession ID | Gene ID | Gene | Gene | Fold Change | P value |
|-------------|---------|------|------|-------------|---------|
| BC021352    | Plod2   | procollagen lysine, 2-oxoglutarate 5-dioxygenase 2 | 26432 | −1.98 | 0.012487 |
| NM_029550   | Keg1    | kidney expressed gene 1 | 64697 | −1.85 | 0.000566 |
| NM_008766   | Slc22a6 | solute carrier family 22 (organic anion transporter), member 6 | 18399 | −1.8 | 0.001602 |
| NM_007376   | Pzp     | pregnancy zone protein | 11287 | −2.24 | 0.022851 |
| NM_008878   | Keg1    | kidney expressed gene 1 | 18816 | −2.54 | 0.001222 |
| NM_010007   | Cyp2j5  | cytochrome P450, family 2, subfamily j, polypeptide 5 | 13109 | −2.12 | 0.042784 |
| NM_011435   | Slc22a6 | solute carrier family 22 (organic anion transporter), member 6 | 20657 | −2 | 0.023677 |
| NM_021788   | Sap30   | sin3 associated polypeptide | 60406 | −1.82 | 0.005361 |
| NM_013478   | Azgp1   | alpha-2-glycoprotein 1, zinc | 12007 | −2.24 | 0.022851 |
| AW105741    | Slc18a2 | solute carrier family 18 (monocarboxylic acid transporters), member 2 | 20502 | −2.32 | 0.007439 |
| BC012637    | Aadat   | aminoadipate aminotransferase | 23923 | −1.97 | 0.007973 |
| NM_008129   | Gclm    | glutamate-cysteine ligase, modifier subunit | 14630 | −2.06 | 0.015486 |
| BC016885    | Ugt8a   | UDP galactosyltransferase 8A | 22239 | −2.77 | 0.004372 |
| L27424      | Timp3   | tissue inhibitor of metalloproteinase 3 | 21859 | −2.34 | 0.006506 |
| NM_027884   | Tns1    | tensin 1 | 21961 | −2.12 | 0.04987 |
| NM_013797   | Slco1a1 | solute carrier organic anion transporter family, member 1a1 | 28248 | −11.51 | 0.014794 |
| NM_008079   | Galc    | galactosylceramidase | 14420 | −2.1 | 0.017597 |
| NM_030721   | Acox3   | acyl-Coenzyme A oxidase 3, pristanoyl | 80911 | −2.68 | 0.006701 |
| NM_007825   | Cyp7b1  | cytochrome P450, family 7, subfamily b, polypeptide 1 | 13123 | −5.04 | 0.009947 |
| NM_015804   | Atp11a  | ATPase, class VI, type 11A | 50770 | −2.76 | 0.012782 |
| BC020534    | Cckar   | cholecystokinin A receptor | 12425 | −2.11 | 0.006061 |
| AB008174    | Hnf1b   | HNF1 homeobox B | 21410 | −1.94 | 0.032091 |
| NM_008016   | Mpp6    | membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6) | 56524 | −2.17 | 0.00856 |
| NM_053097   | Cml3    | camello-like 3 | 93674 | −2.48 | 0.001103 |
| NM_010232   | Fno5    | flavin containing monoxygenase 5 | 14263 | −2.66 | 0.000267 |
| NM_008173   | Nr3c1   | nuclear receptor subfamily 3, group C, member 1 | 14815 | −1.86 | 0.006131 |
| NM_008261   | Hnf4a   | hepatic nuclear factor 4, alpha | 15378 | −2.08 | 0.004817 |
| NM_010517   | Igfbp4  | insulin-like growth factor binding protein 4 | 16010 | −2.37 | 0.00307 |
| NM_010496   | Id2     | inhibitor of DNA binding 2 | 15902 | −1.88 | 0.004033 |
| NM_009203   | Slc22a12| solute carrier family 22 (organic anion/cation transporter), member 12 | 20521 | −2.13 | 0.044714 |
| AK004192    | Cdx3CDx6| antigen | 12491 | −2.07 | 0.009977 |
| NM_00116040 | Galnt1  | UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1 | 14423 | −2.22 | 0.001029 |
| NM_009467   | Ugt2b5  | UDP glucuronosyltransferase 2 family, polypeptide B5 | 22238 | −1.99 | 0.005327 |
| NM_010279   | Gfra1   | glial cell line derived neurotrophic factor family factor receptor alpha 1 | 14585 | −1.86 | 0.029282 |
| BC003451    | Mat2a   | methionine adenosyltransferase II, alpha | 232087 | −2.03 | 0.005257 |
| BC019374    | GlcL    | glutamate-cysteine ligase, catalytic subunit | 14629 | −2.18 | 0.00284 |
| BC025936    | Cyp4a12a| cytochrome P450, family 4, subfamily a, polypeptide 12a | 277753 | −3.57 | 0.005458 |
| U68542      | Cx31    | cut-like homeobox 1 | 13047 | −2.1 | 0.024224 |
| BC013521    | Anxa13  | annexin A13 | 69787 | −1.94 | 0.041203 |
| AY038079    | Fbx11   | F-box and WD-40 domain protein 11 | 103583 | −2.24 | 0.009461 |
| BC003476    | Cdc7CDc7| antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated) | 16149 | −1.9 | 0.012558 |
| AB022340    | Acsm3   | acyl-CoA synthetase medium-chain family member 3 | 20216 | −3.76 | 0.001304 |
| AF213670    | Mtx    | MAX-like protein X | 21428 | −2.08 | 0.007919 |
| NM_01164099 | Add3    | adducin 3 (gamma) | 27360 | −1.84 | 0.002187 |
| BC027063    | Bdh1    | 3-hydroxybutyrate dehydrogenase, type 1 | 71911 | −2.16 | 0.001797 |
| BC023060    | Efemp1  | epidermal growth factor-containing fibulin-like extracellular matrix protein 1 | 216616 | −2.16 | 0.001651 |
| S64539      | Odz1    | ornithine decarboxylase, structural 1 | 18263 | −2.3 | 0.033305 |
| NM_009202   | Slc22a1 | solute carrier family 22 (organic cation transporter), member 1 | 20517 | −1.95 | 0.002763 |
Table 3. Cont.

