Nitrotyrosine-containing Proteins in Diabetic Rat Retina and Müller cells

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OVERVIEW

Introduction: Retinopathy as a complication of diabetes can result in irreversible blindness. Notably, inducible nitric oxide synthase (iNOS), superoxide and nitric oxide are elevated in diabetic retina. Diabetic retinopathy is inhibited and retinal protein nitration is reduced in animals treated with aminoguanidine, an inhibitor of iNOS. Accordingly, reactive nitrogen species (RNS) may play an important role in the molecular mechanisms of diabetic retinopathy. In the present study, we sought the identity of retinal proteins that are nitrated in diabetes and the corresponding nitration sites as an approach to better understanding the pathogenic mechanisms of this retinopathy.

Methods: In vivo studies utilized retinas collected from STZ-diabetic rats (2 month duration), and in vitro studies used a transformed rat retinal Muller cell line (rMC-1) incubated in normal (5mM) and high (25 mM) glucose. Western analysis was used to compare levels of nitrotyrosine. For nitrated protein identification, retinas or cells were homogenized and nitrated proteins immunoprecipitated using anti-nitrotyrosine antibody. Immunoprecipitation products were fractionated by SDS-PAGE, gel bands excised, digested in situ with trypsin and analyzed by QTOF LC MS/MS. Protein identification utilized the ProteinLynx Global Server and Mascot search engines and the Swiss-Protein and NCBI protein databases. Tyrosine-nitration sites were determined by manual examination of MS/MS spectra.

Results: Immunoprecipitation and Western analysis showed that nitrotyrosine immunoreactivity was greater in retinas from diabetic rats than from nondiabetic rats, and a little bit greater in rMC-1 cells incubated in 25 mM glucose than in rMC-1 cells incubated in 5 mM glucose. LC MS/MS analyses of the immunoprecipitation products from diabetic rat retina have localized nitrotyrosine in 13 proteins, including among others insulin-responsive glucose transporter type 4, gamma crystalline B and C, inositol 1,4,5-trisphosphate receptor type 3, and BTB/POZ domain-containing protein. LC MS/MS analyses of immunoprecipitation products from rat Müller cells incubated in 25 mM glucose have identified tyrosine nitration sites in 3 other proteins.

Conclusions: Currently identified nitrotyrosine-containing proteins from diabetic rat retina function in glucose metabolism, signal transduction pathways, redox systems, and transcription/translation regulation. Some of the identified nitration sites appear within important functional domains of the proteins. The present technology offers promise for insights into the mechanisms of diabetic retinopathy.

INTRODUCTION

Diabetic retinopathy is a major complication of diabetes, and remains a leading cause of acquired blindness, but the mechanism of pathogenesis is unclear. A possible role of nitric oxide (NO) in the pathogenesis of diabetic retinopathy has been suggested by findings that the development of diabetic retinopathy in animals can be inhibited by aminoguanidine (Kern and Engerman, 2001), which is known to inhibit iNOS (Misko et al., 1993). Increased production of superoxide (Du et al., 2003) and NO in the retina in diabetes generates peroxynitrite, which can react with tyrosine to produce nitrotyrosine. Nitrotyrosine immunoreactivity appears to be greater in retinas from diabetic rats than from nondiabetic rats and in rMC-1 rat Müller cells grown in high glucose-containing media (Du et al., 2002). In the present study, we sought the identity of retinal proteins that are nitrated in diabetes and the corresponding nitration sites as an approach to better understanding the pathogenic mechanisms of this retinopathy.

METHODS

Sample Preparation: Retinal extracts were prepared and pooled from three STZ-induced diabetic rats (2 month duration) and from three control rats (male, Sprague-Dawley). *In vitro* cell extracts were prepared from the rMC-1 transformed rat Muller cell line (Sarthy et al, 1998) grown in normal (5mM) or in high (25 mM) glucose-containing 10%FBS-DMEM medium. Protein was quantified using a modified Bradford assay.

Immunoprecipitation: Rabbit anti-nitrotyrosine pAb was covalently crosslinked to immobilized protein G with disuccinimidyl suberate. Diabetic rat retinal extract (~500 µg) or high glucose grown Müller cell extract (~1500 µg) was incubated with the antibody-beads with gently rocking/4 °C /overnight. After washing 3x with PBS, bound nitrated proteins were eluted with 62.5 mM Tris-HCl/ 2% SDS at pH 7.0. The eluate was concentrated to 50 µl and applied to 1D SDS-PAGE and Western blot.

SDS-PAGE and Western Analysis: IP-products were subjected to 12% SDS-PAGE, with 50% of each sample used for Coomassie blue detection and 50% used for Western blot with mouse anti-nitrotyrosine mAb. The immunoblot membrane was developed using ECL and the Coomassie blue stained gel was used for protein identification.

