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Pharmacologic intervention targeting glycolytic-related pathways protects against retinal injury due to ischemia and reperfusion

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Abstract

Retinal ischemia contributes to multiple ocular diseases while aminoguanidine (AMG) treatment significantly inhibits the neuronal and vascular degeneration due to acute retinal ischemia and reperfusion (I/R) injury. In the present study, two-dimensional differential in gel electrophoresis (2D DIGE) was applied to profile global protein expression changes due to retinal I/R injury, and the protection effects mediated by AMG. Retinal ischemia was induced by elevated intraocular pressure to 80–90 mmHg for 2 hours, and reperfusion was established afterward. Retinal tissues were collected 2 days after I/R injury. After 2D DIGE analysis, a total of 96 proteins were identified. Among them, 28 proteins were identified within gel spots whose intensities were normalized by AMG pre-treatment, pathway analysis indicated that most were involved in glycolysis and carbohydrate metabolism. Selected enzymes identified by MS/MS within these pathways, including transketolase, triosephosphate isomerase 1, aldolase C, total enolase, and pyruvate kinase were validated by quantitative Western blots. Glycolytic enzymes and other differentially regulated proteins likely play previously unrecognized roles in retinal degeneration after I/R injury, and inhibition of the resulting metabolic changes, using pharmacologically agents such as AMG, serve to inhibit the changes in metabolism and mitigate retinal degeneration. Select glycolytic enzymes may provide novel therapeutic targets for inhibiting the neuronal and vascular degeneration after retinal I/R injury.

Keywords

2D DIGE; Aminoguanidine; Glycolysis; Protein profiling; Retinal ischemia and reperfusion

1 Introduction

Retinal ischemia contributes to visual impairment and blindness in diseases such as acute closed-angle glaucoma, central retinal arterial occlusion, and diabetic retinopathy. Damage to tissues occurs not only during ischemia, but also during reperfusion, where a massive

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release of free radicals occurs [1–3]. Acute ischemia caused by elevation of intraocular pressure (IOP) followed by reperfusion (I/R) has been used to investigate molecular mechanisms and to identify potential therapeutic strategies for retinal ischemia [4]. Recently, we demonstrated vascular degeneration as well as neurodegeneration in this model [5].

Several mechanisms are likely to be involved in the pathogenesis of retinal degeneration in the I/R model, including alteration of AMPA-type glutamate receptor [6,7], up-regulation of chemokine expression [8], and elevation of iNOS [9]. Despite these individual studies, global protein expression changes and pathway analyses in the I/R model has not yet been investigated. Proteomic approaches are promising tools to elucidate changes in signaling and metabolic pathways, both to screen for novel targets relevant to drug discovery and to identify possible therapeutic interventions in retinal vascular and neuronal degeneration.

To gain insights into potential mechanisms that contribute to the retinal degeneration in the I/R model, a 2D DIGE minimal labeling approach was attempted to provide quantitative information on relative levels of protein expression between control and I/R-injured retinas. The proteomic data revealed an altered glycolysis pathway after retinal I/R injury. More interestingly, pretreatment with AMG, a drug known to have neuronal and vascular protection effects in the I/R model [5,9,10], was observed to normalize a number of differently expressed proteins in retina caused by I/R injury, including specific enzymes involved in glycolysis. Of these glycolytic enzymes identified by MS/MS, transketolase, triosephosphate isomerase 1, aldolase C, total enolase and pyruvate kinase were validated by quantitative Western blots. These findings create a foundation for further systems biology explorations of pathways underlying the pathophysiology of neuronal and vascular degeneration in retina, and also provide new potential therapeutic targets for inhibiting ocular diseases in which I/R is a contributing factor.

2 Materials and methods

2.1 Retinal ischemia-reperfusion model

Male Lewis rats weighting 250–300 g were anesthetized. Retinal ischemia was induced as reported previously [5]. Briefly, the anterior chamber of one eye was cannulated with a 30-gauge needle attached to an infusion line of normal saline, and pressure in the eye was regulated to 80–90 mmHg. The duration of ischemia was 2 hours. The other eye of the same rat was set up as control. After ischemia, the needle was withdrawn, and reflow of the retinal circulation documented visually. Some rats were treated with aminoguanidine (AMG, 50 mg/Kg BW in drinking water) from 2 days before the procedure until they were killed (water was measured and changed every day). Rats were sacrificed at 2 days after I/R injury. At least eight animals were included in each experimental group (4 for the 2D DIGE study, and 4–6 for Western blots). All procedures involving the rats are conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2 Sample preparation

Retinas were freshly isolated and sonicated in ice-cold lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, and a cocktail of protease inhibitors (containing leupeptin, pepstatin, PMSF, aprotinin and EDTA- Na_2 ; GE Healthcare)) for 2D DIGE, or sonicated in ice-cold RIPA buffer (Santa Cruz biotechnology, Santa Cruz, CA) for western blots. The supernatants were collected by centrifugation at 12,000g for 15min at 4°C. The total protein concentrations of the samples were determined using an Ettan 2D Quant kit (GE Healthcare) for 2D DIGE or determined using Bio-Rad Protein Assay (Bio-Rad) for western blots.