| Accession ID | Gene                                      | Gene ID | Fold Change | P value  |
|--------------|-------------------------------------------|---------|-------------|----------|
| AK003232     | Cbr3 carboxyl reductase 3                 | 109857  | −3.48       | 0.045187 |
| AK009736     | Gpr137b-ps G protein-coupled receptor 137b, pseudogene | 664662  | −2.01       | 0.00129  |
| AK006387     | Me1 malic enzyme 1, NADP+-dependent, cytosolic | 17436   | −2.8        | 0.011484 |
| AK005023     | Sel1 sel-1 suppressor of lin-12-like (C. elegans) | 20338   | −2.25       | 0.003066 |
| NM_001159375 | Elf4a1 eukaryotic translation initiation factor 4A1 | 13681   | −2.08       | 0.023416 |
| AK002362     | Myo5a myosin VA                           | 17918   | −2.24       | 0.00322  |
| AK003786     | Nfs1 nitrogen fixation gene 1 (S. cerevisiae) | 18041   | −2.05       | 0.009065 |
| AK007618     | Ak3 adenylate kinase 3                    | 56248   | −2.05       | 0.039247 |
| NM_008303    | Hspp1 heat shock protein 1 (chaperonin)   | 15528   | −2.75       | 0.037992 |
| NM_011631    | Hsp90b1 heat shock protein 90, beta (Grp94), member 1 | 22027   | −2.07       | 0.012751 |
| NM_010516    | Cyr61 cysteine rich protein 61            | 16007   | −2.08       | 0.007693 |
| NM_013614    | Odc1 ornithine decarboxylase, structural 1| 18263   | −1.91       | 0.002118 |
| NM_001111289 | Caprin1 cell cycle associated protein 1    | 53872   | −1.93       | 0.041035 |
| NM_023908    | Slco3a1 solute carrier organic anion transporter family, member 3a1 | 108116  | −2.27       | 0.010399 |
| NM_019699    | Fads2 fatty acid desaturase 2             | 56473   | −2.1        | 0.010322 |
| AB046929     | Chst7 carbohydrate (N-acetylglycosaminio) sulfotransferase 7 | 60322   | −2.15       | 0.001535 |
| NM_033564    | Mpv17i Mpv17 transgene, kidney disease mutant-like | 93734   | −2.04       | 0.004172 |
| AK003671     | Car3 carboxic anhydrase 3                 | 12350   | −2.4        | 0.020089 |
| NM_032000    | Trpv1 trichorhinophalangeal syndrome 1 (human) | 83925   | −1.83       | 0.008962 |
| AB031813     | Slco1a1 solute carrier organic anion transporter family, member 1a1 | 28248   | −5.5        | 0.024812 |
| NM_019657    | Hsd17b12 hydroxysteroid (17-beta) dehydrogenase 12 | 56348   | −1.91       | 0.02581  |
| NM_010302    | Gna12 guanine nucleotide binding protein, alpha 12 | 14673   | −1.82       | 0.010264 |
| NM_010232    | Fmo5 flavin containing monoxygenase 5      | 14263   | −3.12       | 0.000843 |
| AF319542     | Kcnk5 potassium channel, subfamily K, member 5 | 16529   | −2.11       | 0.038642 |
| NM_001159555 | Cd36 CD36 antigen                        | 12491   | −3.35       | 0.002187 |
| NM_025903    | Ifdr2 interferon-related developmental regulator 2 | 15983   | −2.47       | 0.005388 |
| AF133669     | Akr6p1 ADP-ribosylation factor-like 6 interacting protein 1 | 54208   | −1.94       | 0.00508  |
| BC022130     | Sct2a1 solute carrier family 26 (sulfate transporter), member 1 | 231583  | −1.85       | 0.008221 |
| BC026422     | Tgm1 transglutaminase 1, K polypeptide     | 21816   | −2.49       | 0.000338 |
| BC026598     | Sct2a7 solute carrier family 22 (organic anion transporter), member 7 | 108114  | −7.3        | 0.007471 |
| M33324       | Ghr growth hormone receptor                | 14600   | −2.58       | 0.005396 |
| M55333       | Ace angiotensin I converting enzyme (peptidyl-dipeptidase A) 1 | 11421   | −2.45       | 0.006298 |
| NM_013876    | Rnf11 ring finger protein 11              | 29864   | −2.38       | 0.008352 |
| NM_01122683  | Bdh1 3-hydroxybutyrate dehydrogenase, type 1 | 71911   | −1.88       | 0.001252 |
| NM_009199    | Slc1a1 solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1 | 20510   | −2.07       | 0.004881 |

