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Angiotensin AT1 receptor antagonism ameliorates murine retinal proteome changes induced by diabetes

Ben-Bo Gao, Joanna A. Phipps, Dahlia Bursell, Allen C. Clermont, and Edward P. Feener*
Research Division, Joslin Diabetes Center, One Joslin Place, Boston, Massachusetts 02215, USA.

Abstract

Diabetic retinopathy is the most common microvascular complication caused by diabetes mellitus and is a leading cause of vision loss among working-age adults in developed countries. Understanding the effects of diabetes on the retinal proteome may provide insights into factors and mechanisms responsible for this disease. We have performed a comprehensive proteomic analysis and comparison of retina from C57BL/6 mice with 2 months of streptozotocin-induced diabetes and age-matched nondiabetic control mice. To explore the role of the angiotensin AT1 receptor in the retinal proteome in diabetes, a subgroup of mice were treated with the AT1 antagonist candesartan. We identified 1,792 proteins from retinal lysates, of which 65 proteins were differentially changed more than 2 fold in diabetic mice compared with nondiabetic mice. The majority (72%) of these protein changes were normalized by candesartan treatment. Most of the significantly changed proteins were associated with metabolism, oxidative phosphorylation, and apoptotic pathways. An analysis of the proteomics data revealed metabolic and apoptotic abnormalities in the retina from diabetic mice that were ameliorated with candesartan treatment. These results provide insight into the effects of diabetes on the retina and the role of the AT1 receptor in modulating this response.

Keywords

angiotensin AT1 receptor; diabetes mellitus; diabetic retinopathy; proteome; retina

Introduction

Diabetic retinopathy (DR) is a sight-threatening disease that develops to some extent in nearly all people with diabetes mellitus. Although DR is primarily characterized as a vascular disease, there is growing evidence that the glial and neural components of retina are also involved. Early vascular changes in DR include increased retinal vascular permeability (RVP), the appearance of microaneurysms and hemorrhages, and changes in vessel diameter and hemodynamics¹. In addition, diabetes can lead to acellular capillaries and pericyte loss, which have been implicated in causing areas of retinal ischemia and the resultant pathological new vessel growth. Diabetes also induces abnormal electroretinogram responses, suggesting early

*Correspondence: Edward P Feener, Joslin Diabetes Center, One Joslin Place, Boston, Massachusetts 02215; TEL: (617)732-2599; FAX: (617)732-2637; edward.feener@joslin.harvard.edu.

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Supporting Information Available: Supporting Information Figure: Representative peptide coverage and MS/MS spectra of Hspa12a and Rdh12.

Supporting Information Table: Retinal proteome in nondiabetic and diabetic mice. This Excel table contains a non-redundant list of IPI, protein name, gene symbol, molecular weight, sequence coverage, total peptides, unique peptides, spectral count of diabetic mice, nondiabetic mice, and diabetic mice treated with candesartan (mean $\pm$ S.E.M. $n = 5$), and redundant protein name for the 1,792 proteins identified in this study. This information is available free of charge via the Internet at <http://pubs.acs.org>.

and regional changes in neuroretinal responses or conduction 2. Although the incidence and progression of DR are reduced with intensive glycemic control, additional therapeutic strategies to prevent this disease are needed.

Experimental and clinical studies suggest that inhibition of the renin-angiotensin system (RAS) may reduce DR³⁻⁴. In animal studies, blockade of RAS was effective in reducing RVP, vascular endothelial growth factor expression, electroretinogram, and hemodynamic abnormalities induced by diabetes⁵⁻⁷. Evidence supporting the importance of RAS inhibition in slowing the progression of retinopathy in humans has been obtained from several trials in both type 1 and type 2 diabetes. The EUCLID (EURODIAB Controlled Trial of Lisinopril in Insulin-Dependent Diabetes Mellitus) Study indicated a beneficial effect of angiotensin-converting enzyme (ACE) inhibitor treatment on DR in people with type 1 diabetes. In addition, the UKPDS (UK Prospective Diabetes Study) demonstrated a reduction in the need for laser photocoagulation in type 2 patients who received an ACE inhibitor⁸. The Diabetic Retinopathy Candesartan Trials (DIRECT) Programme reported that candesartan reduced the incidence of retinopathy in type 1 diabetes by 18% for a 2-step change (EDTRS scale, primary outcome), 35% for a 3-step change, and had no effect on progression of retinopathy in type 1 diabetes⁹. In type 2 diabetes, candesartan treatment resulted in 34% regression of retinopathy¹⁰. Importantly, an overall significant change towards less-severe retinopathy was noted in both type 1 and 2 diabetes. A recent study showed that early blockade of RAS in patients with type 1 diabetes did not slow nephropathy progression but did slow the progression of retinopathy¹¹.

Streptozotocin (STZ) -induced diabetes in rats and mice has been used extensively for analysis of DR. In mouse retina, diabetes increases RVP, apoptosis, and neuronal cell death¹²⁻¹³. Gene expression profiling studies on retina from rats and mice subjected to 1 and 3 months of diabetes revealed upregulation of genes associated with oxidative phosphorylation and protein turnover¹⁴⁻¹⁵. A recent two-dimensional gel-based proteomic analysis of rat retina identified 168 proteins. The study showed that beta catenin, phosphatidylcholine, and aldehyde reductase were increased in retina from rats with STZ-induced diabetes; whereas succinyl CoA ligase and dihydropyrimidinase-related protein were decreased by diabetes¹⁶.

