Diabetic retinopathy, a retinal vascular disease, is inhibited in animals treated with amino-guanidine, an inhibitor of inducible nitric-oxide synthase. This treatment also reduces retinal protein nitration, which is greater in diabetic rat retina than nondiabetic retina. As an approach to understanding the molecular mechanisms of diabetic retinopathy, we sought the identity of nitrotyrosine-containing proteins in retina from streptozotocin-induced diabetic rats and in a rat retinal Müller cell line grown in high glucose (25 mM). Anti-nitrotyrosine immunoprecipitation products from rat retina and Müller cells were analyzed by LC-MS/MS. Ten nitrated proteins in diabetic rat retina and three nitrated proteins in Müller cells grown in high glucose were identified; three additional nitrotyrosine-containing proteins were tentatively identified from diabetic retina. The identified nitrotyrosine-containing proteins participate in a variety of processes including glucose metabolism, signal transduction, and protein nitration/translation. Among the nitrated proteins responsive glucose transporter type 4, which functions in insulin-stimulated glucose uptake of retina (7). A possible role for NO in the pathogenesis of diabetic retinopathy has been implicated previously (8), an inhibitor of iNOS, or by deficiency of diabetes mellitus; exocyst complex component Exo70, which functions in GLUT-4-containing receptor 2, which influences retinal vascularization via fibroblast growth factor signaling. Nitration of tyrosine phosphorylation sites within these proteins, including GLUT-4, exocyst complex component Exo70, protein-tyrosine phosphatase, inositol 1,4,5-trisphosphate receptor, and inositol trisphosphate receptor 3. Quantitation of nitration and phosphorylation at common tyrosine modification sites in GLUT-4 and protein-tyrosine phosphatase η from diabetic and nondiabetic animals suggests that nitration reduced tyrosine phosphorylation ~2x in these proteins from diabetic retina. The present results provide new insights regarding tyrosine nitration and its potential role in the molecular mechanisms of diabetic retinopathy. Molecular & Cellular Proteomics 7:864–874, 2008.

Retinopathy is one of the most common microvascular complications of diabetes mellitus and a leading cause of blindness. Although hyperglycemia is a risk factor in both type 1 and type 2 diabetes, the molecular mechanisms of the disease remain unclear (1, 2). Previous studies demonstrate that diabetic retinopathy involves inflammatory processes (3–5), that nitric oxide (NO)1 and NO-related reactive nitrogen species are important mediators (2, 6), and that increased nitric oxide (NO) and NO occurs in diabetic retinal oxygenation in experimental diabetes (8) and that the development of diabetic retinopathy in animals can be slowed by aminoguanidine (9), an inhibitor of iNOS, or by deficiency of diabetes mellitus; exocyst complex component Exo70, which functions in GLUT-4-containing receptor 2, which influences retinal vascularization via fibroblast growth factor signaling. Nitration of tyrosine phosphorylation sites within these proteins, including GLUT-4, exocyst complex component Exo70, protein-tyrosine phosphatase, inositol 1,4,5-trisphosphate receptor, and inositol trisphosphate receptor 3. Quantitation of nitration and phosphorylation at common tyrosine modification sites in GLUT-4 and protein-tyrosine phosphatase η from diabetic and nondiabetic animals suggests that nitration reduced tyrosine phosphorylation ~2x in these proteins from diabetic retina. The present results provide new insights regarding tyrosine nitration and its potential role in the molecular mechanisms of diabetic retinopathy. Molecular & Cellular Proteomics 7:864–874, 2008.

Retinopathy is one of the most common microvascular complications of diabetes mellitus and a leading cause of blindness. Although hyperglycemia is a risk factor in both type 1 and type 2 diabetes, the molecular mechanisms of the disease remain unclear (1, 2). Previous studies demonstrate that diabetic retinopathy involves inflammatory processes (3–5), that nitric oxide (NO)1 and NO-related reactive nitrogen species are important mediators (2, 6), and that increased nitric oxide (NO) and NO occurs in diabetic retinal oxygenation in experimental diabetes (8) and that the development of diabetic retinopathy in animals can be slowed by aminoguanidine (9), an inhibitor of iNOS, or by deficiency of diabetes mellitus; exocyst complex component Exo70, which functions in GLUT-4-containing receptor 2, which influences retinal vascularization via fibroblast growth factor signaling. Nitration of tyrosine phosphorylation sites within these proteins, including GLUT-4, exocyst complex component Exo70, protein-tyrosine phosphatase, inositol 1,4,5-trisphosphate receptor, and inositol trisphosphate receptor 3. Quantitation of nitration and phosphorylation at common tyrosine modification sites in GLUT-4 and protein-tyrosine phosphatase η from diabetic and nondiabetic animals suggests that nitration reduced tyrosine phosphorylation ~2x in these proteins from diabetic retina. The present results provide new insights regarding tyrosine nitration and its potential role in the molecular mechanisms of diabetic retinopathy. Molecular & Cellular Proteomics 7:864–874, 2008.