Gene Expression in Diabetes with or without Disruption of B<sub>2</sub>R

In B<sub>2</sub>R<sup>−/−</sup> vs. B<sub>2</sub>R<sup>+/+</sup> mice, a total of 43 genes were upregulated and 66 genes were downregulated (Table 4). Among these altered genes: IGFBP, GST, EC-SOD and GHR genes. In a detailed assessment of these genes, expression of IGFBP-1 (3.65 fold) and GST (Yc2, 2.05 fold; omegal1, 2.43 fold) were elevated in the B<sub>2</sub>R<sup>−/−</sup> mice compared to the B<sub>2</sub>R<sup>+/+</sup> mice. On the other hand, gene expressions of Insulin-like growth factor-binding protein-1 (IGFBP-4) (−2.18 fold), EC-SOD (−1.95 fold), FMO2 (−1.94 Fold) and GHR (−2.7 fold) were suppressed in the B<sub>2</sub>R<sup>−/−</sup> mice compared to the B<sub>2</sub>R<sup>+/+</sup> mice, P<0.05. The biological processes depicting genes that are altered in response to diabetes +− B<sub>2</sub>R are shown in Figure 7A & B.

Validation of Specific Gene Expressions by Quantitative Real-time PCR Superoxide Dismutase 3, Extracellular (EC-SOD)

EC-SOD gene encodes a member of the superoxide dismutase (SOD) protein family which are antioxidant enzymes that catalyze the dismutation of two superoxide radicals into hydrogen peroxide and oxygen protecting from oxidative stress. EC-SOD expression tended to be suppressed by diabetes in the wild type mice. Interestingly, in the B<sub>2</sub>R<sup>−/−</sup> mice, EC-SOD expression was increased up to 37% compared to that in the B<sub>2</sub>R<sup>+/+</sup> mice (P<0.05 vs. B<sub>2</sub>R<sup>+/+</sup>, Figure 8A).
Glutathione S-transferase, Alpha 2(Yc2) (GST-Yc2)

GST-Yc2 catalyze the conjugation of reduced glutathiones and a variety of electrophiles, including many known carcinogens and mutagens. Our data indicated that the expression of GST was significantly higher in B2R−/−D mice compared to B2R+/+D mice (*P<0.05 vs. B2R+/+D, Figure 8B).

Flavin Containing Monooxygenase 2 (FMO2)

FMO2 family is NADPH-dependent enzymes that catalyze the oxidation of many drugs and xenobiotics. In the B2R+/+D mice, FMO2 expression was decreased up to 34% compared to that in the controls. However, the expression FMO2 was significantly higher in B2R−/−D mice compared with B2R+/+D mice (*P<0.05 vs. B2R+/+D, Figure 8C).

Figure 7. Biological processes depicting genes that are altered in response to diabetes in wild type control mice and in B2R−/− null mice are shown in pie chart. Data compares genes altered in B2R+/+D vs. B2R−/−D (A) upregulated genes and (B) downregulated genes.