2.3 Cy-dye labeling and 2D DIGE

The samples were labeled using CyDye labeling following the Ettan DIGE User Manual (GE Healthcare). Each of the three experimental groups (control, I/R injured with or without AMG treatment) had 4 replicates (each replicate was extracted from a different animal). Each gel was loaded with 3 samples (2 experimental and 1 internal standard), each labeled with a different dye. Cy3 or Cy5 were used to label experimental samples (50 µg per sample). Cy2 was used to label an internal standard that had equal amounts of retinal extracts from each sample (total of 12) pooled together. Labeling reactions were performed on ice and in the dark for 30 min before being quenched with 10 mM lysine on ice. The combined samples were then diluted with 2x rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG buffer, 20 mM DTT). The 24 cm 3–10 nonlinear IPG strips were subjected to electrophoresis using an Ettan IPGphor II system (GE Healthcare). Focusing was then carried out using the following conditions: (I) 30 V, 12 h, step; (II) 300V, 3h, step; (III) 300–600V, 1350 Vh, gradient; (IV) 600–1000V, 2400 Vh, gradient; (V) 1000–8000V, 13500 Vh, gradient; (6) 8000 V, 56000 Vh. Prior to the second dimension, the IPG strips were equilibrated twice with solutions containing 100 mM Tris-HCl pH 8.0, 6 M urea, 30% glycerol, 2% SDS, and traces of bromophenol blue. DTT (1%) and IAM (4.5%) were added in the solutions of the first and second equilibration steps, respectively. IPG strips were then placed on top of 12.5% polyacrylamide gels that were precast with low-fluorescence glass plates using an Ettan-DALT twelve caster (GE Healthcare). The second-dimensional SDS-PAGE was carried out under the following conditions: 0.2 W/gel for 1 h; 0.4 W/gel for 1 h; 1.8 W/gel for 15 h at room temperature.

2.4 Images and Data analysis

The Cy2 (internal standard), Cy3 and Cy5 components of each gel were individually imaged using excitation/emission wavelengths of 488/520 nm, 532/580 nm and 633/670 nm using a Typhoon 9400 (GE Healthcare), respectively. Gel image analysis was initially processed using DeCyder 6.5 (GE Healthcare). Spot detection was carried out automatically using differential in-gel analysis software module. For gel-to-gel matching and calculation of average abundance and statistics, the biological variation analysis (BVA) software module was used. The internal standard (Cy2-labeled) spot maps were used to match different gels, and the standard: sample volume ratio values were compared enabling the calculation of abundance ratio between samples from different gels, followed by the application of statistical tests [11,12]. Gel spots in which I/R caused a significant intensity change ($p < 0.05$) from normal (by one-way ANOVA) were highlighted as interested spots. Since nonparametric test is more appropriate for comparing multiple experimental groups with small sample numbers, thus Kruskal-Wallis test followed by Mann-Whitney test were used to identify those gel spots in which AMG pre-treatment significantly normalized I/R-induced changes in spot intensities. Some of the left interested gel spots in which have sufficient intensity such that MS identification was likely and can be resolved from adjacent spots were also collected for MS/MS identification. Overall, the experimental design and 2D DIGE approach used here has a power of 0.8 to detect fold changes of plus or minus 30% for 75% at a significance of 0.05 or better for the entire spot distribution [13]. The variance for each spot is individually evaluated, and spots selected for sequencing are based on significance as opposed to fold-change. Thus, some spots were selected for sequencing even though their fold changes were small, as the goal was to identify proteins that might act in concert in important metabolic or signaling pathways [13–15].

2.5 In-gel digestion and protein identification

After image acquisition, one of the gels was subsequently subjected to Deep Purple staining (GE Healthcare) and used for spot picking. The protein pick lists generated from DeCyder were used to robotically excise gel spots using a 96-well plate format using Ettan Spot

Picker (GE Healthcare). After spot picking, the Deep Purple stained image was re-acquired using 488/610 nm wavelengths to confirm the picking sites.

The gel plugs in 96-well plates were equilibrated with 25 mM ammonium bicarbonate for 10 min and then dehydrated for 10-min by incubating with 100% acetonitrile (repeated 2 times). Dehydrated gel plugs were incubated in 10 mM DTT in 25 mM ammonium bicarbonate at 56 °C for 45 min, after cooling to room temperature, incubated in 55 mM IAM in fresh 25 mM ammonium bicarbonate. The dehydrated gel plugs were digested in-gel with porcine modified trypsin (Promega) in 25 mM ammonium bicarbonate for 16 h at 37 °C. Tryptic peptides were then extracted from the gel plugs in two cycles of 60% ACN, 1% formic acid (FA). The combined extract solution for each spot was concentrated to a final volume of 10 µL using a speed-vac.

A high-throughput instrument configuration, the combination of nanoflow multidimensional chromatography (Ettan MDLC, GE Healthcare) and LTQ ion trap mass spectrometer (Thermo), was created and performed for the separation of tryptic peptides and MS/MS analyses. Two Zorbax 300-SB C18 trap columns (5 × 0.3 mm, Agilent) and two nanoscale C18 analytical columns (150 × 0.075mm, Dionex) were set up for the high-through MDLC [16,17]. Mobile phase A consisted of HPLC-grade water with 0.1% FA whereas mobile phase B was 84% HPLC grade ACN with 0.1% FA. 2 µL of 10 µL material was loaded onto the columns and separation was performed at a flow rate of 260 nL/min by applying a linear gradient of 0–60% B in 50 min. Peptide analysis was performed using data dependent acquisition of one MS scan (400 to 2000 *m/z*) followed by MS/MS scans of the five most abundant ions in each MS scan. Some samples were analyzed by using an LCQ Deca XP ion trap mass spectrometer (Thermo, USA) combined to the automated nanoflow reverse-phase LC system (Dionex, USA). Mobile phase A consisted of 5% HPLC grade ACN with 0.1% FA whereas mobile phase B was 95% HPLC grade ACN with 0.1% FA. Digested peptides were separated by an ACN gradient (0–80% B in 40 min) through a reverse phase C18 column at a flow rate of 200 nL/min. Peptide analysis was performed using data dependent acquisition of one MS scan (400 to 2000 *m/z*) followed by MS/MS scans of the three most abundant ions in each MS scan [18].