Retinopathy is one of the most common microvascular complications of diabetes mellitus and a leading cause of blindness. Although hyperglycemia is a risk factor in both type 1 and type 2 diabetes, the molecular mechanisms of the disease remain unclear (1, 2). Previous studies demonstrate that diabetic retinopathy involves inflammatory processes (3–5), that nitric oxide (NO)1 and NO-related reactive nitrogen species are important mediators (2, 6), and that increased nitric oxide (NO) and NO occurs in diabetic retinal oxygenation in experimental diabetes (8) and that the development of diabetic retinopathy in animals can be slowed by aminoguanidine (9), an inhibitor of iNOS, or by deficiency of diabetes mellitus; exocyst complex component Exo70, which functions in GLUT-4-containing receptor 2, which influences retinal vascularization via fibroblast growth factor signaling. Nitration of tyrosine phosphorylation sites within these proteins, including GLUT-4, exocyst complex component Exo70, protein-tyrosine phosphatase, inositol 1,4,5-trisphosphate receptor, and inositol trisphosphate receptor 3. Quantitation of nitration and phosphorylation at common tyrosine modification sites in GLUT-4 and protein-tyrosine phosphatase η from diabetic and nondiabetic animals suggests that nitration reduced tyrosine phosphorylation ~2x in these proteins from diabetic retina. The present results provide new insights regarding tyrosine nitration and its potential role in the molecular mechanisms of diabetic retinopathy. Molecular & Cellular Proteomics 7:864–874, 2008.

1 The abbreviations used are: NO, nitric oxide; BTB/POZ, broad-complex, tramtrack, bric a brac domain, also known as the poxvirus and zinc finger domain; E-value, expectation value; FGF, fibroblast growth factor; FGF2, fibroblast growth factor receptor 2; GLUT-4, insulin-responsive glucose transporter type 4; HSP, heat shock protein; iNOS, inducible nitric-oxide synthase; MIR, mannosyltransferase, inositol 1,4,5-trisphosphate receptor, and ryanodine receptor domain; Tyr(N), nitrotyrosine; PTP-η, protein-tyrosine phosphatase η; rMC-1, retinal Müller cell line 1; BLAST, Basic Local Alignment Search Tool.

*From the ‡Cole Eye Institute and §§Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195, Departments of ¶Medicine, |Ophthalmology, §§Chemistry, and **Center for Diabetes Research, Case Western Reserve University, Cleveland, Ohio 44106, ‡‡Research Service, Cleveland Veterans Affairs Medical Center, Cleveland Ohio 44106, and ¶¶Department of Ophthalmology, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, Ohio 44106

Received, August 31, 2007, and in revised form, December 26, 2007

This paper is available on line at http://www.mcponline.org
the pathophysiology of diabetic retinopathy. As an approach to understanding the pathogenic mechanisms of diabetic retinopathy, we sought the identity of proteins that are nitrated in diabetic rat retina and in rat retinal Müller cells grown in high glucose. Nitrotyrosine-containing proteins were selected by immunoprecipitation, and nitrosylation sites were identified by tandem mass spectrometry. The relative amount of nitration and phosphorylation at a common tyrosine in two proteins from diabetic and non-diabetic retina was compared by peptide mass mapping. The physiological roles of the identified proteins and the localization of nitration sites within structural and functional domains provide new insights into possible molecular mechanisms associated with this blinding disease.

**EXPERIMENTAL PROCEDURES**

**Animal Procedures**—Male Sprague-Dawley rats (225–250 g) were randomly assigned to become diabetic or remain as nondiabetic (11). Diabetes was induced by intraperitoneal injection of a freshly prepared solution of streptozotocin in citrate buffer (pH 4.5) at 55 mg/kg of body weight. Insulin was given as needed to achieve slow weight gain without preventing hyperglycemia and glucosuria (0–2 units of insulin subcutaneously, 0–3 times/week). Diabetic rats were insulin-deficient but not grossly catabolic. All animals had free access to food and water and were maintained under a 14–30 h on/off light cycle for 8–10 weeks. Food consumption and body weight were measured weekly. Just before the animals were sacrificed, serum glucose levels were measured using glucose oxidase-based methods. Hemoglobin (an estimate of the average level of hyperglycemia over the previous 2 months) was measured by affinity chromatography (Glyc-Affin, Pierce) (9). Treatment of animals conformed to the Association for Research in Vision and Ophthalmology Resolution on the Treatment of Animals (12). Experiments were performed using 50% control and 50% diabetic rats, and peptides were extracted, reduced in 100 mM DTT, and alkylated with iodoacetamide (23). The dried tryptic peptide mixture was dissolved in 15 μl of 0.1% formic acid and 5% acetonitrile for mass spectrometric analysis. Tryptic peptide mixtures were analyzed by triple quadrupole electrospray mass spectrometry (LC-MS/MS) using a LTQ XL (ThermoFisher) instrument. The samples were nanoflow infused with gentle shaking (1 h) in a low pH buffer (pH 2.8) to remove non-cross-linked peptides. The immonium ions were fragmented with a high-energy collisional dissociation in the LTQ XL. The bound antibody was then covalently cross-linked to protein G with gentle shaking (1 h) in 0.0025% disuccinimidyl suberate (final concentration, 0.0025%), washed to remove non-cross-linked peptides, and then eluted with 62.5 mM Tris-HCl and 2% SDS at pH 7.0 (60 μl, 60 °C for 30 min). The eluate (~180 μl) was vacuum-concentrated to 50 μl and applied to SDS-PAGE.