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Table 4. Upregulated and Downregulated Genes in B2R<sup>−/−</sup> D vs. B2R<sup>+/+</sup>D.

| Accession ID | Gene ID | Gene ID | Fold Change | P value  |
|--------------|---------|---------|-------------|----------|
| BC009155     | Mgst1   | microsomal glutathione S-transferase 1 | 56615 | 2.01 | 0.025734 |
| NM_019423    | Elovl2  | elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 2 | 54326 | 2.08 | 0.003068 |
| NM_021450    | Trpm7   | transient receptor potential cation channel, subfamily M, member 7 | 58800 | 1.83 | 0.002536 |
| BC027434     | Hbb-b2  | hemoglobin, beta adult minor chain | 15130 | 2.41 | 0.026002 |
| NM_008218    | Hba-a1  | hemoglobin alpha, adult chain | 15122 | 2.73 | 0.027316 |
| AF047838     | Clca1   | chloride channel calcium activated 1 | 12722 | 2.15 | 0.00938 |
| D89669       | Cyp24a1 | cytochrome P450, family 24, subfamily a, polypeptide | 13081 | 6.34 | 0.054554 |
| NM_008341    | Igfbp1  | insulin-like growth factor binding protein 1 | 16006 | 3.65 | 0.030456 |
| NM_027884    | Tns1    | tensin 1 | 21961 | 1.77 | 0.007886 |
| AAD38411     | March7  | membrane-associated ring finger (C3HC4) 7 | 57438 | 1.82 | 0.005006 |
| NM_008182    | Gsta2   | glutathione S-transferase, alpha 2 (Yc2) | 14858 | 2.05 | 0.020506 |
| NM_011313    | S100a6  | S100 calcium binding protein A6 (calcyclin) | 20200 | 2.51 | 0.003671 |
| NM_011169    | Prlr     | prolactin receptor | 19116 | 2.6 | 0.018234 |
| NM_010145    | Ephx1   | epoxide hydrolase 1, microsomal | 13849 | 2.32 | 0.015186 |
| NM_010424    | Hfe      | hemochromatosis | 15216 | 1.85 | 0.005272 |
| NM_001172121 | Rbm3   | RNA binding motif, single stranded interacting protein | 207181 | 2.35 | 0.034332 |
| NM_010279    | Gfra1   | glial cell line derived neurotrophic factor family receptor alpha 1 | 14585 | 1.81 | 0.020694 |
| BC013343     | Hpd     | 4-hydroxophenylpyruvic acid dioxygenase | 15445 | 1.97 | 0.007464 |
| NM_008096    | Gc       | group specific component | 14473 | 3.77 | 0.008264 |
| BC023060     | Efemp1  | epidermal growth factor-containing fibulin-like extracellular matrix protein 1 | 216616 | 2.13 | 0.001006 |
| AK009020     | Clic3   | chloride intracellular channel 3 | 69454 | 2.05 | 0.005867 |
| NM_145942    | Hmgcs1  | 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 | 208715 | 2.75 | 0.005719 |
| NM_019989    | Sh3bgrl | SH3-binding domain glutamic acid-rich protein like | 56726 | 2.04 | 0.037097 |
| NM_010169    | F2r     | coagulation factor II (thrombin) receptor | 14062 | 2.31 | 0.015754 |
| NM_007569    | Btg1    | B-cell translocation gene 1, anti-proliferative | 12226 | 2.03 | 0.025775 |
| NM_001004148 | Slc13a5 | solute carrier family 13 (sodium-dependent citrate transporter), member 5 | 237831 | 1.86 | 0.029255 |
| NM_145569    | Mat2a   | methionine adenosyltransferase II, alpha | 232087 | 1.8 | 0.034233 |
| NM_00110831  | Dnpep   | aspartyl aminopeptidase | 13437 | 1.9 | 0.001443 |
| NM_009837    | Cct4    | chaperonin containing Tcp1, subunit 4 (delta) | 12464 | 2.06 | 0.000479 |
| NM_009242    | Sparc   | secreted acidic cysteine rich glycoprotein | 20692 | 1.8 | 0.020269 |
| NM_008597    | Mgp     | matrix Gla protein | 17313 | 1.91 | 0.002312 |
| NM_019975    | Hac1    | 2-hydroxyacyl-CoA lyase 1 | 56794 | 2.17 | 0.003102 |
| NM_054102    | Ims1abp | influenza virus N51A binding protein | 117198 | 2.1 | 0.01779 |
| NM_013806    | Abcc2   | ATP-binding cassette, sub-family C (CFTR/MRP), member 2 | 12780 | 2.18 | 0.019342 |
| NM_031166    | Id4     | inhibitor of DNA binding 4 | 15904 | 2.36 | 0.013276 |
| BC012874     | Serpina1b | serine (or cysteine) peptidase inhibitor, clade A, member 1B | 20701 | 1.89 | 0.044292 |
| NM_029023    | Scpep1  | serine carboxypeptidase 1 | 74617 | 1.84 | 0.001437 |
| NM_009099    | Rad21   | RAD21 homolog (S. pombe) | 19357 | 2.09 | 0.001454 |
| NM_010362    | Gsto1   | glutathione S-transferase omega 1 | 14873 | 2.43 | 0.008124 |
| NM_016792    | Tnxl    | thio��息印-1 like-1 | 53382 | 1.84 | 0.016406 |
| NM_011701    | Vim     | vimentin | 22352 | 1.95 | 0.026022 |
| NM_007620    | Cbr1    | carbonyl reductase 1 | 12408 | 2.2 | 0.012532 |
| AF108501     | Clca2   | chloride channel calcium activated 2 | 80797 | 2.57 | 0.003658 |
| AF145253     | Sec61a  | Sec61 alpha | 53421 | −2.11 | 0.005453 |
| NM_013560    | Hspb1   | heat shock protein 1 | 15507 | −2.36 | 0.004073 |
| BC021352     | Pld2    | procollagen lysine, 2-oxoglutarate 5-dioxygenase 2 | 26432 | −2 | 0.005958 |