Identification of Nitrated Proteins: For protein identifications, gel slices were excised and digested *in situ* with trypsin, and peptides were analyzed by liquid chromatography electrospray tandem mass spectrometry using a CapLC system and a quadrupole time-of-flight mass spectrometer (QTOF2, Waters Corporation) (Crabb et al., 2002). Protein identifications from MS/MS data utilized MASSLYNX 4.1 software (Waters), the Mascot search engine (Matrix Science) and the Swiss-Prot protein database. Protein modifications were selected as nitration (Y), deamidation (N, Q), oxidation (M), and carbamidomethylation (C). Tyrosine-nitration sites were determined by manual examination of MS/MS spectra (Zhan and Desiderio, 2006). The structural/functional domains and motifs were determined by ScanProsite (http://us.expasy.org/tools/scanprosite/) and MotifScan (http://myhits.isb-sib.ch/cgi-bin/motif_scan).

RESULTS

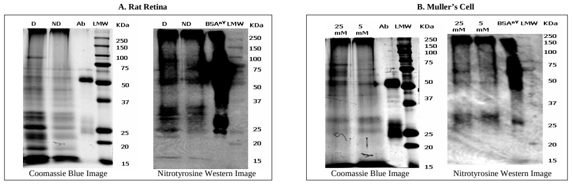


Figure 1. Immunoprecipitation (IP) of nitrotyrosine-containing proteins in (A) diabetic rat retina and (B) Muller's cell grown in high glucose. D = diabetic, ND = non-diabetic; 25 mM = 25 mM glucose (high), 5 mM = 5 mM glocuse (low); Ab = anti-nitrotyrosine antibody (1 µg); BSA^{nY} = nitrated BSA standard; LMW = low molecular weight marker.

These results suggest that nitrotyrosine immunoreactivity is greater in retinas from diabetic rats than from nondiabetic rats (A), and a little bit greater in Muller's cells incubated in 25 mM glucose than in Muller's cells incubated in 5 mM glucose (B).

Table 1. Nitrotyrosine-containing Proteins Identified by LC MS/MS

Protein	Swiss-Prot No.	Nitrotyrosine-containing peptide	Nitrotyrosine-site	E-value Swiss-Prot (229K)	E-value NCBI (4406K)
Diabetic Rat Retina (*In vivo*)					
Gamma crystallin C	P02529	R.^{16}CY*EC$^{\#}$SSDC$^{\#}$PNLQTYFSR^{32}.C	Y^{17}	5e-12	4e-11
Gamma crystallin B	P10066	R.^{16}CY*EC$^{\#}$SSDC$^{\#}$PNLQTYFSR^{32}.C	Y^{17}	5e-12	4e-11
Insulin-responsive glucose transporter type 4	P19357	K.^{51}VIEQSY*N$^{@}$ATWLGR^{63}.Q	Y^{56}	3e-07	2e-06
Sensory neuron synuclein	Q63544	K.^{33}TKEGVMY*VGAKTK^{45}.G	Y^{39}	2e-06	1e-05
Exocyst complex component Exo70	O54922	K.^{403}N$^{@}$DPDKEY*N$^{@}$MPK^{413}.D	Y^{409}	7e-06	5e-05
BTB/POZ domain-containing protein 5	Q9CR40	R.^{322}Y*EFGICVLDQK^{332}.V	Y^{322}	4e-05	3e-04 (1e-04*)
Potassium voltage-gated channel subfamily KQT member 2	O88943	K.^{220}LLGSVVY*AHSK^{230}.E	Y^{226}	3e-04	2e-03
Placental prolactin-like protein K precursor	Q9JKL9	R.^{210}KVNTY*LEVIK^{219}.Y	Y^{214}	6e-04	4e-03
Kynurenine 3-monooxygenase	O15229	R.^{31}N$^{@}$FQ$^{@}$IDVY*EAR^{40}.E	Y^{37}	6e-04	5e-03
Receptor-type tyrosine-protein phosphatase eta precursor	Q64455	K.^{1032}NVY*AIVMLTK^{1041}.C	Y^{1034}	8e-04	5e-03 (2e-03*)
Serine/threonine-protein kinase PLK1	Q62673	K.^{475}KITLLNY*FR^{483}.N	Y^{481}	0.002	0.016
Inositol 1,4,5-trisphosphate receptor type 3	Q63269	R.^{345}NAGEKIKY*R^{353}.L	Y^{352}	0.006	0.039
Transcription termination factor I-interacting protein 5	Q91YE5	R.^{1839}WEEFY*Q$^{@}$GK^{1846}.Q	Y^{1843}	0.009	0.059 (0.026*)
APOBEC1-stimulating protein	Q923K9	K.^{79}IGKIY*EM$^{\$}$R^{86}.M	Y^{83}	0.009	0.062
Rat Müller Cells Grown in High Glucose (*In vitro*)					
Fibroblast growth factor receptor 2 precursor	P21803	R.^{650}DIN$^{@}$NIDY*YKK^{659}.T	Y^{656}	2e-04	2e-03 (7e-04*)
Sensory neuron sodium channel 2	O88457	R.^{5}Y*Y*PVIFPDER^{14}.N	Y^{5}, Y^{6}	6e-05	4e-04
G protein-activated inward rectifier potassium channel 4	P48548	R.^{51}Q$^{@}$RY*M$^{\$}$EKTGK^{59}.C	Y^{53}	0.002	0.012