MS/MS spectra were searched against the *Rattus Norvegicus* database originated from the NCBI database (updated on 07/05/06) and analyzed using TurboSEQUEST Bioworks. In cases where no hits were identified in the *Rattus Norvegicus* database, a *Mus Musculus* database was used. The criteria of peptide cross-correlation scores (Xcorr) of 2.0, 2.5, and 3.75 were set for charge states 1, 2, and 3, respectively. Only proteins with 2 or more peptides meeting above criteria of Xcorr were reported.

2.6 Bioinformatics

Functional genome ontology annotation mapping was performed using PANTHER (Protein analysis through evolutionary relationships) classification system [19,20] (www.pantherdb.org), which classifies the identified proteins categorized by canonical pathways, biological process, and molecular function.

2.7 Western blot analysis

Proteins were fractionated by SDS-PAGE, and probed with antibodies for transketolase (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), aldolase C (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), triosephosphate isomerase 1 (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), enolase (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and pyruvate kinase (1:3000 dilution; Chemicon, Temecula, CA). After extensive washing, protein bands detected by the antibodies were

visualized by enhanced chemiluminescence (ECL, Santa Cruz Biotechnology, Santa Cruz, CA) and evaluated by densitometry (Bio-Rad, Hercules, CA). To confirm equal loading of proteins, transferred nitrocellulose membranes were stained with washable Ponceau S solution (Sigma, St. Louis, MO) and the intrinsic protein β -actin (1:10000 dilution, Sigma, St. Louis, MO) were performed by Western blots. The densitometry results of western blots were expressed as the mean $\pm$ SD. Expression is reported relative to β -actin in the same sample, and normalized to control retinas. The data were analyzed by the nonparametric Kruskal-Wallis test followed by Mann-Whitney test. Differences were considered statistically significant when the p values were less than 0.05.

3 Results

3.1 2-DE of retinal extracts

From the total of 12 samples (4 from each of 3 groups), six gels were run, each gel having an internal standard (pool of all samples) and 2 different experimental samples. A dye swapping strategy was used to avoid dye-labeling bias. The paired control and I/R injured retinas were run on the same gel. Using this technique, well-resolved and reproducible spots were obtained for each gel, and ~2300 spots per gel were detected within the TIFF images using the Decyder software platform; >1600 spots were matched in all 6 gels.

3.2 Protein identification by ESI-MS/MS analysis

3.2.1 MS analysis of I/R injury-induced differently expressed proteins that can be normalized by AMG pre-treatment—The Decyder image analysis platform was used to provide statistical analysis using its Differential Image Analysis (DIA) and Biological Variation Analysis software modules. These analyses revealed 165 spots that were significantly altered after retinal I/R injury (see Materials and Methods). AMG treatment previously has shown significant inhibition of degenerative morphological changes in neuronal structures of retina as well as inhibition of vascular cell death after I/R injury [5], suggesting AMG may block or potentiate the expression of certain proteins that play critical roles in neuronal and vascular degeneration subsequent to retinal I/R injury. For eighteen of the above 165 spots, the altered expression caused by I/R was reversed by AMG based on Deep Purple post-staining (Figure 1). MS/MS analysis successfully identified 28 proteins in 17 of the 18 spots (listed in Table 1). In over half of the spots (9 out of 17), only single protein hits were identified, thus the significant fold changes due to I/R, and their reversal by AMG, were uniquely associated with the functions of these proteins. These included transketolase, glucose phosphate isomerase, actin-like 6A, septin 7, NADH dehydrogenase Fe-S 2, aldolase C, phosphoglycerate kinase, and triosephosphate isomerase 1 (in two separate spots). For the other 8 spots with multiple protein hits, the proteins specifically associated with the significant fold changes cannot be identified, but these are shown for completeness in Table 1. Among the 17 spots where proteins were identified, 11 spots (16 proteins) were down-regulated by greater than 1.2 fold, and 6 spots (12 proteins) were up-regulated by greater than 1.2 fold. Examples of relative spot densities of each sample for selected spots of interest are shown in Figure 2.

3.2.2 Identification of additional proteins that were differentially expressed after I/R injury—AMG treatment significantly, but not completely, inhibits neuronal and vascular degeneration after I/R [5,9,10]. Thus, it is likely that other proteins not inhibited by AMG might also contribute to the retinal degeneration in retinal I/R injury. We thus extended our study to consider other spots showing significant expression changes after retinal I/R injury. One hundred forty-seven spots showed significant changes between control retina and I/R injured retina but were not significantly reversed by AMG. Two criteria were applied to these spots (see Materials and Methods). Ninety-three gel spots that

fit both criteria were excised from the gel and subjected to MS/MS analysis. The selected gel spots are indicated in Figure 3, and protein identification results are summarized in Table 2 (Two proteins that identified based on a single peptide in two spots are not included in Table 2). In most cases, a single protein was identified from each spot; these included 51 proteins specifically associated with retinal I/R injury whose expression was not significantly reversed by AMG. Many of these proteins exhibited isoforms having significant fold changes that were internally consistent. In some cases multiple proteins were identified within a spot, in these cases the proteins specifically associated with the significant fold changes cannot be identified, but these are shown for completeness (Table 2).

3.3 Biological roles of identified proteins

All altered proteins identified in the present study were categorized using the PANTHER classification system by their representation in: (i) canonical pathways, (ii) biological processes, and (iii) molecular functions (Figure 4). We included all the proteins identified by mass spectrometry, including cases where multiple proteins were identified with a spot, to provide the greatest possible coverage for the bioinformatics analysis. Of the 96 total proteins input into the PANTHER, there were 64 hits in the pathway group, 147 hits in the biological processes group, and 118 hits in the molecular function group.