In our study, we characterized the mouse retinal proteome and examined the effects of diabetes in the absence or presence of treatment with the angiotensin AT1 receptor blocker, candesartan, on protein expression profiles in the retina.

Materials and Methods

Experimental Animals

Animals were group-housed with full access to standard laboratory chow and water at a room temperature of 23 ± 1 °C in a 12-h light/12-h dark cycle. To induce diabetes, 7–8 weeks old male C57BL/6 mice were injected intraperitoneally with 45 mg/kg STZ (Sigma–Aldrich, St. Louis, MO) in 10 mM citrate buffer vehicle over a 5 day interval. Nondiabetic mice (NDM, $n = 11$) were injected with the citrate buffer only. Animals with blood glucose levels greater than 250 mg/dl 72 h later were considered diabetic mice (DM, $n = 21$). Diabetic mice assigned to the treatment group (DMC, $n = 11$) received candesartan-cilexetil (Astrazeneca) *ad libitum* in drinking water at the dose of 10 mg/kg/day beginning 3 days after the completion of the STZ injection protocol and confirmation of diabetes onset. Blood pressure was measured by a noninvasive tail cuff sensor and monitoring system (Visitech Systems, Inc., Apex, NC). Blood glucose levels were measured using an Advantage II Accu-Check monitor. Two months after injection, mice were deeply anaesthetized by an intraperitoneal injection of sodium pentobarbital (Nembutal), and subsequently euthanized by cervical dislocation. Eyes were

immediately enucleated, the retina isolated from the retinal pigment epithelium and then immediately frozen in liquid nitrogen.

Identification of Retinal Proteins by LC-MS/MS

Both retina from each mouse were combined and retinal protein extracted by sonication in ice-cold lysis buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 4 mM EDTA, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 100 mM NaF, 2 mM Na_3VO_4 , 1 mM PMSF, 0.1 mg/ml aprotinin, 10% glycerol and 1% Triton X-100. Lysates were centrifuged at 20,000 *g* for 30 min. The protein concentration in the supernatant was determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Retinal lysates (200 μg) from 5 mice in each group were separated by 10 % SDS-PAGE. The SDS-PAGE gel was stained with Coomassie Brilliant Blue G-250 stain (Bio-Rad, Hercules, CA) and destained in 10% glacial acetic acid and 40% methanol. The entire lane for each sample was divided into 40 slices. Gel slices were individually digested with trypsin (Promega, Madison, WI). Gel tryptic digests were analyzed by tandem mass spectrometry using a LTQ linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA).

Assignment of MS/MS data was performed using X!Tandem (version 2006.09.15, The Global Proteome Machine Organization) search against the International Protein Index (IPI) mouse sequence database (IPI_MOUSE_v3.39, 52777 sequences, European Bioinformatics Institute) and a randomized version of the same IPI database generated by a Perl script, decoy.pl (Matrix Science, London, UK). The default X!Tandem search parameters were used, except for the following: a maximum valid expectation value of 0.1; potential residue mass modification of +16.0 Da for oxidized methionine and +71.0 Da for acrylamide alkylated cysteine; spectrum parameters including a fragment monoisotopic mass error of ± 0.4 Da and a precursor monoisotopic mass error of ± 0.5 Da.

Compilation of Search Results

Search results were compiled into a MySQL database and analyzed using MS Results Manager, a proteomics computational analysis software based on the PHP-MySQL-Apache platform¹⁷. Data processing occurred in five steps: i) File parsing: X!Tandem search results were parsed into the MySQL database. ii) Summarizing: The search results from each sample, generated from 40 gel slices, were combined based on IPI identifier (ID). iii) Protein-level filtering: The redundant proteins, contaminants, and proteins that did not have 2 unique peptides identified from a single slice or adjacent slices were filtered. iv) Reporting: Proteins that were identified in at least 2 samples were compiled into one reporting table. If multiple IDs were assigned for the same peptide match, a uniform ID was selected for the comparison of proteins identified between samples. The false discovery rate (FDR) of protein identification was calculated by dividing the number of random sequences by the sum of “random” and “real” sequences and multiplying by 100. Spectral count (the total number of observations of spectral-peptides matches) for each protein was calculated by the summation of peptides matched.

Bioinformatics Analysis

Gene Ontology (GO) annotations were extracted from Gene Ontology Annotation Database (GOA Mouse 49.0)¹⁸ and generic GO slim provided by European Bioinformatics Institute and the Gene Ontology¹⁹.

Statistics

Students' t-tests and one-way ANOVA were performed by in-house PHP script based on PHP statistics extension, or GraphPAD Prism (GraphPAD Software, San Diego, CA). The results represent the total spectral count (mean $\pm$ S.E.M). Values of $P < 0.05$ were considered significantly different.