**Nitrotyrosine Immunoprecipitation**—Nitrotyrosine immunoprecipitations were performed essentially as described previously (22). Briefly rabbit anti-nitrotyrosine polyclonal antibody (90 μg; Chemicon International, Temecula, CA) was coupled with immunopurified immobilized protein G beads (400 μg; Pierce) for 2 h with gentle shaking in 0.14M NaCl, 0.008 M sodium phosphate, 0.01 M triethylammonium bicarbonate, and 0.01 M KCl at pH 7.4 and then washed three times with the unbound antibody (Pierce molecular biology grade) at pH 7.4. The bound antibody was then covalently cross-linked to protein G beads and incubated overnight with the retinal proteins, and bound proteins were eluted as described above.

**SDS-PAGE and Western Analysis**—Nitrotyrosine immunoprecipitation products (50%, v/v) of each sample were also subjected to Western analysis (17) using 12% acrylamide SDS-PAGE, polyvinylidene fluoride membrane (Millipore), mouse anti-nitrotyrosine monoclonal antibody (Millipore), and chemiluminescence detection (GE Healthcare). Western analysis with anti-Glut-4 and anti-PTP-γ antibodies utilized the same methods but with 10% acrylamide gels.

**Protein Identification by LC-MS/MS**—Following SDS-PAGE, immunoprecipitation products were subjected to in situ trypptic digestion, and peptides were extracted, reduced in 100 mM DTT, and alkylated with iodoacetamide (23). The dried tryptic peptide mixture was dissolved in 15 μl of 0.1% formic acid and 5% acetonitrile for mass spectrometric analysis. Tryptic peptide mixtures were analyzed by on-line LC-MS/MS on a QTOF2 mass spectrometer (Waters) using a
Cap LC system (Waters), a 0.3 x 3-mm trapping column (C18 PepMap 100, LC Packings), a reverse phase separating column (75 μm x 5 cm, Vydac C18), and a flow rate of 250 nl/min (24). Gradient LC separation was achieved with aqueous formic acid/acetonitrile solvents. The QTOF2 mass spectrometer was operated in standard MS/MS switching mode with the three most intense ions in each survey scan subjected to MS/MS analysis. Instrument operation and data acquisition utilized MassLynx 4.1 software (Waters). Initial protein identifications from MS/MS data utilized the Mascot search engine (Matrix Science, version 2.1.03) and the Swiss Protein database (release 51.1, November 17, 2006). The Swiss Protein database search parameters included all mammals (~47,000 total sequence entries), two missed tryptic cleavage sites allowed, precursor ion mass tolerance of 1.2 Da, fragment ion mass tolerance of 0.8 Da, and protein modifications for Tyr nitration, Asn and Gln deamidation, Met oxidation, and Cys carbamidomethylation. Select gel slice MS/MS data sets were also analyzed by the same search engine and parameters against all mammals in the National Center for Biotechnology Information (NCBI) database (release 20070818, August 18, 2007, −669,000 total sequence entries). A minimum Mascot ion score of 25 was used for accepting all peptide MS/MS spectra except for nitrotyrosine-containing peptides, which were examined manually as described previously (22). The uniqueness of the nitrated protein identifications was evaluated by BLAST search of the NCBI database (release cited above) for rat sequences (−70,000 total entries) and mouse sequences (−139,000 total entries). BLAST searches were also performed using rat sequences (−6300 total entries) and mouse sequences (−13,600 total entries) in the Swiss Protein database (release 54.1, August 21, 2007).

Protein Identification by Peptide Mass Mapping—Coomassie stained SDS-PAGE bands from retinal extracts that contained immunoreactive bands for GLUT-4 at ~55 kDa or with immunoreactive bands for PTP-11 at ~100 kDa were excised from the gel and analyzed by LC-MS/MS as described above. The target proteins were not detected by MS/MS; therefore the following identification strategy was utilized. All theoretical tryptic peptides from GLUT-4 and PTP-11 were generated in silico with PeptideProphet software (25) using the databases of monoisotopic, one missed tryptic cleavage, mass cutoff of 500 Da, and all known post-translation modifications for Tyr nitration, Asn and Gln deamidation, Met oxidation, and Cys carbamidomethylation. Each theoretical tryptic peptide was matched manually to the singly and doubly charged precursor ions that were generated in silico to obtain the observed tryptic peptide masses [M + H]+ and [M + 2H]+. The observed tryptic peptide masses ([M + H]+ and [M + 2H]+) were then searched for protein identification using the Mascot peptide mass fingerprint search engine (Matrix Science, version 2.1.03) and the Swiss Protein database (release 54.6x, December 14, 2007). The Swiss Protein database search parameters included all mammals (~57,000 total sequence entries), one missed tryptic cleavage site allowed, singly charged monoisotopic ion, peptide mass tolerance of 100 ppm, and protein modifications for Tyr nitration and Tyr phosphorylation. The relative amounts of nitration or phosphorylation in select peptides from GLUT-4 and PTP-11 were estimated based on the ion intensity of the modified and unmodified peptides essentially as described previously for nitration (17) and glycosylation (25).