The pathway altered most after retinal I/R was glycolysis (8.3%; based on total number of identified proteins (96); Figure 4A). This enrichment of glycolysis appears not to be due to random chance, since in a random selection of a different 96 proteins, only 1 hit for glycolysis is shown. To minimize bias in the choice of the decoy, we randomly selected from cDNA sequences in the rat genome that had values of the codon adaptation index in the top 25% of expected gene expression, reflective of and consistent with the relative abundance of expression for the classes of proteins represented in a 2-D gel study [21, 22]. The frequencies of pathway “hits” for glycolytic enzymes after retinal I/R injury was significant by a Fisher Exact test, compared to the randomly selected group ($p < 0.0001$). The biological processes most changed after retinal I/R were related to protein metabolism and modification (18.8%), carbohydrate metabolism (16.7%), transport (14.6%), and signal transduction (13.5%; Figure 4B). Oxidoreductase was one of the main protein groups involved in molecular function that was altered after retinal I/R (10.4%; Figure 4C), and changes included several subunits from the electron transport chain (such as ubiquinol-cytochrome C reductase core protein I and II, NADH dehydrogenase (ubiquinone) flavoprotein 1, NADH dehydrogenase (ubiquinone) Fe-S protein 2 and 3, NADH dehydrogenase (ubiquinone) 1 beta subcomplex) and enzymes that involved in TCA cycle (malate dehydrogenase 1, and lactate dehydrogenase B). Other enzymes involved in molecular function that were altered after retinal I/R included enzymes from the regulatory molecule (11.5%), cytoskeletal protein (9.4%) and transferase (9.4%) groups.

3.4 Western blotting validation

1-D Western blotting of several identified proteins was performed to validate the protein quantitation and protein identification results of 2-DE and MS/MS, particularly for protein expression changes found to be reversed by AMG. Western blot densitometric quantitation results, as well as representative expression levels of several glycolytic enzymes, including transketolase, triosephosphate isomerase 1, aldolase C, total enolase (including α , β and γ) and pyruvate kinase are shown in Figure 5. For transketolase, triosephosphate isomerase 1, pyruvate kinase, aldolase C and total enolase, I/R injury caused -2.9, -2.8, -1.8, -2.0, and -1.9 fold change in expression levels, respectively (all $P < 0.05$). Treatment with AMG followed by retinal I/R injury increased expression of these proteins by 3.1, 1.6, 1.7, 1.6 and 1.2 fold, respectively ($P < 0.05$, except total enolase). The comparison between Western

blots results and 2-DE results are listed at Table 3 (where the same protein is identified from multiple spots from 2-DE, all the fold changes were listed). The directions of expression change were identical for the 2-DE and Western blot data while the magnitudes of the fold changes were similar.

4 Discussion

Experimentally-induced retinal I/R has been proven to be a useful model to investigate neurodegeneration and the relationship between neuronal and vascular damage in retina. In this model, neurons in the retinal ganglion cell layer are rapidly damaged following reperfusion, and vascular degeneration occurs subsequently [5]. Two days after retinal I/R injury was judged to be an appropriate time interval for the present study, since neuron degeneration already is established, vascular degeneration is just beginning, and there still is a significant population of cells left in the retina. From a clinical perspective, identifying proteins and pathways that are altered soon after I/R injury likely will have more beneficial effects for therapeutic intervention than identifying those which change at a later stage, presumably after damage has become irreversible.

To have a comprehensive understanding of global protein changes that occur after retinal I/R injury, high-resolution 2-D gel and MS/MS analysis were performed. Beyond identifying protein expression changes associated with I/R injury, the present study also examined the effects of pharmacological reversal or protection from injury mediated by AMG, a drug known to mitigate the histopathology of retinal I/R [5,9,10]. Our analyses revealed an altered protein expression profile in which differential expressions of nearly 60 proteins were uniquely associated with retinal I/R injury. Among them, only a subset was specifically normalized by the AMG treatment, a known drug that can inhibit the retinal degeneration, and many of these latter targets were validated. Thus, many of the protein changes detected after the retinal injury apparently seem unrelated to developing the histopathology. Critical changes in protein expression were detected by including a therapy that inhibits the histopathology.

The predominant pathway altered after retinal I/R was found to be glycolysis (Figure 4A). Glucose is known to be an important energy source for the retina [23, 24]. Winkler et al demonstrated that the isolated rat retina can maintain most of its ATP requirement in the absence of oxygen, as long as glucose is abundant, supporting the idea that the anaerobic glycolysis may represent an important energy source for the ischemic retina [24]. Glycolysis (Figure 6) involves 2 separate phases: the first phase requiring energy to convert glucose to fructose 1,6-biophosphate (FDP), and the second generating energy phase via degradation of FDP to pyruvate by aldolase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase and pyruvate kinase. The present study demonstrated that many enzymes involved in the second phase of glycolysis were downregulated after retinal I/R (Table 1 and 2). Total enolase (including α , β and γ) tended to be decreased after retinal I/R (Figure 5), even though MS/MS analysis demonstrated that α -enolase was increased and γ -enolase was decreased after retinal I/R. Transketolase, an enzyme that regulates crosstalk between glycolysis and the pentose phosphate pathway (Figure 6), also was significantly down regulated after retinal I/R (Table 1 and Figure 5). Preservation of the pentose phosphate pathway also is critical during ischemia, because of the NADPH generated by this pathway. Thus, alterations in expression of glycolytic and glycolytic related enzymes may play critical roles in retinal dysmetabolism and degeneration after I/R injury.

Expression of a number of these enzymes, such as aldolase C, triosephosphate isomerase, total enolase, and transketolase were completely or partially normalized by AMG treatment

(Table 1 and Figure 5). Phosphohexose isomerase, an enzyme involved in the energy consumption step of glycolysis, was increased after retinal I/R injury, but its expression likewise was inhibited by AMG treatment. Up regulation of an enzyme involved in ATP consumption and down regulation of other enzymes involved in ATP generation in glycolysis are consistent with energy deficits and associated NADH and/or ATP depletion after retinal I/R injury. Consistent with our finding of impaired glycolysis after I/R, in a recently performed microarray study on rats 12 hours after retinal I/R injury by Yoshimura et al, a 4.4-fold downregulation in mRNA for the enzyme that involved in the first step of glycolysis pathway (hexokinase) was detected [25].