| NM_029550    | Keg1    | kidney expressed gene 1 [Mus musculus ] | 64697 | −1.8 | 0.031526 |
| AK146840     | Amd1    | S-adenosylmethionine decarboxylase 1 | 11702 | −2 | 0.014904 |
| NM_030706    | Trim2   | tripartite motif-containing 2 | 80890 | −2 | 0.048874 |
| Accession ID | Gene ID | Gene | Gene | Fold Change | P value |
|--------------|---------|------|------|-------------|---------|
| NM_010274    | Gpd2    | glycerol phosphate dehydrogenase 2, mitochondrial | 14571 | −1.9 | 0.008751 |
| NM_008878    | Serpin2 | serine (or cysteine) peptidase inhibitor, clade F, member 2 | 18816 | −1.8 | 0.027152 |
| NM_011435    | Sod3    | superoxide dismutase 3, extracellular | 20657 | −2 | 0.00248 |
| BC006716     | Vdr     | vitamin D receptor | 22337 | −1.8 | 0.05968 |
| NM_019444    | Ramp2   | receptor (calcitonin) activity modifying protein 2 | 54409 | −3.2 | 0.027003 |
| AF067806     | Pde8a   | phosphodiesterase 8A | 18584 | −1.9 | 0.036591 |
| AF012834     | Kcnj15  | potassium inwardly-rectifying channel, subfamily J, member | 56379 | −2.1 | 0.025618 |
| NM_007788    | Csnk2a1 | casein kinase 2, alpha 1 polypeptide | 12995 | −2 | 0.033254 |
| L27424       | Timp3   | tissue inhibitor of metalloproteinase 3 | 21859 | −2.1 | 0.0122 |
| NM_008079    | Galc    | galactosylceramidase | 14420 | −1.9 | 0.045887 |
| NM_023646    | Dnaja3  | DNAJ (Hsp40) homolog, subfamily A, member 3 | 83945 | −1.9 | 0.007017 |
| NM_030721    | Acox3   | acyl-Coenzyme A oxidase 3, pristanoyl | 80911 | −2 | 0.018101 |
| NM_018760    | Sla2    | solute carrier family 4 (anion exchanger), member 4 | 54403 | −2.1 | 0.029658 |
| NM_010890    | Mus musculus neural precursor cell expressed, developmentally down-regulated 4 (Nedd4) | 17999 | −1.9 | 0.000172 |
| NM_015017    | Igfbp4  | insulin-like growth factor binding protein 4 | 16010 | −2.2 | 0.018949 |
| NM_008397    | Itga6   | integrin alpha 6 | 16403 | −2.3 | 0.03713 |
| NM_009203    | Slc2a12 | solute carrier family 2 (metabolite transporter), member 12 | 20521 | −1.9 | 0.010432 |
| NM_018881    | Fmo2    | flavin containing monoxygenase 2 | 55990 | −1.9 | 0.000286 |
| NM_011851    | Ntse  | nucleotidase, ecto | 23959 | −2 | 0.020878 |
| NM_001164733 | Gclc    | glutamate-cysteine ligase, catalytic subunit | 14629 | −1.9 | 0.046958 |
| BC025936     | Cyp4a12 | cytochrome P450, family 4, subfamily A, member 2 | 277753 | −2.2 | 0.050246 |
| BC021452     | Ddx6    | DEAD (Asp-Glu-Ala-Asp) box polypeptide 6 | 13209 | −1.8 | 0.002793 |
| NM_016870    | Acsm3   | acyl-CoA synthetase medium-chain family member 3 | 20216 | −2.7 | 0.073051 |
| AF213670     | Mlx     | MAX-like protein X | 13430 | −2.1 | 0.002547 |
| NM_001164099 | Add3    | adducin 3 (gamma) | 27360 | −1.8 | 0.003839 |
| NM_001167745 | Wasl    | Wiskott-Aldrich syndrome-like (human) | 73178 | −2.3 | 0.005224 |
| BC027063     | Bdh1    | 3-hydroxybutyrate dehydrogenase, type 1 | 79119 | −2.7 | 0.0021 |
| AK003232     | Cbr3    | carbonyl reductase 3 | 109857 | −2.57 | 0.049803 |
| AK014338     | Manf    | meningeal astrocyte-derived neurotrophic factor | 74840 | −1.9 | 0.027373 |
| NM_001159375 | Ef4a1   | eukaryotic translation initiation factor 4A1 | 13681 | −2.2 | 0.006428 |
| NM_010911    | Nfs1    | nitrogen fixation gene 1 (S. cerevisiae) | 18041 | −2.1 | 0.002002 |
| AK015410     | Dnmt2   | DNA methyltransferase 2 | 13430 | −2.1 | 0.004377 |
| AK013376     | Aplp2   | amyloid beta (A4) precursor-like protein 2 | 11804 | −2.3 | 0.007349 |
| AK007618     | Ak3     | adenylate kinase 3 | 56248 | −2.2 | 0.026914 |
| NM_031843    | Dpp7    | dipeptidylpeptidase 7 | 83768 | −1.8 | 0.017748 |
| EDL19081     | Actb    | actin, beta | 11461 | −1.88 | 0.006181 |
| NM_010477    | Hsp1    | heat shock protein 1 (chaperonin) | 15510 | −2.72 | 0.029635 |
| NM_011631    | Hsp90b1 | heat shock protein 90, beta (Grp94), member 1 | 22027 | −2.17 | 0.009681 |
| NM_00111289  | Caprin1 | cell cycle associated protein 1 | 53872 | −1.88 | 0.005003 |
| NM_080555    | Ppap2b  | phosphatidic acid phosphatase type 2B | 67916 | −2.08 | 0.001515 |
| NM_011390    | Slc12a7 | solute carrier family 12, member 7 | 20499 | −1.88 | 0.010937 |
| NM_010302    | Gna12   | guanine nucleotide binding protein, alpha 12 | 14673 | −1.83 | 0.001605 |
| NM_019664    | Kcnj15  | potassium inwardly-rectifying channel, subfamily J, member 15 | 16516 | −2.17 | 0.012327 |
| U41465       | Bcl6    | B-cell leukemia/lymphoma 6 | 12053 | −1.82 | 0.009357 |
Insulin-like Growth Factor Binding Protein (IGFBP-1)