Results

Blood Glucose and Blood Pressure

DM had significantly higher blood glucose (530.5 ± 26.0 vs. 142.9 ± 8.9 mg/dl, $P < 0.001$, Fig. 1a) and less body weight gain (24.3 ± 0.7 vs. 33.4 ± 1.3 g, $P < 0.001$, Fig. 1b) compared to NDM. Blood pressure was similar in DM and NDM mice (Fig. 1c and d). Candesartan treatment had no effect on blood glucose (476.7 ± 26.1 vs. 530.5 ± 26.0 mg/dl, Fig. 1a) or body weight (23.3 ± 0.7 vs. 24.3 ± 0.7 g, Fig. 1b) but did significantly reduce both systolic blood pressure (93.4 ± 1.0 vs. 69.7 ± 8.2 mmHg, $P < 0.01$, Fig. 1c) and diastolic blood pressure (59.0 ± 2.6 vs. 42.0 ± 3.6 mmHg, $P < 0.01$, Fig. 1d) compared with untreated DM group.

Identification of the Proteins in Mouse Retina

The retina from NDM, DM and DMC ($n = 5$) were subjected to proteomics analysis. Two hundred μ g of retinal tissue lysate were fractionated by 10% SDS-PAGE. Each lane was excised into 40 slices followed by tryptic digestion and the digests were analyzed by LC-MS/MS. The resultant data was analyzed by X!Tandem (Supporting Information Figure shows two representative MS/MS spectra) and MS Result Manager. The analysis of 15 samples and 600 gel slices led to the identification of a total of 1,792 proteins (Supporting Information Table) with a FDR (for protein identification) of 0.28%. In order to get an overview of the ontology content, Gene Ontology (GO) slim terms were used to categorize the retinal proteome. The GOA Mouse database was also categorized by GO slim terms for comparison (Table 1). There are 52,777 proteins in IPI_MOUSE_v3.39 database and 33,691 proteins (64%) that are annotated proteins in GOA Mouse 49.0 database. If multiple IDs were assigned for the same peptide match, the ID that had more GO term annotations was selected. This led to a high percentage (95.76%) of annotated proteins in the retinal proteome. Compared to the proteins in the GOA mouse database, the analyses of the biological process terms distribution in the observed retinal proteome showed greater than 2 fold distribution of proteins with GO term categories related to amino acid and derivative metabolism (2.64 fold), biosynthesis (2.14 fold), catabolism (2.65 fold), and electron transport (5.45 fold). These analyses of the cellular component terms distribution showed there are more cytoplasm proteins (2.49 fold) and less cell surface proteins (0.33 fold). The analyses of the molecular function terms distribution showed there are more proteins related to isomerase activity (3.41 fold), ligase activity (2.43 fold), lyase activity (2.85 fold), oxidoreductase activity (2.15 fold), translation regulator activity (2.77 fold), protein transporter activity (2.69 fold) and less proteins related to signal transducer activity (0.24 fold), receptor activity (0.11 fold), transcriptional regulator activity (0.40 fold), and channel or pore class transporter activity (0.32 fold) (Table 1).

Previous reports indicate that spectral counting can be used as a semi-quantitative measurement of protein abundance^{20–21}. The most abundant proteins in the retina include the glycolytic enzymes: alpha-enolase, pyruvate kinase isozymes M1/M2, glyceraldehyde-3-phosphate-dehydrogenase isoform 1, and fructose-bisphosphate aldolase A; S-arrestin, one of the major proteins of the rod photoreceptor; tubulin alpha-1C, a major constituent of microtubules, and beta-globin (Supporting Information Table).

Differentially Expressed Proteins in DM Retina

The protein compositions of retina from the NDM and DM group were compared by spectral counting for each protein from each sample. Each protein was usually identified in multiple slices, which were visualized by an output of spectral counts per slice as grayscale digital images (Fig. 2). We identified 170 proteins that were differentially expressed in DM retina with $P < 0.05$. The proteins changed with $P < 0.05$ may be caused by type 1 errors in multiple comparisons. The coefficient of variation generated from spectral counting is high when there are a few peptides identified in each protein¹⁷. Therefore, we introduced another FDR for