Bioinformatics Analyses—Protein structural and functional domain analysis was performed with ScanProsite, Motifscan, InterProScan, ProDom, and Pfam. Analysis of possible metabolic networks involving the identified nitrotyrosine-containing proteins was performed with Ingenuity Pathways Analysis software (Ingenuity® Systems).

RESULTS

Characteristics of the Diabetic Rats—The streptozotocin-induced diabetic rats used in this study were hyperglycemic with mean serum glucose and glycated hemoglobin concentrations (301 ± 45 mg/dl and 9.0 ± 1.4%, respectively; n = 8) that were significantly greater than observed in nondiabetic rats (57 ± 7 mg/dl and 3.3 ± 0.4%, respectively; n = 8). The diabetic rats also failed to gain weight at a normal rate; mean body weights at 8–10 weeks of the study for diabetic rats and nondiabetic animals were 234 ± 55 and 448 ± 78 g, respectively (n = 8 per category).

SDS-PAGE and Western Analysis following Nitrotyrosine Immunoprecipitation—Anti-nitrotyrosine immunoprecipitation products from rat retina and rMC-1 Müller cells were analyzed by SDS-PAGE and Western blot. Coomassie Blue staining was significantly more intense for rMC-1 Müller cells grown in either 5 or 25 mM glucose. D, nondiabetic; Ab, anti-nitrotyrosine antibody (1 μg); BSA, low molecular weight markers, which include BSA (~1 μg) at −75 kDa not chemically modified with tetranitromethane (17).
rMC-1 cells grown in either 25 or 5 mM glucose (Fig. 1B).

**Nitrotyrosine-containing Proteins**—Following SDS-PAGE and tryptic digestion, immunoprecipitation product peptides were subjected to LC-MS/MS. Tandem mass spectra provided the sequence of 13 nitrotyrosine-containing peptides from diabetic rat retina and three nitrotyrosine-containing peptides from rat retinal Müller cells grown in high glucose (Table I), one of which contained two nitrated tyrosines. Each nitrotyrosine-containing peptide sequence was matched to a protein by Mascot analysis of all mammalian sequences in the Swiss Protein database and confirmed by BLAST analysis of rat and mouse sequences in both the Swiss Protein and NCBI protein databases. The expectation values (E-values) derived from the BLAST analyses of the rat and mouse sequences in the NCBI database are shown in Table I and support the statistical significance of the majority of alignments and protein identifications (E-values < 0.046). E-values reflect the number of higher scoring alignments expected to occur by chance. BLAST analyses of the larger mouse database yielded lower significance alignments (E-values between 0.07 and 0.18) for inositol 1,4,5-trisphosphate receptor type 3, transcription termination factor I-interacting protein 5, and APOBEC1-stimulating protein. To further support these three alignments, the MS/MS data from the relevant gel slices were also analyzed by Mascot search against all 669,000 mammalian sequences in the NCBI database; this search identified the same three proteins from the peptide sequences shown in Table I.

A representative MS/MS spectrum is shown in Fig. 2, and the data interpretation used to identify the nitrotyrosine-containing protein is described here containing 13-amino acid peptide from GLUT-4. In the MS spectrum (scan number 2H\[11001\]) of the peptide that eluted at retention time 23.9 min, the singly charged b and y product ions (y1, y3, y4, y10, y11, b1, b4, b6, b9, b10, m/z 73) were observed in the MS/MS spectrum with the corresponding amino acid sequence VIEQSYN\[^{N\text{a}}\]ATWLGR shown where Y* is nitrotyrosine and N\[^{\text{a}}\] is deamidated Asn. This peptide sequence matched exactly to residues 51–63 of the protein, and nitrotyrosine was assigned to Tyr\[^{56}\] (Table I). The loss of NH\[^{3}\] from the singly charged b9 and b10 ions was also detected as labeled in the MS/MS spectrum. The E-values generated from the BLAST searches of the determined peptide sequence support the protein identification. Similar approaches were used to interpret the 12 other MS/MS spectra from diabetic rat retina shown in supplemental Fig. 1 and the three MS/MS spectra from Müller cells grown in high glucose shown in supplemental Fig. 2.

**Nitration at Tyrosine Phosphorylation Sites in GLUT-4 and PTP-\(\eta\)**—Nitration of tyrosine was identified at phosphorylation sites in several proteins, including GLUT-4 peptide 51VIEQSYN\[^{ATWLRG}\[^{63}\] and PTP-\(\eta\) peptide 1039\[^{NVYAVMV-}\]LTK\[^{1041}\]. To investigate whether tyrosine nitration possibly competes with phosphorylation in these peptides, GLUT-4 and PTP-\(\eta\) from diabetic and nondiabetic retina (from seven rats each) were excised from SDS-PAGE (supplemental Fig. 3), and tryptic peptides were analyzed by LC-MS/MS. Neither protein was detected by MS/MS due to low abundance, but peptide mass mapping provided 19–25% sequence coverage with average errors of 26–34 ppm and E-values of e\[^{-10}\] to e\[^{-11}\], confirming the presence of the target proteins in the excised gel bands (supplemental Table 1). For the above two peptides, singly, doubly, and triply charged ions were quantified by ion intensities for the nitrated, phosphorylated, and unmodified species, all of which were found in both diabetic and nondiabetic retina (supplemental Table 2). Relative amounts are summarized in Fig. 3 and presented in detail in supplemental Table 2. Similar amounts of unmodified peptide were detected in diabetic and nondiabetic retina and averaged ~47% for the GLUT-4 peptide and ~61% for the PTP-\(\eta\) peptide. For both peptides, diabetic retina contained relatively more of the nitrated (24–42%) than phosphorylated (10–13%) species. In nondiabetic retina, the amount of phosphorylated peptides (20–26%) was higher relative to diabetic retina (10–13%). Notably in diabetic and nondiabetic retina, the amount of nitrotyrosine-containing peptides (26%) was significantly greater than the phosphorylated species (10–13%).

