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Diabetic Retinopathy and Transcriptional regulation of a Small Molecular Weight G-Protein, Rac1

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Abstract

In diabetic retinopathy, increased cytosolic reactive oxygen species, produced by NADPH oxidase (Nox), damage mitochondria, and this accelerates apoptosis of retinal capillary cells, resulting in the histopathology. Activation of Nox2 is mediated by a small molecular weight GTPase, Rac1, and retinal Rac1 is activated in diabetes. Our goal is to investigate the molecular mechanism responsible for transcriptional activation of *Rac1* in the development of diabetic retinopathy. Using retinal microvessels, the site of histopathology associated with diabetic retinopathy, from streptozotocin-induced diabetic rats, we investigated the binding of the nuclear transcriptional factor- κ B (NF- κ B) at *Rac1* promoter. Since activation of NF- κ B is regulated by its acetylation-deacetylation, the role of acetylation in *Rac1* transcription was confirmed in the retina from diabetic mice overexpressing a deacetylase, Sirtuin 1. Diabetes increased the binding of p65 subunit of NF- κ B at the *Rac1* promoter. Overexpression of *Sirtuin 1* prevented hyper-acetylation of p65, decreased its binding at the *Rac1* promoter and ameliorated Rac1-Nox2 mediated mitochondrial damage. Thus, in diabetes *Rac1* transcriptional activation in the retina is mediated by acetylation of NF- κ B, and modulation of acetylation during the early stages of diabetic retinopathy has potential to inhibit/retard its development.

Keywords

Acetylation; Diabetic retinopathy; Rac1; Sirtuin 1; Transcriptional regulation

1. Introduction

Oxidative stress is one of the major metabolic abnormalities implicated in the development of diabetic retinopathy. Increased production of cytosolic reactive oxygen species (ROS) during the early stages of this progressive disease, initiate mitochondrial damage, and subsequent cytochrome c leakage into the cytosol activates the apoptotic machinery (Brownlee, 2005; Kowluru and Kowluru, 2014; Kowluru et al., 2015; Kowluru et al., 2014a). Animal models have clearly documented that capillary cell apoptosis precedes the

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development of diabetic retinopathy (Kern et al., 2000; Kowluru and Chan, 2010). Membrane-bound NADPH oxidase (Nox) represents as one of the major sources of cytosolic ROS production, and membrane-bound subunits Nox1, Nox2 (gp91phox), Nox4 and p22phox are present throughout the body, including in the vasculature (Kowluru and Kowluru, 2014; Kowluru et al., 2014a). Diabetes activates Nox2 and Nox4 in the retina and its vasculature, and we have shown that Nox2 activation precedes mitochondrial damage (Ibrahim et al., 2015; Li et al., 2010; Kowluru and Kowluru, 2014; Kowluru et al., 2014a). A small molecular weight GTPase, Rac1 is considered essential for the assembly of Nox2 holoenzyme (Sarfstien et al., 2004). In diabetes, Rac1 is activated in the retina, and Rac1-mediated signaling is implicated in activation of Nox2 (Kowluru et al., 2014a; Kumar et al., 2015). However, putative molecular mechanisms responsible for retinal Rac1 activation in the diabetes remain to be identified.

Recent literature has clearly documented a role of epigenetic modifications in the transcriptional regulation of several genes associated with normal development, and also with diseases (Kato and Natarajan, 2014; Kowluru et al., 2015; Kowluru and Mishra, 2015; Maghbooli et al., 2015; Stankov et al., 2013). Diabetes brings in epigenetic modifications in genes associated with major metabolic abnormalities implicated in the development of diabetic retinopathy, including oxidative stress and inflammation. Diabetes alters global acetylation of retinal histones and activates the acetylation-deacetylation machinery (Zhong and Kowluru, 2010), and it also regulates acetylation of many transcriptional factors including activator protein 1 (Ap1) and nuclear factor- κ B (NF- κ B) (Kowluru et al., 2014b; Mishra et al., 2015). However, whether posttranslational modifications have any role in maintaining retinal *Rac1* transcription remains unclear.