Recently, the relationship between glycolysis and neurodegeneration has been explored. Moderate inhibition of glycolysis at glyceraldehyde-3-phosphate dehydrogenase induced death of cultured hippocampal neurons by increasing calcium influx through NMDA receptors and calcium release from intercellular ER stores [26]. Conversely, enhanced glycolysis also has been demonstrated to regulate cellular life span by reducing oxidative stress [27]. Injection of pyruvate, the end-product of glycolysis, inhibited neuronal degeneration even 12 hours after retinal I/R injury [28]. In addition, providing additional substrates for glycolysis (via chronic [diabetes] or acute increase in glucose [systemically or directly in the eye]) all markedly reduced the functional and structural damages after retinal I/R injury, while 2-deoxyglucose, a glycolytic inhibitor, exacerbated the damage [29]. Preservation of glycolysis protects from I/R induced injury also in other tissues, as illustrated by the neuroprotective and cardioprotective effects of FDP injections in brain [30] and myocardium [31] after I/R injury.

Alterations of retinal function due to blockage of glycolysis after I/R are further exacerbated by changes in expression of enzymes of other energy generation pathways such as TCA cycle (Figure 6). Down regulation of isocitrate dehydrogenase and malate dehydrogenase after retinal I/R injury were also identified by MS/MS. Dysfunction of electron transport chain, which also involved in ATP generation, likewise was identified by MS/MS after retinal I/R injury, especially altered expression of some subunits of complex I, III and V. Consistent with this, Nucci et al demonstrated that intraocular administration of coenzyme Q10 (CoQ10), a cofactor of the electron transport chain, had neuroprotection effects after retinal I/R [32]. Previous studies demonstrated that I/R-induced impairment of electron transport chain complex I is due in part to poly(ADP-ribose) polymerase (PARP) activation [33]. PARP activation is a known sequelae of retinal I/R injury [5,34], and inhibition of PARP after the I/R injury prevented retinal morphological damage in rats [34]. Interestingly, repletion of intracellular NAD⁺ after PARP activation in cultured mouse astrocytes completely restored glycolytic capacity and prevented cell death, suggesting that NAD⁺ depletion might be a cause of glycolytic failure after PARP hyperactivation [35].

I/R of the retina caused also a striking over expression of calgranulin B (S100a9; 7.6 fold; Table 2). Calgranulin B is a calcium-binding protein, and in conjunction with calgranulin A (S100a8), is a prominent player in innate immunity [36]. The contribution of the innate immunity to ischemia and reperfusion injury is increasingly recognized and investigated [37–39]. The transcription factor, NF-kappa B, is a critical regulator involved in innate immunity and inflammation, and has been demonstrated to play role in retinal degeneration after I/R injury [40,41]. We did not detect alterations in transcription factors in the present study, but such alterations are not easily detectable by such proteomics approaches [42].

Neuronal cell death mediated partially by apoptosis has been proposed as one of causal mechanisms of neurodegeneration in retinal I/R injury. Increased numbers of TUNEL positive cells and cleavage forms or activities of capase-1 and 3 have been demonstrated in retinal I/R injury [5,43–45]. Surprisingly, these classic apoptotic proteins were not identified

in the present proteomics study. Moreover, several other proteins previously identified as important in retinal I/R (including iNOS and COX-2 [5,9,10]) also were not identified by the strategy used in the present study. This apparent lack of sensitivity of our proteomic approach likely is due to a variety of factors involving poor detection of low abundance proteins, and poor resolution of membrane-bound or high molecular weight proteins, basic proteins, or post-translational modifications.

Retinal I/R results in a degenerative process that probably reflects complex relationships between several pathophysiological pathways. Accordingly, using techniques such as proteomics that do not depend on prior expectations are useful. Impaired glycolytic and mitochondrial energetics systems are important changes caused by the retinal I/R, and these changes in expression of critical proteins likely contributed to the neuronal and vascular degeneration in the retina. Further mining of the database generated in these studies is expected to provide additional insight into other metabolic pathways impacted by I/R.

Abbreviations

2-DE	2-dimensional electrophoresis
ACN	acetonitrile
AMG	aminoguanidine
ANOVA	analysis of variance
BVA	biological variation analysis
DIA	differential in-gel analysis
DIGE	differential in gel electrophoresis
ECL	enhanced chemiluminescence
FA	formic acid
FDP	fructose 1,6-diphosphate
IAM	iodoacetamide
IOP	intraocular pressure
I/R	ischemia and reperfusion
MDLC	multi-dimensional liquid chromatography
PANTHER	protein analysis through evolutionary relationships
PARP	poly(ADP-ribose) polymerase

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Figure 1.

2D gel migration patterns of proteins that normalized by AMG treatment after retinal I/R injury. Cy dye image from samples run on pH 3–10 nonlinear gradient IPG strips and 12% polyacrylamide gel is shown. Numbers in the figure are master spot number that used to match every gel, and spot areas are highlighted. Refer to table 1 for fold changes in expression that calculated using DeCyder software.

Figure 2. Identified, differentially expressed proteins that normalized by AMG treatment after retinal I/R injury were shown by DeCyder output. (A) AMG treatment bring back the protein levels that were decreased after retinal I/R (B) AMG treatment inhibit retinal I/R-induced some proteins' overexpression. Values on the y-axis are the relative arbitrary units. Circles indicate the value of individual samples. The crosses indicate the average values of each group. (C: control retinas; I/R: ischemia/reperfusion injured retinas; I/R+AMG: AMG pre-treated ischemia/reperfusion injured retinas)

Figure 3.