IGFBP-1 gene is a member of the insulin-like growth factor binding protein (IGFBP) family and encoding proteins with an IGFBP domain and a thyroglobulin type-I domain. It binds both insulin-like growth factors (IGFs) I and II and circulates in the plasma prolonging the half-life of the IGFs. In our work, the deletion of B2R didn’t change the expression of IGFBP-1. However, IGFBP-1 expression was decreased up to 33% by

Table 4. Cont.

| Accession ID | Gene Description                                      | Gene ID | Fold Change | P value    |
|--------------|-------------------------------------------------------|---------|-------------|------------|
| AF319542     | Kcnk5 potassium channel, subfamily K, member 5        | 16529   | −2.05       | 0.003671   |
| NM_008261    | Hnf4a hepatic nuclear factor 4, alpha                 | 15378   | −1.8        | 0.00581    |
| NM_001159555 | Cd36 CD36 antigen                                      | 12491   | −2.58       | 0.021768   |
| NM_016697    | Gpc3 glypican 3                                        | 14734   | −2.43       | 0.001794   |
| BBS40964     | Ifnd2 interferon-related developmental regulator 2    | 15983   | −2.09       | 0.036417   |
| BC022130     | Slc26a1 solute carrier family 26 (sulfate transporter), member 1 | 231583 | −1.81       | 0.011694   |
| M33324       | Ghr growth hormone receptor                            | 14600   | −2.71       | 0.020174   |
| NM_013876    | Rnf11 ring finger protein 11                           | 29864   | −2.37       | 0.002724   |
| NM_026147    | Rps20 ribosomal protein 520                            | 67427   | −1.97       | 0.023604   |
| NM_008538    | Marcks myristoylated alanine rich protein kinase C substrate | 17118 | −1.96       | 0.011844   |
| NM_009199    | Slc1a1 solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1 | 20510 | −2.21       | 0.008478   |
| U13836       | Atp6v0a1 ATPase, H+ transporting, lysosomal V0 subunit A1 | 11975 | −1.9        | 0.007939   |

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Figure 8. Renal expression of (A) Superoxide dismutase 3, extracellular (EC-SOD), (B) Glutathione S-transferase, (GST), (C) Flavin containing monoxygenase (FMO) and (D) Insulin-like growth factor binding protein (IGFBP-1) in B2R<sup>+/−</sup>D and B2R<sup>−/−</sup>D. Renal cortex mRNA levels were measured by real time PCR. Data presented in the bar graph demonstrates that disruption of B2R results in significant increases in anti-oxidant enzymes as well as IGFBP-1 (*P<0.05 B2R<sup>−/−</sup>D vs. B2R<sup>+/+</sup>D, n = 3).