Diabetic retinopathy is a major complication of diabetes involving the retina vasculature. In early stages of the disease, microaneurysms form in the retina that can allow serum to leak into the retina, causing edema and decreased vision. In the late stages, known as proliferative diabetic retinopathy, retinal and optic nerve head neovascularization occurs, generating hemorrhaging, and associated fibrovascular tissue may contract, leading to scarring and retinal detachment. Without timely diagnosis and treatment, blindness may ensue. Previous studies suggest that inflammatory processes involving nitric oxide mediators and the generation of nitrotyrosine-containing proteins may play a role in the disease (2, 9, 11, 26). Diabetes also appears to contribute to the development of other ocular disorders such as glaucoma (2). Notably aminoguanidine inhibition of iNOS not only slows the progression of diabetic retinopathy in rats, it also protects rat retina from injury from ischemia (27) and glaucoma (28). To probe the role tyrosine nitration may play in diabetic retinopathy, we sought the identity of nitrotyrosine-containing proteins in diabetic rat retina and in rat retinal Müller cells cultured in the presence of high glucose.

Nitrotyrosine immunoaffinity precipitation was utilized to preferentially enrich for nitrated proteins, and SDS-PAGE
<table>
<thead>
<tr>
<th>Swiss-Prot accession number</th>
<th>Protein</th>
<th>Sequence coverage</th>
<th>Peptide matches</th>
<th>Nitrotyrosine-containing peptide</th>
<th>Tyr(\text{N}) site</th>
<th>E-values</th>
<th>E-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NCBI</td>
<td>NCBI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>rat</td>
<td>mouse</td>
</tr>
<tr>
<td>Diabetic rat retina</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P02529</td>
<td>γ-Crystallin C</td>
<td>11</td>
<td>R ↓ CY*EC^5SSDC^6PN^8LQ^9TYFSR ↓ C</td>
<td>Tyr^{17}</td>
<td>5e^{-11}</td>
<td>1e^{-10}</td>
<td></td>
</tr>
<tr>
<td>P10066</td>
<td>γ-Crystallin B</td>
<td>8</td>
<td>R ↓ CY*EC^5SSDC^6PN^8LQ^9TYFSR ↓ C</td>
<td>Tyr^{17}</td>
<td>5e^{-11}</td>
<td>1e^{-10}</td>
<td></td>
</tr>
<tr>
<td>P19357</td>
<td>Insulin-responsive glucose transporter type 4</td>
<td>1</td>
<td>K ↓ VIEQSY*N^7ATWLGR ↓ Q</td>
<td>Tyr^{26}</td>
<td>3e^{-6}</td>
<td>6e^{-6}</td>
<td></td>
</tr>
<tr>
<td>Q63544</td>
<td>Sensory neuron synuclein</td>
<td>1</td>
<td>K ↓ TKEGVMY^9VGAKTK ↓ G</td>
<td>Tyr^{25}</td>
<td>2e^{-9}</td>
<td>3e^{-10}</td>
<td></td>
</tr>
<tr>
<td>O54922</td>
<td>Exocyst complex component Exo70</td>
<td>1</td>
<td>K ↓ N^2DPDKEY*N^3MPK ↓ D</td>
<td>Tyr^{29}</td>
<td>7e^{-9}</td>
<td>1e^{-4}</td>
<td></td>
</tr>
<tr>
<td>Q9CR40</td>
<td>BTB/POZ domain-containing protein 5</td>
<td>1</td>
<td>R ↓ Y^3EFGCIVLDQK ↓ Y</td>
<td>Tyr^{22}</td>
<td>2e^{-2}</td>
<td>4e^{-5}</td>
<td></td>
</tr>
<tr>
<td>O88943</td>
<td>Potassium voltage-gated channel subfamily KQT member 2</td>
<td>6</td>
<td>K ↓ LLGSVY^5AHSK ↓ E</td>
<td>Tyr^{23}</td>
<td>3e^{-3}</td>
<td>6e^{-3}</td>
<td></td>
</tr>
<tr>
<td>Q9JKL9</td>
<td>Placental prolactin-like protein K</td>
<td>625</td>
<td>R ↓ KVNT^*LEVIK ↓ Y</td>
<td>Tyr^{25}</td>