2D gel migration patterns of additional proteins that altered after retinal I/R injury. Cy dye image from samples run on pH 3–10 nonlinear gradient IPG strips and 12% polyacrylamide gel is shown. Numbers in the figure are master spot number, and spot areas are highlighted. Refer to table 2 for fold changes in expression that calculated using DeCyder software.

Figure 4. PANTHER classification by pathway (A), biological process (B) and molecular function (C). (Heterotrimeric G-protein signaling pathway-1* is G_i alpha and G_s alpha mediated; Heterotrimeric G-protein signaling pathway-2* is G_q alpha and G_o alpha mediated; Heterotrimeric G-protein signaling pathway-3* involves in rod outer segment phototransduction)

Figure 5.

Western blots of several glycolytic enzymes with densitometric quantitation results on top and representative protein bands at the bottom. Expression is reported relative to β -actin in same sample, and normalized to control retinas, set as 1 fold. (C: control retinas, I/R: ischemia/reperfusion injured retinas; I/R+AMG: AMG pre-treated ischemia/reperfusion injured retinas; * $p < 0.05$ compared to control retinas; ** $p < 0.05$ compared to I/R injured retinas)

Figure 6.

Alteration of glycolysis and pathways that related to glycolysis after retinal I/R injury. Bold; decreased expression after retinal I/R injury. Italics; increased expression after retinal I/R injury. (LDH, lactate dehydrogenase; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase)

Table 1

Protein levels that normalized in the AMG treated I/R-injured retinas.

Master spot no.	Protein name	Accession no. (GI#)	No. of matched peptide	Sequence coverage (%)	I/R vs. C (fold change)	AMG vs. I/R (fold change)
a. Spot intensity increased in I/R-injured group and normalized by AMG pre-treatment						
541	Fibrinogen beta chain precursor	1706801	13	34.4	2.6	-1.4
	Adenylate cyclase-associated protein 1	59709467	5	16.1	2.6	-1.4
	D-3-phosphoglycerate dehydrogenase	1944614	7	13.5	2.6	-1.4
	leucine aminopeptidase 3 (predicted)	62660568	18	39.6	2.6	-1.4
517	Glucose phosphate isomerase	38303865	15	20.2	1.9	-1.4
937 ^a	Glutamine synthetase 1	76496512	12	31.1	1.8	-1.2
	Laminin receptor	38014840	17	56.3	1.8	-1.2
733	Actin-like 6A	68533651	10	29.1	1.3	-1.2
579	Protein kinase C and casein kinase substrate in neurons 1	22256946	7	20.1	1.2	-1.1
	Tubulin, alpha	38328248	24	62.3	1.2	-1.1
1061	Phosphoserine aminotransferase	61889115	6	21.6	1.2	-1.4
	Annexin A1 (Lipocortin I)	113947	9	34.1	1.2	-1.4
b. Spot intensity decreased in I/R-injured group and normalized by AMG pre-treatment						
997	Aldolase C	71122313	17	52.3	-1.7	1.2
889 ^a	Phosphoglycerate kinase	38649310	11	30.7	-1.6	1.3
1546	Triosephosphate isomerase 1	12621074	21	70.4	-1.5	1.3
1585	Ubiquitin carboxy-terminal hydrolase L1 [*]	25058057	4	33.6	-1.5	1.3
	Glyoxylase	38181954	4	29.4	-1.5	1.3
	RAB3A, member RAS oncogene family [*]	31566349	4	19.6	-1.5	1.3
746	Septin 7	12018296	16	28.7	-1.4	1.2

Master spot no.	Protein name	Accession no. (Gi#)	No. of matched peptide	Sequence coverage (%)	I/R vs. C (fold change)	AMG vs. I/R (fold change)
1538	Triosephosphate isomerase 1	12621074	16	62.9	-1.4	1.2
296 ^a	Transketolase	12018252	17	31.7	-1.3	1.3
1083	GTP-binding protein Gt alpha-1 chain	62654264	13	32.0	-1.3	1.3
	GTP-binding protein alpha o	8394152	6	18.9	-1.3	1.3
	Heterogeneous nuclear ribonucleoprotein C	66910561	13	32.2	-1.3	1.3
1349	Guanine nucleotide binding protein, beta ploypeptide 2- like 1	18543331	5	20.5	-1.3	1.2
	Voltage-dependent anion channe 2	13786202	4	12.5	-1.3	1.2
756	NADH dehydrogenase (ubiquinone) Fe-S protein 2	51980672	19	45.3	-1.2	1.1
1119	ATPase, H+ transporting, V0 subunit D isoform	62665162	8	16.0	-1.2	1.2
	SEC13-like	54261633	3	15.8	-1.2	1.2

All the data listed in here were gel spots that have significant intensity changes by I/R injury ($p < 0.05$) and those intensity changes, except very few indicated spots, can be significantly normalized by AMG pre-treatment ($p < 0.05$).