significance to evaluate the peptide number cutoff. The FDR for significance was calculated by dividing the significantly changed protein number of a random group by the experiment group and then multiplying by 100. We randomly divided the samples into 3 groups and performed statistics. The FDR for significance dropped from 24.7% (all matched proteins) to 17.32% and 15.5% when the peptide cutoff was set to 3 and 6, respectively (Fig. 3a). No further decreases were found with increased peptide cutoffs. Based on these results, we selected a peptide cutoff of at least 3 peptides and no more than 6 peptides in this study. The selection of fold change also affected FDR for significance. The FDR for significance dropped from 16.16% to 14.24% and 9.23% when the fold change was set to ≥ 1.5 and ≥ 2.0 , respectively (Fig. 3b). Therefore, the criteria for differentially expressed proteins used for further analysis were defined for this study as: $P < 0.05$, fold change ≥ 2.0 , and the average total peptides >5 for either group. We identified 65 proteins (3.62% of total proteins) that were differentially expressed in the DM retina compared with the NDM group (Table 2), comprised of 55 proteins that were increased and 10 proteins that were decreased. After gene-annotation enrichment analysis and functional annotation clustering analysis by DAVID, 23 proteins were enzymes, 40 proteins were related to metabolic processes, 19 proteins were related to protein metabolic processes, and 5 proteins were related to lipid metabolic processes. Sixteen proteins related to protein metabolism that were increased in the DM retina are: prefoldin subunit 2 (Pfdn2), coatomer subunit zeta-1 (Copz1), eukaryotic translation elongation factor 1 delta (Eef1d), eukaryotic translation initiation factor 2 subunit 1 (Eif2s1), sarcolemmal membrane-associated protein (Slmap), glycyl-tRNA synthetase (Gars), AP2-associated protein kinase 1 (Aak1), heat shock 70 kDa protein 12A (Hspa12a), 60S ribosomal protein L23a (Rpl23), 60S ribosomal protein L21 (Rpl21), 9S ribosomal protein L12 (Mrpl12), 26S protease regulatory subunit S10B (Psmc6), dual specificity protein phosphatase 3 (Dusp3), FK506-binding protein 4 (Fkbp4), MAGUK p55 subfamily member 2 (Mpp2), and AP-3 complex subunit beta-2 (Ap3b2). Three proteins related to protein metabolic processes that were decreased were: ubiquitin-conjugating enzyme E2N (Ube2n), protein phosphatase methylesterase 1 (Ppme1), and FK506-binding protein 2 (Fkbp2). Increased levels of proteins in the DM retina related to lipid metabolic processes included: fatty acid synthase (Fasn), long-chain-fatty-acid--CoA ligase 3 (Acsl3), estradiol 17-beta-dehydrogenase 8 (H2-Ke6), retinol dehydrogenase 12 (Rdh12), NADH-cytochrome b5 reductase 3 (Cyb5r3). Three proteins associated with apoptosis were increased in retina from diabetic mice, including: apoptosis inhibitor 5 (Api5), dynamin-like 120 kDa protein (Opa1), and NADH-ubiquinone oxidoreductase 75 kDa subunit (Ndufs1)²². Conversely, astrocytic phosphoprotein PEA-15 (Pea15a) was decreased.

Effect of Candesartan on Expressed Proteins in DM Retina

In order to examine the effect of candesartan on the proteome change, proteome analysis was performed on the candesartan-treated DM (DMC) retina. Eighty-nine proteins were differentially expressed in retina from DMC compared with the untreated DM group. However, only 31 proteins were differentially expressed between NDM and DMC retina. Of the 65 proteins that were differentially expressed in DM retina compared with the NDM group, 24 proteins were reversed (DM vs. DMC, $P < 0.05$; Fig. 4a) and 23 proteins were reversed by at least 50% ($P > 0.05$) with the treatment of candesartan. Six proteins were unchanged by the treatment of candesartan (Fig. 4b). This result suggests that candesartan ameliorated abnormalities in 72% of the proteins altered in diabetic mice. Proteins related to lipid metabolism (Fasn, Acsl3, H2-Ke6, Rdh12, Cyb5r3) and proteins related to apoptosis regulation (Api5, Opa1, Pea15a) were normalized after candesartan treatment.

Discussion

In this report we have 1) generated a murine retinal proteomic inventory, 2) identified a group of proteins that are altered in mice subjected to 2 months of diabetes, and 3) evaluated the effect

of angiotensin AT1 receptor antagonism on the diabetic retinal proteome. A recent study using the 2-DE technique detected 168 proteins in the normal rat retina and identified in gels displaying retinal proteins from diabetic rats 24 unique proteins, 37 absent proteins, 8 spots with increased expression, and 27 with decreased expression¹⁶. In this study, we performed a comprehensive proteomics analysis of the mouse retina under diabetic conditions and with candesartan treatment using a 1D-PAGE-LC/MS/MS technique. Using this approach, we identified 1,792 unique proteins in the murine retinal proteome with an FDR of 0.28%. Comparing this result to the SAGE (serial analysis of gene expression) study in which 3,834 unique known genes have been found to be expressed in the retina²³, the proteome list provided in this study is an informative resource, particularly in relation to retinal protein changes in diabetes. The GO term analysis provided a broad view of the retinal proteome, with several notable traits in the proteome list compared with all annotated mouse genes. In the retinal proteome, we observed more cytoplasm proteins and more proteins related to metabolism, electron transport, translational regulator activity, and protein transporter activity. We also observed less cell surface proteins and fewer proteins related to signal transducer activity, receptor activity, transcription regulator activity, and channel transporter activity. There are several possible explanations for these results. Firstly, the signal transducer, receptor, transcription regulator, and channel proteins are usually relatively low in abundance and require additional enrichment methods for detection from lysates by LC-MS/MS. Secondly, the retina is the most metabolically active tissue in the body and therefore may be enriched in metabolic proteins. Lastly, the tissue extraction method used may have a higher efficiency of recovering cytosolic proteins compared to membrane associated proteins.

Previous studies reported that a number of genes for inflammatory processes were altered during the first week of STZ-induced diabetes in rat retina²⁴. After 1 and 3 months of STZ injection, most of the upregulated genes could be classified into two functional categories: oxidative phosphorylation and protein turnover¹⁵. However, large-scale analyses of mRNA expression and protein abundance data showed that the correlation between mRNA and protein levels was insufficient to predict protein expression levels from quantitative mRNA data because of differences in translational and post-translational processing^{25–26}. Quantitative proteomic approaches based on the number of spectral counts have been widely applied on large scale and complex samples^{20–21}. Quantitative analysis based on spectral count was also successfully applied on 1D-PAGE LC/MS/MS-based proteomics in our previous human vitreous proteomics studies^{27–28}. In this study, the changes in retinal protein levels were examined in NDM and DM mice 2 months after the injection of STZ. We found that 65 proteins were differentially changed more than 2-fold in DM retina compared with NDM. After GO annotation analysis, the categories of these proteins were: protein metabolism, lipid metabolism, apoptosis, and vision.