The promoter region of *Rac1* has binding sites for many transcriptional factors including for NF- κ B and specific protein 1 (Sp1). NF- κ B is activated in the retina in diabetes, and activated NF- κ B is implicated in increased capillary cell apoptosis (Kowluru et al., 2003; Romeo et al., 2002). Acetylated form of NF- κ B is transcriptionally active, and its activity is suppressed by removal of acetyl group. Sirtuin-1 (Sirt1), an NAD-dependent deacetylase, plays an important role in maintaining acetylation of NF- κ B (Jung et al., 2012; Pavlova et al., 2013). Our recent work has shown that the activity of Sirt1 in the retina is inhibited in diabetes, and p65 subunit of NF- κ B is hyper-acetylated (Kowluru et al., 2014b). The role of acetylation of NF- κ B in maintaining *Rac1* activation in the retina in diabetes remains to be investigated.

Our goal is to investigate the molecular mechanism responsible for transcriptional activation of *Rac1* in the development of diabetic retinopathy. Using retinal microvessels from diabetic rats, we investigated the binding of NF- κ B with *Rac1* promoter. The role of acetylation-deacetylation in Rac1 transcription was confirmed in the retina from diabetic mice overexpressing *Sirt1*.

2. Methods

2.1. Animal models

Retina was isolated from male Wistar rats (BW 200g) after maintaining them diabetic (streptozotocin-induced) for 6-8 months, and their age-matched normal rats served as controls (Kowluru et al., 2014a). Streptozotocin-induced diabetic wild-type C57BL/6J (WT) or *Sirt1* overexpressing (C57BL/6-Actbtm3.1, *Sirt1* Npa/J, Jackson Laboratory, Bar Harbor, Maine, USA) mice were maintained for 6 months and age-matched normal mice were used as controls. As previously reported (Mishra et al., 2015), retina from these *Sirt1* overexpressing (*S1*) mice had ~60% increase in *Sirt1* expression compared to the wildtype mice. Each group had 7-10 animals, and the methods used to maintain animals are routinely employed in our laboratory (Kowluru et al., 2014a; Mishra et al., 2015). The treatment of rats/mice followed the guidelines of the National Institute of Health Principles of Laboratory Animal Care and were approved by Wayne State University's Animal Care and Use Committee.

2.2. Retinal microvessels

Retinal microvasculature was prepared by incubating the retina in distilled water for one hour at 37°C and the vasculature was isolated under a microscope by repetitive inspiration and ejection through Pasteur pipettes. As reported earlier, the resulting retinal vasculature was devoid of nonvascular preparations (Kowluru et al., 2014a). Chromatin immunoprecipitation (ChIP) was performed in retinal microvessels by ChIP assay using a kit from Millipore Cor. Temecula, CA (Mishra et al., 2015; Zhong and Kowluru, 2011). Briefly, immuno-precipitated protein-DNA complex (100-120µg) was incubated with antibody against p65 subunit of NF-κB (Cat #3045, Cell Signaling Technology, Danvers, MA). DNA fragments were recovered by phenol: chloroform: isoamyl alcohol, ethanol precipitated and analyzed for *Rac1* estimation by q-PCR. Normal rabbit IgG was used as negative antibody control and DNA from the input (25-30µg protein-DNA complex) as an internal control. Each ChIP measurement was made in 5-6 samples/group.

2.3. Gene expression

SYBR green-based real time q-PCR was performed using gene and species-specific primers (Table I). Denaturation was performed at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, and annealing and extension was done at 60°C for 60 seconds. Dissociation was performed at 95°C for 15 seconds, 60°C for 60 seconds, 95°C for 15 seconds and 60°C for 15 seconds. SYBR green single melting curve and correct-size product on a 1.2% agarose gel were analyzed to confirm the PCR products. For ChIP assay, values obtained from the immunoprecipitate were normalized to the Ct value from the input sample, and for mRNA, to the Ct value from *β-actin* (rat) or *18S* (mice) in the same sample using the ddCt method (Mishra et al., 2015; Zhong and Kowluru, 2011).

2.4. Rac1 activity

G-LISA colorimetric assay kit (Cytoskeleton, Denver, CO, USA) was used to measure the activity of Rac1, as described previously (Kowluru et al., 2014a; Kumar et al., 2015).

2.5. Nox2 activity

The reaction mixture for Nox2 activity consisted of lucigenin as an electron acceptor and NADPH as substrate. Each measurement was made in the presence and absence of 0.2nM apocynin (Kowluru et al., 2014a; Kumar et al., 2015; Veluthakal et al., 2015).

2.6. Acetylation of NF- κ B

Retinal homogenate was immunoprecipitated with acetyl-lysine antibody (ab22550, Abcam, Cambridge, MA), and then incubated with Protein A/G Plus agarose beads. After washing the beads, the proteins were separated by SDS-PAGE, and western blotted using p65 antibody (Kowluru et al., 2014b; Mishra et al., 2015).