<td>6e^{-10}</td>
<td>1e^{-2}</td>
<td></td>
</tr>
<tr>
<td>Q15229</td>
<td>Kynurenine 3-monoxygenase</td>
<td>651</td>
<td>R ↓ N^8FQ^8DVY^9RAR ↓ E</td>
<td>Tyr^{27}</td>
<td>1e^{-2}</td>
<td>2e^{-2}</td>
<td></td>
</tr>
<tr>
<td>Q64555</td>
<td>Protein-tyrosine phosphatase η</td>
<td>599.3</td>
<td>K ↓ N^5V^4ANMLTK ↓ C</td>
<td>Tyr^{104}</td>
<td>3e^{-3}</td>
<td>7e^{-3}</td>
<td></td>
</tr>
<tr>
<td>Q62673</td>
<td>Serine/threonine-protein kinase PLK1</td>
<td>606.8</td>
<td>K ↓ KITLNN^*FR ↓ N</td>
<td>Tyr^{25}</td>
<td>2e^{-2}</td>
<td>4e^{-5}</td>
<td></td>
</tr>
<tr>
<td>Q63269</td>
<td>Inositol 1,4,5-trisphosphate receptor type 3</td>
<td>561.8</td>
<td>R ↓ NAGEKKY{R} ↓ L</td>
<td>Tyr^{2}</td>
<td>5.8e^{-1}</td>
<td>1.1e^{-1}</td>
<td></td>
</tr>
<tr>
<td>Q91YE5</td>
<td>Transcription termination factor I-interacting protein 5</td>
<td>566.7</td>
<td>R ↓ WEEFY^3Q^3GK ↓ Q</td>
<td>Tyr^{143}</td>
<td>3.9e^{-2}</td>
<td>7e^{-2}</td>
<td></td>
</tr>
<tr>
<td>Q923K9</td>
<td>APOBEC1-stimulating protein</td>
<td>535.8</td>
<td>I^6GKIY^*EM^5R ↓ M</td>
<td>Tyr^{2}</td>
<td>9e^{-2}</td>
<td>1.8e^{-1}</td>
<td></td>
</tr>
<tr>
<td>Rat Müller cells grown in 25 mM glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P21803</td>
<td>Fibroblast growth factor receptor 2</td>
<td>665.9</td>
<td>R ↓ N^8NIDY^*YKK ↓ T</td>
<td>Tyr^{20}</td>
<td>1e^{-2}</td>
<td>3e^{-2}</td>
<td></td>
</tr>
<tr>
<td>O88457</td>
<td>Sensory neuron sodium channel 2</td>
<td>695.4</td>
<td>R ↓ YY^9PVFPDER ↓ N</td>
<td>Tyr^{2}</td>
<td>6e^{-2}</td>
<td>3e^{-3}</td>
<td></td>
</tr>
<tr>
<td>P48548</td>
<td>G protein-activated inward rectifier potassium channel 4</td>
<td>602.3</td>
<td>R ↓ Q^8R^9Y^8M^8EKTGK ↓ C</td>
<td>Tyr^{2}</td>
<td>1.8e^{-2}</td>
<td>3e^{-2}</td>
<td></td>
</tr>
</tbody>
</table>
fractionation was used to simplify the complexity of the immunoprecipitates for LC-MS/MS analysis. To select for low abundance nitrated peptides, ion scores in the Mascot data-base searches were disregarded, and 1256 candidate spectra were compiled, including 821 spectra from diabetic rat retina and 435 spectra from Müller cells cultured in 25 mM glucose. Manual inspection and interpretation of all the candidate MS/MS spectra led to the identification of 16 nitrotyrosine-containing peptides (Table I). These methods provided statistically significant identification of tyrosine nitration sites in 10 retinal proteins from in vivo retina and three proteins from cultured retinal Müller cells. The sequence of one of the nitrotyrosine-containing peptides is common to both γ-crystallins. It is not clear whether both or only one of these proteins is nitrated. Three other potential nitrotyrosine-containing proteins were identified. Alignments are considered tentative for inositol 1,4,5-trisphosphate receptor type 3, transcription termination factor I-interacting protein 5, and APOBEC1-stimulating protein because of lower significance E-values from the BLAST searches of NCBI mouse sequences. However, all the MS/MS spectra provided reliable peptide sequence information, and the tentative protein assignments are considered in the following discussion of structure, function, and pathways.