Metabolic abnormalities are integral in the development of retinopathy in diabetic mice²⁹. In our study, 40% of the differentially expressed proteins in the retina from diabetic mice were related to metabolic processes, of which 19 proteins were related to protein metabolic processes. This result is consistent with previous studies that reported genes involved in energy production and protein synthesis were highly expressed in the retina³⁰ and genes for protein turnover were upregulated in the retina after 2 months of STZ-induced diabetes¹⁵. In addition, several proteins related to lipid metabolism were also increased in the DM retina including: fatty acid synthase (Fasn), long-chain-fatty-acid--CoA ligase 3 (Acsl3), estradiol 17-beta-dehydrogenase 8 (H2-Ke6), retinol dehydrogenase 12 (Rdh12), and NADH-cytochrome b5 reductase 3 (Cyb5r3).

Hyperglycemia causes increased production of mitochondrial reactive oxygen species (ROS), which has been implicated in contributing to the clinical complications associated with diabetes and obesity³¹. We found that two oxidative phosphorylation-related proteins (NADH-

ubiquinone oxidoreductase 75 kDa subunit (Ndufs1) and NADH dehydrogenase ubiquinone iron-sulfur protein 2 (Ndufs2)) were increased in the retina from diabetic mice.

Apoptosis of retinal neural cells and vascular pericytes and the proliferation of endothelial cells have been well established as early changes in DR 32–34. The mechanisms by which diabetes influences apoptosis in the retina are not fully understood. In our study, three proteins related to apoptosis regulation (apoptosis inhibitor 5 (Api5), dynamin-like 120 kDa protein (Opa1), and NADH-ubiquinone oxidoreductase 75 kDa subunit (Ndufs1) 22) were increased. Another apoptosis-related protein, astrocytic phosphoprotein PEA-15 (Pea15a), was decreased in DM retina. Api5 was initially isolated as a gene whose expression promoted cell survival following serum deprivation 35. Api5 was identified as a suppressor of E2F-dependent apoptosis *in vivo*³⁶. Opa1 is highly expressed in rat and human retina^{37–38}, and mutation in Opa1 is associated with autosomal dominant optic atrophy³⁹. PEA-15 is a small protein (15 kDa) that was first identified as an abundant phosphoprotein in brain astrocytes and is an endogenous protein that inhibits FAS and TNFR1-mediated apoptosis⁴⁰. PEA-15 may play a major role in signal integration and has been shown to modulate signaling pathways that control apoptosis and cell proliferation. In particular, PEA-15 protects astrocytes from TNF α -triggered apoptosis and regulates the actions of the ERK MAP kinase cascade by binding to ERK and altering its subcellular localization 41.

Experimental and clinical studies have suggested that the RAS plays a role in the pathogenesis of DR. ACE inhibition and angiotensin receptor blockade may reduce the progression of DR. The beneficial effects of inhibiting angiotensin AT1 receptor signaling may include: inhibiting leukocyte adhesion to the retinal vasculature⁴², reducing overexpression of vascular endothelial growth factor and hyperpermeability⁴³, normalizing retinal blood flow 44, and ameliorating neuronal dysfunction⁴⁵. The results from our proteomics study show that 72% of the differentially abundant proteins isolated from STZ-induced diabetic mice retina could be prevented by candesartan treatment. In particular, we found that the proteins related to lipid metabolism (Fasn, Acsl3, H2-Ke6, Rdh12, Cyb5r3) and apoptosis regulation (Api5, Opa1, Pea15a) were normalized with candesartan treatment.

In summary, we have provided a proteomic inventory of the mouse retina with 65 proteins involved in metabolic processes and apoptosis regulation that are altered in DM mice retina. The majority of the upregulated proteins are classified under four categories: metabolism, oxidative phosphorylation, and apoptosis. Treatment of mice with the angiotensin AT1 receptor antagonist, candesartan, prevented the majority of protein changes induced by diabetes. The proteomics data identified proteins involved in metabolism and apoptosis that are abnormally abundant in the retina from diabetic mice, and they may contribute to the development of DR. These results provide insight into new possible preventative and therapeutic interventions of DR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Characteristics of diabetic mice (DM) and nondiabetic mice (NDM)

Blood glucose (a) body weight (b) systolic blood pressure (c) diastolic blood pressure (d) NDM, DM, and diabetic mice treated with candesartan (DMC). ** $P < 0.01$ and *** $P < 0.001$ compared with NDM, ## $P < 0.01$ compared with DM. Error bars represent standard error of the mean (S.E.M). $n = 6-11$.

Figure 2. Extracted spectral counting of Hspa12a and Rdh12 from retina of diabetic mice (DM), nondiabetic mice (NDM), and diabetic mice treated with candesartan (DMC) displayed as grayscale digital image

The spectral counting from each slice was converted to 256 intensities (i.e., shades of gray). The black represents the strongest intensity and the white represents the weakest intensity.

Figure 3. The selection of peptide count (a) and fold change (b) affected the false discovery rate
The false significant rate was calculated by dividing the significantly changed protein number of a random group by the experiment group and multiplying by 100.