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diabetes in the wild type mice (P<0.05). Interestingly, in B2R−/−D mice, IGFBP-1 expression was upregulated significantly: up to 2.7-fold increase compared to that in B2R+/+D (*P<0.05 vs. B2R+/+D, Figure 8D).

We next performed a targeted analysis to identify the involvement of these selected validated genes in the most highlighted altered pathways (apoptosis, oxidative stress and inflammation). These genes were shown to be highly related to the aforementioned pathways as shown in Figure 9.

Systems Biology Analysis of Altered Genes in B2R−/−D and B2R+/+D mice

Pathway Studio 9.0 (2011, Ariadne Genomics, Rockville, MD) was also used to search for potential altered cellular processes, and related pathways for associations with gene alterations in our diabetic mice in the presence or absence of B2R. The network was generated using the “direct interaction” algorithm with the filters of “Cellular process and Protein” as Entity Type while the Relation Type parameter was set to “Regulation Analysis” to map altered pathways regulated by the identified (downregulation vs. upregulation) subsets of genes. Several processes believed to be central to the pathogenesis of DN included oxidative stress mechanisms (ROS generation & oxidative stress), cardiac injury mechanisms along with pronounced inflammatory process with a marked alteration in the pro-apoptotic genes as illustrated in Figure 10.

Figure 9. Pathways influenced by the validated targeted genes. Targeted system biology analysis of the biological process and molecular function of the 4 validated genes (Superoxide dismutase 3, extracellular (EC-SOD), Glutathione S-transferase, alpha 2(Yc2) (GST-Yc2), Flavin containing monoxygenase 2 (FMO2), Insulin-like growth factor binding protein (IGFBP-1). Similar to the identified altered pathways, these 4 proteins are shown to be related to the identified molecular pathways (apoptosis, oxidative stress and inflammation).
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Discussion

A pivotal event initiated by DN is glomerular injury, characterized by mesangial deposition and podocyte loss. The degree of podocyte loss and mesangial expansion are strongly correlated with the clinical manifestations of DN, such as albuminuria and decreased GFR [3,4,15]. Microalbuminuria, an early marker of DN, signifies high risk for progressive renal failure and cardiovascular disease [16]. Microalbuminuria has also been associated with increased cardiovascular mortality in diabetic and non-diabetic populations and with generalized and glomerular endothelial dysfunction [17]. Identifying biomarkers and risk factors that contribute to the development of microalbuminuria may provide insights into the mechanisms of diabetic renal injury.

Few interventions have been shown to slow the progression of renal disease in diabetic patients. These include intensive glycemic control, blood pressure control and treatment with angiotensin converting enzyme inhibitors (ACEI) or angiotensin receptor blockers (ARBs) [6,18]. Despite these interventions and beneficial effects, diabetic patients progress with time to develop end stage renal disease. It is of significance to note here that a recent Interventional study aimed at blockade of the renin-angiotensin system (RAS) with ACE-inhibitors or ARBs, in patients with type 1 diabetes, did not slow nephropathy progression [19]. However, the

Figure 10. Molecular & Biological Pathway Interaction Map Analysis upon Diabetes induction with or without disruption of B_2_R. Using Pathway Studio 9.0, altered genes relevant to diabetic induction with or without disruption of B_2_R were analyzed. In B_2_R^+/+ D vs. B_2_R^−/− D mice, a total of 109 genes were found to be altered (43 upregulated and 66 downregulated). The network was generated using "direct interaction" algorithm to map cellular processes and interactions among altered genes. Of interest, global Pathway analysis revealed association of these genes to oxidative stress mechanisms (ROS generation & oxidative stress), cardiac injury mechanisms along with pronounced inflammatory process with a marked alteration in the pro-apoptotic genes. The upregulated genes are shown in green and downregulated genes are in red.
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exact factors responsible for these maladaptive signals leading to renal failure are poorly defined.