As an approach to determining the significance of tyrosine nitration in diabetic retinopathy, metabolic network systems involving the nitrotyrosine-containing proteins were sought using bioinformatics methods. Pathway analysis suggested that 10 of the nitrated proteins could function in metabolic networks involving cellular growth and proliferation, connective tissue development and function, and/or musculoskeletal system development. Of these networks, cellular growth and proliferation is consistent with the neovascularization manifested in diabetic retinopathy. The 10 proteins highlighted in the cellular growth and proliferation pathway included GLUT-4, sensory neuron synuclein, exocyst complex component Exo70, potassium voltage-gated channel KQT 2, PTP-η, serine/threonine-protein kinase PLK1, inositol 1,4,5-trisphosphate receptor 3, transcription termination factor I-interacting protein 5, fibroblast growth factor receptor 2, and G protein-activated inward rectifier potassium channel 4.
Bioinformatics analysis of protein domains was also pursued for insights into the physiological significance of the identified tyrosine nitration sites. All of the identified nitration sites occur in structural or functional domains as shown schematically in Fig. 4. Of particular interest, five of the identified nitration sites appear to be targets of tyrosine phosphorylation. For example, nitrotyrosine (Tyr(N)352) was detected in the MIR 4 domain of inositol 1,4,5-trisphosphate receptor type 3 (Fig. 4A), a multipass membrane protein. The MIR domain refers to “mannosyltransferase, inositol 1,4,5-trisphosphate receptor, and tyrosine receptor” domain common to several signal transduction-associated proteins and functions in binding and catalysis. Tyr352 is a component of the tyrosine kinase phosphorylation motif KviEqsY (residues 344–352; lower-case denotes variable residues) and therefore also a target of phosphorylation. In GLUT-4 (Fig. 4B), a 12-pass membrane protein, nitrotyrosine (Tyr(N)56) was detected in an extracellular domain adjacent to a glycosylation site. Tyr56 is a component of the tyrosine kinase phosphorylation motif KvEqSvY (residues 50–56) and a target of phosphorylation. In PTP-η (Fig. 4C), a single pass membrane protein, nitrotyrosine (Tyr(N)1034) was detected in the catalytic tyrosine-protein phosphatase domain. Tyr1034 is a component of the tyrosine kinase phosphorylation motif RmvwEknvY (residues 1026–1034) and a target of phosphorylation. Nitration of sensory neuron synuclein (Fig. 4D) was detected at Tyr(N)39 in repeat 2 of an 11-amino acid tandem repeat. Tyr39 is a component of the tyrosine kinase phosphorylation motif RnvMWKvNvY (residues 32–39) and a target of phosphorylation. Nitration of fibroblast growth factor receptor 2 (Fig. 4E), a single pass membrane protein, was identified in the N-terminal cytoplasmic domain. Two adjacent nitrotyrosines were detected in the N-terminal cytoplasmic domain. Tyrosine nitration was detected in Greek key 1 of γ-crystallin B and/or γ-crystallin C (Fig. 4K), homologous cytosolic proteins that contain four β/γ Greek key domains composed of four antiparallel β-strands. In BTB/POZ domain-containing protein 5 (Fig. 4L), nitrotyrosine was detected in Kelch protein interaction domain 1. The BTB domain (broad-complex, tramtrack, and bric a brac), also known as the POZ domain (poxvirus and zinc finger), is a homodimerization domain in proteins containing multiple zinc fingers of the C2H2 type. In placental prolactin-like protein K (Fig. 4M), a secreted protein, Tyr(N)413 was detected adjacent to a throneine phosphorylation site (Thr13) within the casein kinase II phosphorylation motif TyIE (residues 213–216). Several other phosphorylation sites are localized within the C-terminal region. Tyrosine nitration in serine/threonine-protein kinase PLK1 (Fig. 4N) was identified at Tyr(N)481 immediately adjacent to protein interaction motifs and our nitrotyrosine MS/MS data, the results suggest a relative reduction in tyrosine phosphorylation in GLUT-4 and PTP-η in the diabetic animals due to tyrosine nitration. GLUT-4 is the main glucose transporter activated by insulin in skeletal muscle and adipocytes. Based on immunodetection, others have reported that GLUT-4 is not expressed in human ocular tissues (29); however, our data supports GLUT-4 expression in rat retina by both immunodetection and peptide mass mapping. Nitration of GLUT-4 could impact glucose transport into retinal cells and play a role in diabetic retinopathy (30, 31). PTP-η converts protein tyrosine phosphatase to protein tyrosine, contributing to a variety of cell signaling process. This phosphatase has recently been shown to modulate vascular endothelial growth factor signaling (32), further suggesting that nitration of a tyrosine phosphorylation site in the catalytic domain could impact retinal angiogenesis in diabetic retinopathy.

We identified tyrosine nitration sites in several other types of structural or functional domains (Fig. 4). Tyrosine nitration was localized to the FAD-dependent oxidoreductase domain of kynurenine 3-monooxygenase (Fig. 4F), a two-pass mitochondrial outer membrane protein. In potassium voltage-gated channel subfamily KQT member 2 (Fig. 4G), nitrotyrosine was detected at Thr213 in the cation channel region of the protein. The C2H2 zinc finger of the C-terminal region (Fig. 4H) was identified in the C-terminal region. Tyrosine nitration was detected at Tyr(N)481 immediately adjacent to protein interaction motifs and our nitrotyrosine MS/MS data, the results suggest a relative reduction in tyrosine phosphorylation in GLUT-4 and PTP-η in the diabetic animals due to tyrosine nitration. GLUT-4 is the main glucose transporter activated by insulin in skeletal muscle and adipocytes. Based on immunodetection, others have reported that GLUT-4 is not expressed in human ocular tissues (29); however, our data supports GLUT-4 expression in rat retina by both immunodetection and peptide mass mapping. Nitration of GLUT-4 could impact glucose transport into retinal cells and play a role in diabetic retinopathy (30, 31). PTP-η converts protein tyrosine phosphatase to protein tyrosine, contributing to a variety of cell signaling process. This phosphatase has recently been shown to modulate vascular endothelial growth factor signaling (32), further suggesting that nitration of a tyrosine phosphorylation site in the catalytic domain could impact retinal angiogenesis in diabetic retinopathy.