Figure 4. Effect of candesartan on proteins in diabetic mouse retina

Diabetic mice (DM), nondiabetic mice (NDM), and diabetic mice treated with candesartan (DMC). 24 proteins were normalized by the treatment of candesartan (a) ($P < 0.05$, NDM vs. DM and DM vs. DMC) and 6 proteins were not reversed by the treatment of candesartan (b) ($P < 0.05$, NDM vs. DM and NDM vs. DMC). Error bars represent standard error of the mean (S.E.M). $n = 5$ mice.

Table 1

Frequency of gene ontology terms in murine retinal proteome annotation

GO Term	Retina		GOA	
	n	%	n	%
biological_process; GO:0008150				
%behavior; GO:0007610	65	3.8	992	2.9
%cellular process; GO:0009987	1266	73.8	21704	64.4
%cell communication; GO:0007154	220	12.8	6860	20.4
%cell differentiation; GO:0030154	82	4.8	1814	5.4
%cellular physiological process; GO:0050875	1193	69.5	17128	50.8
%transport; GO:0006810	365	21.3	3910	11.6
%cell death; GO:0008219	67	3.9	987	2.9
%cell motility; GO:0006928	33	1.9	590	1.8
%membrane fusion; GO:0006944	4	0.2	24	0.1
%development; GO:0007275	172	10.0	3833	11.4
<cell differentiation; GO:0030154	82	4.8	1814	5.4
%physiological process; GO:0007582	1282	74.7	19992	59.3
%cellular physiological process; GO:0050875	1193	69.5	17128	50.8
%transport; GO:0006810	365	21.3	3910	11.6
%cell death; GO:0008219	67	3.9	987	2.9
%cell motility; GO:0006928	33	1.9	590	1.8
%extracellular structure organization and biogenesis; GO:0043062	16	0.9	188	0.6
%metabolism; GO:0008152	903	52.6	13176	39.1
%amino acid and derivative metabolism; GO:0006519	75	4.4	557	1.7
%biosynthesis; GO:0009058	247	14.4	2269	6.7
%catabolism; GO:0009056	128	7.5	948	2.8
%electron transport; GO:0006118	10	0.6	36	0.1
%macromolecule metabolism; GO:0043170	580	33.8	7381	21.9
%nucleobase\, nucleoside\, nucleotide and nucleic acid metabolism; GO:0006139	299	17.4	5547	16.5
%response to stimulus; GO:0050896	140	8.2	3755	11.2
%secretion; GO:0046903	42	2.5	369	1.1
%regulation of biological process; GO:0050789	279	16.3	6169	18.3
cellular_component; GO:0005575				
%cell; GO:0005623	1422	82.9	22930	68.1
<cell surface; GO:0009986	7	0.4	419	1.2
<intracellular; GO:0005622	1281	74.7	15109	44.9
<chromosome; GO:0005694	32	1.9	587	1.7
<cytoplasm; GO:0005737	1027	59.9	8113	24.1
<nucleus; GO:0005634	390	22.7	6264	18.6

GO Term	Retina		GOA	
	n	%	n	%
<membrane; GO:0016020	521	30.4	10929	32.4
%extracellular; GO:0005576	96	5.6	2479	7.4
<extracellular matrix; GO:0005578	51	3.0	778	2.3
<extracellular space; GO:0005615	19	1.1	455	1.4
%unlocalized; GO:0005941	14	0.8	111	0.3
molecular_function; GO:0003674				
%antioxidant activity; GO:0016209	6	0.4	69	0.2
%binding; GO:0005488	1180	68.8	20206	60.0
%nucleic acid binding; GO:0003676	258	15.0	5668	16.8
%protein binding; GO:0005515	558	32.5	8530	25.3
%catalytic activity; GO:0003824	688	40.1	9330	27.7
%helicase activity; GO:0004386	19	1.1	249	0.7
%hydrolase activity; GO:0016787	269	15.7	3588	10.7
%isomerase activity; GO:0016853	41	2.4	236	0.7
%kinase activity; GO:0016301	61	3.6	1736	5.2
%ligase activity; GO:0016874	74	4.3	597	1.8
%lyase activity; GO:0016829	37	2.2	255	0.8
%oxidoreductase activity; GO:0016491	144	8.4	1315	3.9
%transferase activity; GO:0016740	147	8.6	3328	9.9
%enzyme regulator activity; GO:0030234	55	3.2	1186	3.5
%motor activity; GO:0003774	17	1.0	276	0.8
%signal transducer activity; GO:0004871	69	4.0	5549	16.5
%receptor activity; GO:0004872	23	1.3	4262	12.7
%structural molecule activity; GO:0005198	119	6.9	1329	3.9
%transcription regulator activity; GO:0030528	43	2.5	2098	6.2
%translation regulator activity; GO:0045182	31	1.8	220	0.6
%transporter activity; GO:0005215	167	9.7	2503	7.4
%carrier activity; GO:0005386	96	5.6	999	3.0
%channel or pore class transporter activity; GO:0015267	11	0.6	674	2.0
%electron transporter activity; GO:0005489	46	2.7	475	1.4
%ion transporter activity; GO:0015075	82	4.8	1210	3.6
%protein transporter activity; GO:0008565	14	0.8	102	0.3
Total	1716	100	33691	100