Y* = Nitrated tyrosine. C# = Carbamidomethylated Cys. N@ = deamidated Asn. Q@ = Deamidated Gln. M$ = Oxidated Met.
E-Value = Expectation value, the number of alignments with scores ≥ than the associated score expected to occur in a database search by chance. For E-values, the smaller the better. E-value increases as the database gets larger, and decreases as the alignment gets longer. The (229K) and (4406K) means the total number of sequences in the database at the time of the search.

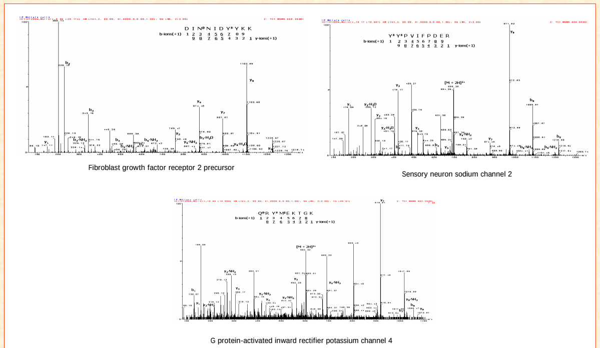


Figure 2. MS/MS spectrum of nitrotyrosine-containing peptide from rat Muller cells grown in high glucose. Y* = Nitrated tyrosine. N@ = deamidated Asn. Q@ = Deamidated Gln. M$ = Oxidated Met.

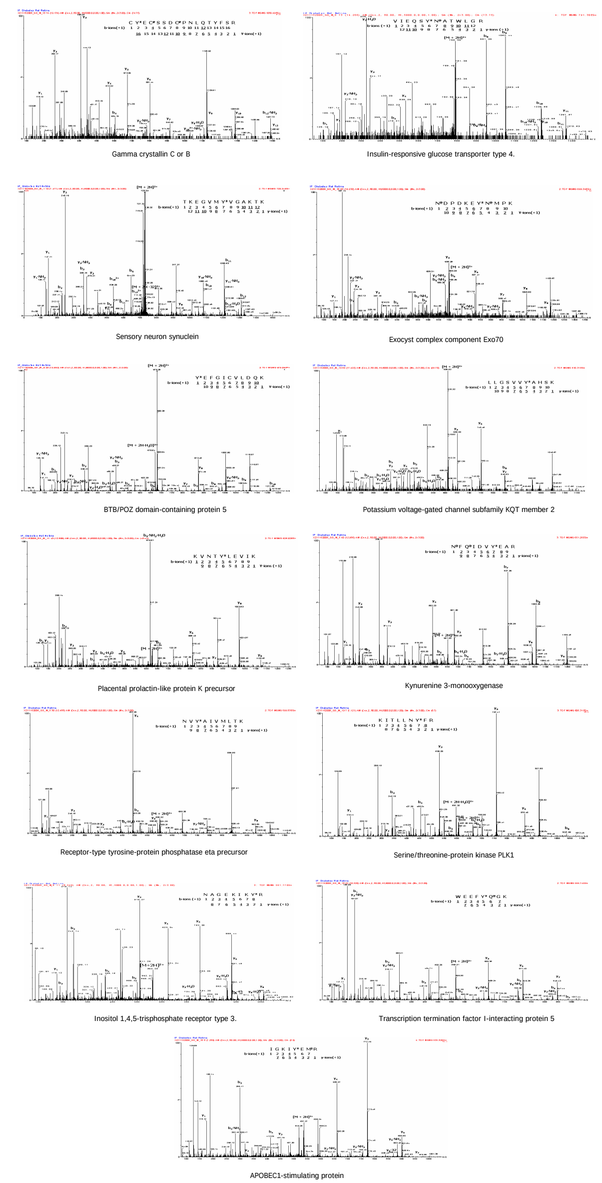
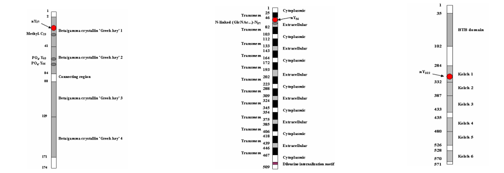


Figure 3. MS/MS spectrum of nitrotyrosine-containing peptide from diabetic rat retina. Y* = Nitrated tyrosine. C# = Carbamidomethylated Cys. N@ = deamidated Asn. Q@ = Deamidated Gln. M$ = Oxidated Met.