We identified tyrosine nitration sites in several other types of structural or functional domains (Fig. 4). Tyrosine nitration was localized to the FAD-dependent oxidoreductase domain of kynurenine 3-monooxygenase (Fig. 4F), a two-pass mitochondrial outer membrane protein. In potassium voltage-gated channel subfamily KQT member 2 (Fig. 4G), nitrotyrosine was detected in the cation channel region of the protein. The C2H2 zinc finger of the C-terminal region (Fig. 4H) was identified in the C-terminal region. Tyrosine nitration was detected at Tyr(N)481 immediately adjacent to protein interaction motifs and our nitrotyrosine MS/MS data, the results suggest a relative reduction in tyrosine phosphorylation in GLUT-4 and PTP-η in the diabetic animals due to tyrosine nitration. GLUT-4 is the main glucose transporter activated by insulin in skeletal muscle and adipocytes. Based on immunodetection, others have reported that GLUT-4 is not expressed in human ocular tissues (29); however, our data supports GLUT-4 expression in rat retina by both immunodetection and peptide mass mapping. Nitration of GLUT-4 could impact glucose transport into retinal cells and play a role in diabetic retinopathy (30, 31). PTP-η converts protein tyrosine phosphatase to protein tyrosine, contributing to a variety of cell signaling process. This phosphatase has recently been shown to modulate vascular endothelial growth factor signaling (32), further suggesting that nitration of a tyrosine phosphorylation site in the catalytic domain could impact retinal angiogenesis in diabetic retinopathy.
Tyrosine Nitration in Diabetic Retina

domain POLO box 1 (residues 417–480). In transcription termination factor I-interacting protein 5 (Fig. 4O), a nuclear protein, Tyr(N)\textsuperscript{1843} was detected in the C-terminal region near the protein interaction bromodomain (residues 1755–1825). Tyrosine nitration of APOBEC1-stimulating protein (Fig. 4P), which shuttles between the nucleus and cytoplasm, was detected in the RRM1 domain, an RNA recognition motif.

The biological significance and relevance to diabetic retinopathy of the identified nitration events have yet to be determined; however, the functions of the other nitrotyrosine-containing proteins in Table I and Fig. 4 all appear to be susceptible to possible modulation by nitration. Nitration of \(\gamma\)-crystallin B and \(\gamma\)-crystallin C may enhance the chaperone function of these proteins as reported for oxidative modification of \(\alpha\)-crystallin and HSP 70 (33, 34). Sensory neuron synuclein may be involved in modulating axonal architecture and neurofilament network integrity (35), and although its function remains unclear (36), nitrination of the observed tyrosine phosphorylation site could have biological consequences. Exocyst complex component Exo70 functions in the docking of exocytic vesicles with specific sites on plasma membranes (37) and appears to play a role in insulin-stimulated glucose uptake of GLUT4-containing vesicles (38); nitration of the observed tyrosine phosphorylation site could alter its activity in diabetic retinopathy. BTB/POZ domain-containing proteins are implicated in a variety of processes including DNA binding and organization of macromolecular structures (39); the observed nitration in the N-terminal cytoplasmic domain could impact the channel subfamily KQT 2 is a multipass membrane protein that functions in neuronal excitability (40). Its current can be dependent upon ligand binding rather than voltage (48); the two adjacent nitrotyrosine observed near the N terminus could influence ligand/protein interactions. G protein-activated inward rectifier potassium channel 4 forms a heteromultimeric pore that allows faster flow of potassium into than out of the cell (49). The observed nitration in the C terminus of Kir4.1 could influence the signaling properties of the channel.

In summary, the present study provides new insights for tyrosine nitration in diabetic disease. Identification of the localization sites we identified provides new insight into the biological consequences of tyrosine nitration at these sites in diabetic retina. The phosphorylation at two of these sites appears to be greater in diabetic retina, whereas the phospho-serine/serine appears to contain relatively more phosphorylation. The results suggest that nitration reduced tyrosine phosphorylation at these sites in diabetic retina and implicate potential consequences to signal transduction processes. All of the identified tyrosine nitration sites occur in protein domains that may modulate other protein functions and cellular processes. Further investigation is required to determine the biological consequences of the identified tyrosine nitration events and their relevance to the pathogenic mechanisms of diabetic retinopathy.

* This work was supported, in whole or in part, by National Institutes of Health Grants EY00300, EY11373, EY14239, and EY15638. This work was also supported by Ohio Biomedical Research Technology Transfer Grant 05-29, The Foundation Fighting Blindness; a Research to Prevent Blindness (RPB) Challenge Grant (to the Cole Eye Institute), an RPB Senior Investigator Award (to J. C.), a Steinbach Award (to J. W. C. and The Cleveland Clinic Foundation). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\[\text{SUPPLEMENTAL MATERIAL}\]

"The online version of this article (available at http://www.mcpオンライン.ORG) contains supplemental material.

To whom correspondence should be addressed: Cole Eye Inst. (J.C.), Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Tel.: 216-445-0425; Fax: 216-445-3670; E-mail: crabbj@ccf.org."
REFERENCES


ney, gastrointestinal tract, and other tissues. *J. Biol. Chem.* 268, 11356–11363