Table 2
Proteomic differences in retina from diabetic mice (DM) compared with nondiabetic mice (NDM)

IP1	Protein Name	Gene Symbol	NDM	DM	Location	Type
IP100131063	Isoform A of Methyl-CpG-binding protein 2	Mecp2	1.5 ± 0.4	6.5 ± 0.7 ^{***}	N	Transcription regulator
IP100420706	Leucine-rich PPR motif-containing protein, mitochondrial precursor	Lrpprc	2.2 ± 0.7	8.8 ± 2.1 [*]	C	other
IP100117657	Isoform 1 of Dynamin-like 120 kDa protein, mitochondrial precursor	Opal	1.4 ± 0.8	5.3 ± 1.1 [*]	C	enzyme
IP100133713	Isoform 1 of D-tyrosyl-tRNA(Tyr) deacylase 1	Dtd1	1.8 ± 0.6	6.7 ± 2.0 [*]	C	enzyme
IP100624363	Isoform 1 of Sarcolemmal membrane-associated protein	Slmap	2.4 ± 0.7	8.4 ± 0.9 ^{***}	M	other
IP100125971	26S protease regulatory subunit S10B	Psmc6	1.6 ± 0.7	5.7 ± 1.2 [*]	N	peptidase
IP100321266	Isoform 1 of Paraspeckle component 1	Pspe1	2.1 ± 0.7	7.1 ± 1.5 [*]	N	other
IP100310240	annexin A6 isoform b	Anxa6	2.1 ± 0.9	7.1 ± 1.6 [*]	M	other
IP100458612	Isoform 1 of AP2-associated protein kinase 1	Aak1	3.4 ± 0.7	11.3 ± 2.1 ^{**}	C	kinase
IP100115454	Sideroflexin-1	Sfxn1	2.4 ± 1.1	7.8 ± 1.5 [*]	C	transporter
IP100266463	Isoform A of Arsenite-resistance protein 2	Ars2	1.8 ± 0.7	5.6 ± 1.0 ^{**}	N	other
IP100461456	60S ribosomal protein L23a	Rpl23	3.1 ± 0.9	9.7 ± 1.7 ^{**}	C	other
IP100115598	Isoform Short of Estradiol 17-beta-dehydrogenase 8	H2-K66	1.7 ± 0.8	5.1 ± 1.0 [*]	C	enzyme
IP100279443	Heat shock 70 kDa protein 12A	Hspa12a	9.7 ± 2.7	28.6 ± 3.3 ^{**}	U	other
IP100169772	Long-chain-fatty-acid-CoA ligase 3	Acs13	2.9 ± 1.1	8.7 ± 1.7 [*]	C	enzyme
IP100118963	39S ribosomal protein L12, mitochondrial precursor	Mrpl12	3.0 ± 1.3	8.9 ± 0.8 ^{**}	C	other
IP100130147	Isoform 2 of RNA-binding protein	Raly	2.3 ± 0.8	6.5 ± 0.7 ^{**}	N	other
IP100126115	Isoform 1 of Sideroflexin-3	Sfxn3	7.3 ± 2.1	20.0 ± 2.1 ^{**}	C	transporter
IP100112223	EF-hand domain-containing protein 2	Efh2	2.1 ± 0.9	5.6 ± 0.8 [*]	U	other
IP100117281	Isoform Mitochondrial of Phospholipid hydroperoxide glutathione peroxidase, mitochondrial precursor	Gpx4	2.5 ± 1.0	6.6 ± 1.0 [*]	C	enzyme