2.7. Mitochondrial damage

Genome specific quantitative extended length PCR was performed to assess mtDNA damage using the methods reported previously (Kowluru et al., 2011). Briefly, mtDNA was amplified with 24 cycles, PCR products were resolved on agarose gel, and relative amplification was quantified by normalizing the intensity of the long product to the short product (mtDNA=13.4kb/210bp); decrease in the ratio corresponds to increase in mtDNA damage.

Gene transcripts of mtDNA-encoded *Cytochrome b (Cytb)* and *NADH-ubiquinone oxidoreductase chain 6 (ND6)* were quantified by SYBR green based q-PCR.

2.8. Statistics analysis

Data are presented as mean $\pm$ standard deviation. Multiple group comparisons were made by Oneway ANOVA followed by Student-Newman-Keuls test (data with normal distribution) or by Kruskal-Wallis one-way analysis followed by Dunn's test (numbers that did not have normal distribution). A p value < 0.05 was considered statistically significant.

3. Results

3.1 Effect of diabetes on NF- κ B binding at *Rac1* promoter in retinal microvessels

Retinal microvessels from diabetic rats had 3 fold increase in p65 binding at the *Rac1* promoter compared to the retinal microvessels from the age-matched normal rats; in each sample, IgG control values were less than 0.5% of those obtained from p65 antibody (Figure 1A).

Consistent with the increased binding, microvessels from diabetic rats also had 2-3 fold increase in *Rac1* transcription and the enzyme activity (Figure 1B). In the same animals, *Nox2* transcripts were also elevated by about 2 fold in their retinal microvasculature compared to the values obtained from normal rats (Figure 1C).

3.2. Inhibition of acetylation of NF- κ B and its binding at *Rac1* promoter

Acetylated form of NF- κ B is transcriptionally active (Yang et al., 2010), and diabetes inhibits the activity of deacetylase Sirt1 in the retina (Kowluru et al., 2014b; Mishra et al., 2015). The effect of overexpression of *Sirt1* on *Rac1* transcriptional activation was

determined in mice overexpressing *Sirt1*. Figure 2A shows that *Sirt1* overexpression prevented diabetes-induced increase in p65 binding at the *Rac1* promoter. In the same *Sirt1*-overexpressing diabetic mice, acetylation of p65 was also ameliorated (Figure 2B). The values obtained from the retina of age-matched *Sirt1* overexpressing diabetic and non diabetic mice, and wildtype normal mice were not different from each other.

3.3. Overexpression of *Sirt1* and Rac1-Nox2 activation

Role of acetylation in Rac1-Nox2 activation was investigated in mice overexpressing *Sirt1*. Although *Rac1* mRNA levels were elevated by 3 fold in the retina of wildtype diabetic mice compared to normal mice, overexpression of *Sirt1*, as expected, also protected *Rac1* transcription, further confirming the role of acetylation in retinal *Rac1* transcription in diabetes (Figure 2C). Consistent with *Rac1* transcription, activation of Nox2 was also ameliorated, and the values obtained from diabetic mice overexpressing *Sirt1* were not significantly different from those observed from wildtype or *Sirt1* overexpressing normal mice (Figure 2D). Figure 2E is included to show that the retina of *Sirt1* overexpressing mice had >50% increase in *Sirt1* expression (protein and mRNA).

3.4. *Sirt1* and mitochondrial damage

Since increase in Rac1-Nox2, via producing cytosolic ROS, damages mitochondria; the effect regulation of Rac1 on mitochondrial damage was investigated. As shown in figure 3A, overexpression of *Sirt1* attenuated increase in retinal mtDNA damage initiated by diabetes, and also ameliorated decrease in its transcription; the transcripts of *Cytb* and *ND6* in diabetic mice overexpressing *Sirt1* were significantly higher compared to age-matched wildtype diabetic mice (Figure 3B).