^a AMG vs. I/R $p = 0.06$

* blast result from *Mus Musculus* genome

I/R: ischemia/reperfusion injured retinas

C: control retinas

AMG: aminoguanidine pre-treated I/R-injured retinas

Table 2

Additional proteins that altered by retinal I/R injury

Master spot no	Protein name	Accession no. (G#)	No. of matched peptide	Sequence coverage (%)	I/R vs. C (fold change)	p value
a. Spot intensity increased in I/R-injured group						
2037	Hemoglobin alpha, adult chain 1	37748075	5	50.9	9.7	5.3E-04
2051	Beta-globin	984679	7	42.9	8.4	1.5E-03
2043	Beta-globin	984679	8	51.9	8.1	3.9E-04
2082	S100 calcium binding protein A9	16758364	2	24.6	7.6	8.7E-06
2035	Beta-globin	984679	4	28.6	7.5	2.0E-03
860	Preprocathepsin D	55882	5	15.7	3.6	0.010
	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin)	38014574	3	10.2	3.6	
2077	Similar to germinal histone H4 gene	109504921	6	33.7	2.8	3.5E-05
1494	Galectin-3	57870645	5	31.5	2.6	1.8E-07
840	Ckb protein	71121736	7	24.2	2.5	3.9E-03
489	Heterogeneous nuclear ribonucleoprotein K isoform b	14165435	2	7.2	2.4	1.7E-04
1636	Tumor protein, translationally-controlled 1	16758734	2	8.4	2.4	2.1E-04
507	Vimentin	14389299	14	35.8	2.1	4.3E-04
	Tubulin, alpha	38328248	16	43.8	2.1	
697	Enolase1	38649320	2	5.8	2.1	8.1E-03
699	Enolase1 (predicted)	62648074	5	24.1	2.0	4.0E-03
838	Ckb protein	71121736	7	20.9	2.0	9.6E-03
1332	Annexin V	4521315	8	30.7	2.0	1.5E-04
2052	Similar to germinal histone H4 gene	109504921	2	16.4	2.0	3.2E-03
137	Aconitase 2, mitochondrial	38541404	6	10.4	1.9	0.048
	MAP2 RNA trans-acting protein MARTA1	12584134	8	16.8	1.9	
	Serotransferrin precursor (Transferrin)	6175089	11	19.4	1.9	

Master spot no	Protein name	Accession no. (Gi#)	No. of matched peptide	Sequence coverage (%)	I/R vs. C (fold change)	p value
447	Protein disulfide-isomerase A3 precursor	1352384	17	32.4	1.9	5.7E-04
845	Gamma-actin	109489498	11	28.0	1.9	1.5E-03
2056	Beta-globin	984679	4	28.5	1.9	0.017
221	Tumor necrosis factor type 1 receptor associated protein	84781723	12	19.7	1.8	8.6E-03
710	NADH dehydrogenase (ubiquinone) flavoprotein 1	55741424	2	9.8	1.8	1.8E-03
711	Enolase 1 (predicted)	62643850	3	12.2	1.8	2.4E-03
713	Enolase 1 (predicted)	62643850	3	12.2	1.8	7.1E-04
1104	Transaldolase 1	37747886	2	6.7	1.8	0.016
705	Enolase 1	38649320	3	10.0	1.7	3.9E-03
843	Gamma-actin	109489498	10	25.6	1.7	2.5E-03
1365	Microtubule-associated protein, RPEB family, member 1	51859267	3	16.8	1.7	1.6E-04
1423	Chloride intracellular channel 1	50657380	7	36.8	1.7	
	Similar to phospholysine phosphohistidine inorganic pyrophosphate phosphatase	57528359	4	17.6	1.7	2.0E-04
219	Tumor necrosis factor type 1 receptor associated protein	84781723	16	25.0	1.5	0.0159
586	ATPase, H ⁺ transporting, V1 subunit B, isoform 1 *	19527064	8	15.2	1.5	2.0E-06
725	Proteasome 26S subunit ATPase 4 *	15215225	5	18.4	1.5	2.1E-05
	Proteasome 26S subunit ATPase 3	2492523	19	44.9	1.5	
1475	Proteasome subunit, alpha type 4	8394069	2	13.8	1.5	4.6E-03
2045	Beta-globin	984679	4	35.2	1.5	5.0E-04
540	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, alpha 1 *	6980956	17	59.7	1.4	2.0E-03
	Glutamate dehydrogenase 1	6980956	13	30.3	1.4	
498	Prolyl 4-hydroxylase, beta polypeptide	38197382	21	49.4	1.3	8.3E-04

Master spot no	Protein name	Accession no. (Gi#)	No. of matched peptide	Sequence coverage (%)	I/R vs. C (fold change)	p value
450	Protein disulfide-isomerase A3 precursor	1352384	19	37.1	1.2	3.2E-03
583	Leucine aminopeptidase 3	58865398	20	43.1	1.2	4.2E-05
1640	Tumor protein, translationally-controlled 1	16758734	2	8.4	1.2	2.2E-05
a. Spot intensity decreased in I/R-injured group						
1511	Carbonic anhydrase 2	9506445	4	23.1	-2.7	0.011
	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation, zeta	62990183	3	17.6	-2.7	
175	N-ethylmaleimide sensitive fusion protein	13489067	13	19.2	-2.3	1.5E-07
414	Guanosine diphosphate dissociation inhibitor 1	71534276	5	11.7	-2.0	1.4E-03
769	Enolase 2, gamma	37805239	18	54.8	-2.0	7.5E-03
992	Aldolase C	71122313	8	31.4	-2.0	1.1E-05
1575	Ubiquitin carboxy-terminal hydrolase L1 *	25058057	5	39.3	-2.0	4.1E-05
977	Aldolase A	6978487	11	36.3	-1.8	6.1E-05
1493	Calretinin	55853	5	16.8	-1.8	0.012
1553	Similar to biliverdin reductase B	109461493	2	20.8	-1.8	0.012
1669	Glutathione S-transferase, pi 2	34849843	2	17.0	-1.8	0.017
299	Transketolase	12018252	14	28.8	-1.7	2.6E-03
524	UDP-glucose pyrophosphorylase 2	66910575	2	5.2	-1.7	7.3E-07
935	Glutamate oxaloacetate transaminase, cytosolic	91997	14	38.3	-1.7	4.9E-04
	Similar to proteasome 26S ATPase subunit 6	109502281	6	22.3	-1.7	
969	Poly(rC) binding protein 3 * (predicted)	10947014	3	16.1	-1.7	1.2E-03
1069	GTP-binding protein alpha o	8394152	5	17.7	-1.7	8.9E-05
1193	Malate dehydrogenase 1, NAD (soluble)	15100179	6	24.3	-1.7	8.3E-03