IP1	Protein Name	Gene Symbol	NDM	DM	Location	Type
IP100315548	60S ribosomal protein L21	Rpl21	1.9 ± 0.8	5.0 ± 1.0 *	C	other
IP100322828	Phenylalanyl-tRNA synthetase beta chain	Farsb	2.3 ± 0.8	6.0 ± 1.4 *	C	enzyme
IP100464317	glutaminase isoform 1	Gls	7.4 ± 2.1	18.9 ± 3.9 *	C	enzyme
IP100751759	Isoform 1 of Polypyrimidine tract-binding protein 2	Ptbp2	2.6 ± 0.9	6.7 ± 0.8 **	N	other
IP100331541	6-phosphofructokinase, muscle type	Pfkm	6.0 ± 2.0	15.4 ± 1.8 **	C	kinase
IP100754418	Apoptosis inhibitor 5	Api5	5.2 ± 1.7	13.2 ± 3.0 *	C	other
IP100230139	FK506-binding protein 4	Fkbp4	2.4 ± 0.9	6.1 ± 1.2 *	N	enzyme
IP100308882	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor	Ndufs1	17.4 ± 3.1	43.2 ± 8.7 *	C	enzyme
IP100119545	Prefoldin subunit 2	Pfdn2	2.9 ± 0.8	7.1 ± 0.9 **	C	other
IP100111151	Rabphilin-3A	Rph3a	8.8 ± 1.1	21.3 ± 4.7 *	M	transporter
IP100474446	Eukaryotic translation initiation factor 2 subunit 1	Eif2s1	3.1 ± 0.9	7.5 ± 1.2 *	C	Translation regulator
IP100118875	eukaryotic translation elongation factor 1 delta isoform a NADH dehydrogenase [ubiquinone]	Eef1d	2.2 ± 0.7	5.2 ± 0.8 *	C	Translation regulator
IP100128023	iron-sulfur protein 2, mitochondrial precursor	Ndufs2	3.5 ± 1.5	8.2 ± 1.0 *	C	enzyme
IP100473646	70 kDa protein	Rhot2	3.7 ± 0.7	8.4 ± 1.1 **	C	enzyme
IP100816884	heterogeneous nuclear ribonucleoprotein H3	Hnrph3	3.2 ± 1.1	7.2 ± 1.2 *	N	other
IP100113686	Isoform 1 of General transcription factor II-1	Gtf2i	3.0 ± 0.8	6.9 ± 1.1 *	N	Transcription regulator
IP100109870	Gamma-soluble NSF attachment protein	Napg	4.2 ± 1.3	9.5 ± 1.5 *	C	transporter
IP100229534	Myristoylated alanine-rich C-kinase substrate	Marcks	28.4 ± 6.0	63.9 ± 7.8 **	M	other
IP100110990	Dual specificity protein phosphatase 3	Dusp3	7.7 ± 1.8	17.3 ± 1.5 **	C	enzyme
IP100139637	Coatomer subunit zeta-1	Copz1	3.1 ± 1.5	7.0 ± 0.3 *	C	transporter
IP100331146	UMP-CMP kinase	Cmpk	4.3 ± 1.1	9.7 ± 1.0 **	N	kinase
IP100125147	Isoform 1 of MAGUK p55 subfamily member 2	Mpp2	7.9 ± 2.5	17.4 ± 3.1 *	M	kinase
IP100395157	Isoform 1 of Protein 4.1	Epb4.1	9.2 ± 1.5	19.8 ± 2.0 **	M	other
IP100420426	Isoform 1 of AP-3 complex subunit	Ap3b2	3.7 ± 1.0	8.0 ± 0.9 **	C	transporter

IPI	Protein Name	Gene Symbol	NDM	DM	Location	Type
	beta-2					
IP000113223	Fatty acid synthase	Fasn	10.1 ± 3.0	21.4 ± 3.5*	C	enzyme
IP000554933	Glutathione S-transferase theta-1	Gstt1	2.6 ± 0.4	5.4 ± 0.4***	C	enzyme
IP000121079	Isoform 1 of NADH-cytochrome b5 reductase 3	Cyb5r3	3.3 ± 1.0	6.8 ± 0.7*	C	enzyme
IP000227144	Retinol dehydrogenase 12	Rdh12	19.6 ± 4.6	40.9 ± 5.7*	C	enzyme
IP000653664	Isoform 1 of Enolase-phosphatase E1	Enoph1	3.9 ± 1.4	8.1 ± 1.0*	C	enzyme
IP000115663	Isoform 1 of Dynactin subunit 1	Dctn1	11.6 ± 2.2	24.0 ± 3.7*	C	other
IP000515140	Arsenite methyltransferase	As3mt	2.9 ± 0.6	6.1 ± 0.6**	C	enzyme
IP000224152	DNA-(apurinic or apyrimidinic site) lyase	Apex1	7.2 ± 2.0	14.7 ± 1.8*	N	enzyme
IP000112555	Glycyl-tRNA synthetase	Gars	7.5 ± 1.5	15.3 ± 2.6*	C	enzyme
IP000649886	Isoform Ia of Synapsin-1	Syn1	17.5 ± 2.3	35.4 ± 5.8*	M	transporter
IP000123281	Leucine-rich repeat-containing protein 59	Lrc59	3.2 ± 0.6	6.5 ± 0.6**	U	other
IP000165854	Ubiquitin-conjugating enzyme E2 N	Ube2n	40.4 ± 5.3	20.3 ± 4.1*	C	enzyme
IP000788505	Isoform 1 of Interphotoreceptor matrix proteoglycan 1 precursor	Impg1	28.8 ± 2.4	13.8 ± 4.6*	U	other
IP000108189	Histidine triad nucleotide-binding protein 1	Hint1	70.2 ± 11.2	32.3 ± 8.2*	N	enzyme
IP000415908	Protein phosphatase methyltransferase 1	Ppme1	6.3 ± 1.5	2.6 ± 0.4*	U	enzyme
IP000322749	Small nuclear ribonucleoprotein Sm D1	Snrpd1	42.0 ± 9.4	16.6 ± 4.4*	N	other
IP000121013	Isoform 1 of Astrocytic phosphoprotein PEA-15	Pea15a	38.8 ± 5.8	14.7 ± 3.1**	C	transporter
IP000230766	Parvalbumin alpha	Pvalb	30.1 ± 5.7	11.2 ± 4.9*	C	other
IP000463193	Novel protein similar to odorant binding protein Ia Obpl1a	EG546335	14.1 ± 3.1	3.7 ± 2.2*	U	other
IP000130922	Beta-crystallin S	Crygs	67.8 ± 18.3	16.6 ± 3.4*	U	other
IP000133948	FK506-binding protein 2 precursor	Fkbp2	10.9 ± 2.8	2.3 ± 0.7*	C	enzyme

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

$P < 0.001$ comparison between NDM and DM group

N: Nucleus; C: Cytoplasm; M: Membrane; U: Unknown.

Value expressed as mean $\pm$ S.E.M, $n = 5$ mice