GAMMA CRYSTALLIN C

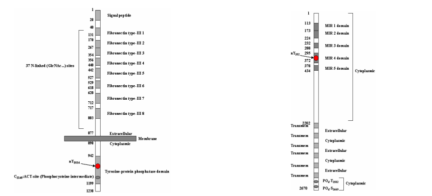
Function: a structural protein in lens with 4 beta/gamma "Greek key" domains, each composed of 4 antiparallel beta-strands. There are two phosphorylation sites in "Greek key" 2, and one methyl-Cys in "Greek key" 1. Tyrosine nitration was detected in "Greek key" 1.

INSULIN-RESPONSIVE GLUCOSE TRANSPORTER TYPE 4

Function: a twelve-pass membrane protein that serves as an insulin-regulated facilitative glucose transporter. This protein is a candidate for post-receptor defects in non-insulin-dependent diabetes mellitus. N_{57} in the extracelluar region is glycosylated; tyrosine nitration was detected at Y_{56}.

BTB/POZ DOMAIN-CONTAINING PROTEIN 5

Function: many BTB proteins are transcriptional regulators that are thought to act through the control of chromatin structure. The BTB domain (Broad-Complex, Tramtrack and Bric a brac), also known as the POZ domain (POxvirus and Zinc finger), are homodimerization domains occurring at the N-terminus of proteins containing multiple zinc fingers of the C_2H_2 type. Tyrosine nitration was detected at Y_{322} in a Kelch protein-protein interaction domain.

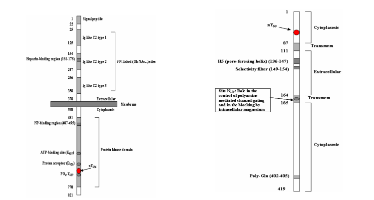


RECEPTOR-TYPE TYROSINE-PROTEIN PHOSPHATASE ETA

Function: a single-pass membrane protein containing 8 fibronectin type-III domains and a tyrosine-protein phosphatase domain. Fibronectin type-III domains exhibit functional and structural modularity involving cell adhesion, cell morphology, thrombosis, and cell migration. Tyrosine nitration was detected at Y_{1034} in the tyrosine-protein phosphatase domain and could affect phosphatase activity.

INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR TYPE 3

Function: a homotetramer receptor for inositol 1,4,5-trisphosphate that mediates the release of intracellular calcium. Tyrosine nitration was detected at Y_{352} in the MIR 4 domain. MIR refers to mannosyltransferase, inositol 1,4,5-trisphosphate receptor (IP3R), and ryanodine receptor (RyR) domain found in some signal transduction-associated proteins.



FIBROBLAST GROWTH FACTOR RECEPTOR 2

Function: a single-pass membrane protein and receptor for acidic and basic fibroblast growth factors, with protein tyrosine phosphorylation activity. Y_{657} is a phosphorylation site in the protein kinase domain and tyrosine nitration was detected at Y_{656}, which could affect kinase activity. Studies have implicated FGF signaling in choroidal angiogenesis and retinal vascularization (Rousseau, et al., 2003).

G PROTEIN-ACTIVATED INWARD RECTIFIER POTASSIUM CHANNEL 4

Function: a two-pass membrane protein and potassium channel protein with a greater tendency to allow potassium to flow into the cell rather than out. Tyrosine nitration (nY_{53}) was detected in the cytoplasmic region.

Figure 4. Localization of tyrosine nitration in functional/structural domains of select proteins

CONCLUSIONS

- The identified nitrotyrosine-containing proteins from diabetic rat retina (13) and from Müller cells (3) function in multiple pathways, including glucose metabolism, signal transduction, and redox system.
- Tyrosine nitration occurred within important functional/structural domains of the identified proteins; however, the significance of these nitration events remains to be determined.
- Mass spectrometric technology offers promise for both the identification of nitration sites as well as quantification of the differences in the proteome between diabetic and non-diabetic retina. Efforts are now directed toward iTRAQ quantitative proteomic analysis of diabetic rat retina.

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