Metabolic imbalances associated with high tissue glucose and abnormal lipid levels in the diabetic state influence many pathways that contribute to the pathogenesis of DN [20,21]. The modifiable factors engaged in these processes are yet to be identified but there is evidence for promotion of chronic low-grade inflammation, oxidative stress, endothelial dysfunction, stimulation of proliferative/apoptotic pathways, and deposition of extracellular matrix [22–24]. Importantly, inflammatory mediators and growth factors are increasingly recognized as key players in the pathogenesis of DN [25–27].

Our published work has provided evidence for the involvement of the kallikrein-kinin system (KKS) in the initiation of DN [7,13]. In the current work, we performed longitudinal data analysis to assess the rate of change in AER levels over time among the 4 different groups. Our data indicated that targeted deletion of B2R in mice interferes with the progression of DN. Diabetic B2R+/− mice display reduced AER compared to diabetic B2R+/+ mice. Other investigators have also implicated a role for B2R in DN. They have shown that in DN [30,31].

Contrary to the aforesaid findings, Kakoki and Smithies have reported a protective role for B2R in DN. They have shown that when using the Insulin Akita mouse is the propensity for these mice to develop mesangial deposits of IgG [34].

To investigate the underlying mechanisms and involved pathways linking the role of B2R genotype to the development/progression of DN, we examined the contribution of B2R genotype on the global genomics level. We performed a global microarray study comparing gene expression profiles among four groups of mice [32,33]. Other factors contributing to these apparent differences in the role of B2R in DN may be attributed to differences in the model of DN studied, genetic background of the animal models studied, severity and metabolic control of the diabetic state, specifics of the experimental design, the end points measured. It is noteworthy to point here that a confounding factor to be considered when using the Insulin Akita mouse is the propensity for these mice to develop mesangial deposits of IgG [34].

Findings from this work highlighted the role of several altered pathological pathways involved in the development of diabetes in the B2R+/−/D mice vs. B2R+/−/C mice which included: endothelial injury, oxidative stress, and insulin and lipolipid metabolism.

A detailed analysis of the top scoring biological processes data [ Panther Analysis] reflected the central role of B2R to increased immune response/inflammation along with other cellular functions (transport, systems process and response to stimulus which can be linked to protective/compensatory mechanism. This is in accordance with a previous study by Bascands et al, in which a global microarray renal gene expression changes were examined in lipopolysacharide-treated wild-type and kinin B1 receptor-knockout mice to investigate underlying mechanisms of renal inflammation reflected the role of acute phase response and inflammatory process [33].

This is in contrast to the sole effect of diabetes induction in wild type mice which reflected more pronounced metabolic/cellular processes changes (metabolites precursor generation, cellular adhesion, and cellular communication) rather than inflammatory immune response mediated response. Of interest, is the upregulation of one of the genes, aquaporin 4, (AQP4, 2.24) due to diabetes. AQP4 functions as a water transport channel in the kidney and has been shown to be downregulated in mice lacking B2R [36].

These results validate existing published literature linking renal inflammation to early events of renal disease [37–39]. Furthermore, a global systems biology analysis among the diabetic mice with or without disruption of B2R (B2R+/−/D vs. B2R+/+D) illustrated the role of oxidative stress mechanisms (ROS generation & oxidative stress), along with inflammatory process with a marked alteration in the pro-apoptotic genes. Indeed, these results may reflect a pathologic exacerbative role of B2R in inducing cellular vascular injury mediated via apoptotic pathways in the presence of diabetes. These findings are in concert with other microarray studies involving B1 and B2 receptor knockout mice [40,41].

Taken together, the finding of this study investigates the contributing role of B2-receptors in either exacerbating or at least enhancing the occurrence of diabetic nephropathy. In conclusion, the present study investigates the impact B2R deletion on the development of DN. A critical analysis of the data hints that renal function is preserved in the B2R+/−/D mice especially at the early stages of DN, compared to that of B2R+/−/D mice; these data were substantiated by the genomics/systems biology analysis. To the best of knowledge, this represents the first study that utilizes wide scale genomic/systems biology analysis in B2R+/−/D mice. Finally, several of the identified genes (EC-sod, GST, IGFBP1 and FMO) were validated with RT-PCR to confirm gene alteration. Further studies including immunohistological analysis and assessment of protein levels and the activities of the antioxidants identified are certainly necessary to further evaluate the contributing role of the disruption of the B2-receptors.

Author Contributions
Wrote the paper: AAJ. Conceived the design of the study: AAJ. Performed all biostatistical analysis of the data, longitudinal data analysis of AER, plasma glucose, BW and power analysis: MJ. Performed the global gene pathway analysis and were responsible for generating the graphics: FK MAH IC. Wrote the sections related to data analysis and methods: MJ. Helped in writing the manuscript: AE ENZ. Responsible for the overall preparation of the manuscript and for the work accomplished: AAJ.

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