Master spot no	Protein name	Accession no. (Gi#)	No. of matched peptide	Sequence coverage (%)	I/R vs. C (fold change)	p value
1557	Triosephosphate isomerase 1	12621074	11	53.7	-1.7	9.6E-05
475	Pyruvate kinase 3 isoform 3	109518934	17	37.5	-1.6	9.7E-03
521	Vacuolar H ⁺ ATPase B2	17105370	15	28.9	-1.6	6.5E-05
1492	Phosphoglycerate mutase type B subunit	8248819	9	43.5	-1.6	2.3E-03
1545	Triosephosphate isomerase 1	12621074	9	53.4	-1.6	9.6E-06
370	rCRMP-3	1399540	3	9.4	-1.5	3.3E-03
	Alpha isoform of regulatory subunit A, protein phosphatase 2 *	8394027	15	28.4	-1.5	
460	Pyruvate kinase 3 isoform 3	109518934	11	28.1	-1.5	5.9E-03
890	Phosphoglycerate kinase	38649310	6	21.8	-1.5	3.7E-03
	Glutamine synthetase 1	76496512	4	11.9	-1.5	
959	Aldolase A	6978487	10	36	-1.5	1.6E-04
964	Aldolase A	6978487	13	45.1	-1.5	3.9E-04
986	Aldolase C	71122313	9	34.9	-1.5	
	Poly(rC) binding protein 1 * (predicted)	6754994	5	24.3	-1.5	2.6E-04
995	Aldolase C	71122313	7	21.1	-1.5	2.2E-04
1127	Lactate dehydrogenase B	37590241	10	32.1	-1.5	8.6E-04
1184	Pyridoxal (pyridoxine, vitamin B6) kinase	13929082	9	32.7	-1.5	9.6E-04
1276	Phosphatidylinositol transfer protein	47477900	4	18.3	-1.5	1.9E-03
1287	N-ethylmaleimide sensitive fusion protein attachment protein beta *	29789104	8	20.7	-1.5	2.0E-03
1537	Triosephosphate isomerase 1	12621074	7	40.2	-1.5	2.5E-04
1696	Phosphatidylethanolamine binding protein	38649317	3	28.9	-1.5	2.3E-03
1312	N-ethylmaleimide sensitive fusion protein attachment protein alpha	38648848	9	34.5	-1.5	3.3E-05
1488	Phosphoglycerate mutase type B subunit	8248819	5	30.4	-1.4	0.023

Master spot no	Protein name	Accession no. (Gi#)	No. of matched peptide	Sequence coverage (%)	I/R vs. C (fold change)	p value
1544	Triosephosphate isomerase 1	12621074	9	49.1	-1.4	2.9E-03
451	Pyruvate kinase 3 isoform 3	109518934	13	32.7	-1.3	0.015
651	Septin 11	50401563	6	20.3	-1.3	4.1E-03
971	Ubiquitin-like domain containing CTD phosphatase 1	58400827	4	13.5	-1.3	4.0E-03
	Poly(rC) binding protein 2 (predicted)	66911068	3	14.5	-1.3	
1007	Glutamate oxaloacetate transaminase, mitochondrial	38197424	14	36.8	-1.3	0.013
1060	Isocitrate dehydrogenase 3 (NAD+)	16758446	9	25.9	-1.3	8.9E-04
1191	Malate dehydrogenase 1, NAD (soluble)	15100179	6	17.8	-1.3	8.8E-03
1295	Voltage-dependent anion channel 1	75773332	2	10.4	-1.3	5.0E-03
	Voltage-dependent anion channel 2	13786202	2	12.4	-1.3	
1300	Voltage-dependent anion channel 1	75773332	13	57.5	-1.3	1.2E-04
1638	DJ-1 protein	16924002	9	69.7	-1.3	0.034
739	Ubiquinol-cytochrome c reductase core protein 1	51259340	14	33.0	-1.2	8.7E-03
816	Ominthine aminotransferase	11968102	6	14.6	-1.2	
	Similar to thymidylate kinase family LPS-inducible	109479207	4	12.4	-1.2	9.2E-03
1079	Isocitrate dehydrogenase 3 (NAD+)	16758446	7	22.9	-1.2	5.1E-03
1086	Isocitrate dehydrogenase 3 (NAD+)	16758446	2	6.2	-1.2	2.9E-03
1498	Similar to NADH dehydrogenase (ubiquinone) Fe-S protein 3	109470303	8	37.5	-1.1	0.021

* : blast result from *Mus Musculus* genome
I/R: ischemia/reperfusion injured retinas
C: control retinas

The observed molecular weights and PI values of identified proteins on 2-DE image were checked with their theoretical molecular weights and PI values, and allowed 10 Kda and 1–2 PI shift due to possible post-translational modifications.

Table 3

Fold change comparison between western blots and 2-DE on some of the identified proteins.

	I/R vs. C (fold change)		I/R+AMG vs. I/R (fold change)	
	WB	2-DE	WB	2-DE
Transketolase	-2.9	-1.3, -1.7	3.1	1.3, 1.1
Triosephosphate isomerase 1	-2.8	-1.4, -1.5, -1.5, -1.4, - 1.6, - 1.7	1.6	1.2, 1.2, 1, 1.1, 1.1, 1.2
Pyruvate kinase	-1.8	-2.0, -1.3, -1.5, -1.6	1.7	1.2, 1.1, 1.2, 1.3
Aldolase C	-2.0	-1.7, -1.5, -2.0, -1.5	1.6	1.2, 1.1, 1, 1.2
Enolase ^a	-1.9	-2.0	1.2	1

^a only enolase γ expression level was used in 2-DE result for the comparison