4. Discussion

Retinopathy is one of the most devastating complications of diabetes, and due to multifactorial etiology, the molecular mechanism of its development remains unclear. The retina experiences many metabolic abnormalities, and gene expressions are altered (Kowluru et al., 2015). Our previous study has shown that Rac1-mediated Nox2 activation in the retina is an early event in the pathogenesis of diabetic retinopathy, and it leads to the mitochondrial damage and initiation of capillary cell apoptosis (Kowluru et al., 2014a; Kumar et al., 2015). *Rac1* promoter has binding sites for many transcriptional factors including NF- κ B and Sp1, and here we present exciting results showing that in diabetes, the binding of p65 subunit of NF- κ B at *Rac1* promoter is increased in the retinal microvasculature (the site of histopathology of diabetic retinopathy), resulting in the activation of Rac1-Nox2. Furthermore, overexpression of deacetylase *Sirt1* attenuates diabetes-induced acetylation of p65, decreases its binding at the *Rac1* promoter, and prevents the activation of Rac1-Nox2 mediated mitochondrial damage. Taken together, these results suggest that the molecular mechanism of retinal Rac1 transcriptional activation in diabetes involves p65 binding at its promoter, and acetylation-deacetylation status modulates this binding.

The transcriptional activity of NF- κ B is increased in the retina in diabetes, and NF- κ B activation is implicated in the accelerated apoptosis of retinal capillary cells, a phenomenon

that precedes the development of histopathology characteristic of diabetic retinopathy (Kern et al., 2000; Kowluru et al., 2003; Romeo et al., 2002). Posttranslational modifications are considered as one of the major contributors in altering the metabolic machinery and transcription of a gene, and diabetic environment is associated with many such modifications, including methylation, phosphorylation and acetylation (Goldberg et al., 2006; Helzer et al., 2015; Reddy and Natarajan, 2011; Santos et al., 2014). In diabetic retinopathy, acetylation of transcriptional factors Ap1 and NF- κ B in the retina are implicated in activation of matrix-metalloproteinase-9 (MMP-9), which damages mitochondria, and initiates the apoptotic process (Kowluru et al., 2014b; Mishra et al., 2015). Sirt1-mediated deacetylation of p65 decreases its transactivation potential (Yang et al., 2010), and is associated with a multitude of NF- κ B-driven pathways implicated in inflammation (Kauppinen et al., 2013; Yang et al., 2010). In diabetes, retinal Sirt1 is inhibited and Ap1 and p65 are hyper-acetylated (Kowluru et al., 2014b; Mishra et al., 2015). The results presented here clearly show that *Sirt1* overexpression in diabetic mice also protects *Rac1* transcriptional activation, and this is mediated via inhibition of p65 binding at *Rac1* promoter, suggesting a critical role of this deacetylase in *Rac1* transcriptional activation, seen in the retina in diabetes.

Rac1 is a key regulator of Nox2 activation, and Rac1-Nox2 mediated cytosolic ROS are implicated in damaging the mitochondria, and disturbing mitochondrial homeostasis (Kowluru et al., 2015; Kowluru et al., 2014a). The role of acetylation-mediated activation of p65 in *Rac1* transcriptional activation is further supported by our results showing that in the same *Sirt1* overexpressing mice, diabetes-induced activation of Nox2 is also ameliorated. The damaged mtDNA compromises its transcription and the electron transport chain system becomes suboptimal, which continues to propagate the futile cycle of ROS (Kowluru et al., 2015; Madsen-Bouterse et al., 2010). Our previous study has documented that *Sirt1* overexpression in retinal endothelial cells ameliorates their accelerated apoptosis induced by hyperglycemic milieu (Mishra et al., 2015). The results presented here show that *Sirt1* overexpression prevents diabetes-induced mtDNA damage and protects decrease in its transcription, as determined by transcripts of mtDNA-encoded *Cytb* of complex III and *ND6* of complex I of the electron transport chain, further strengthening the role of NF- κ B acetylation-mediated Rac1 activation in mitochondrial homeostasis, and eventually, in the development of diabetic retinopathy.

Taken together, here we provide a novel mechanism for transcriptional activation of Rac1 in the retinal microvasculature in diabetes. Our data demonstrate that in diabetes, transcription of retinal *Rac1* is regulated by NF- κ B, and due to its hyper-acetylation, the binding at *Rac1* promoter is increased. Activated Rac1, via Nox2, increases cytosolic ROS, and damages mitochondria. The damaged mitochondria initiate the apoptotic machinery, and accelerated apoptosis of retinal capillary cells leads to the development of diabetic retinopathy. Moreover, damaged mtDNA compromises its transcription and electron transport chain system becomes suboptimal, resulting in a futile cycle of ROS, and the retinopathy continues to progress (Figure 4). Thus, regulation of NF- κ B binding at *Rac1*, by modulating its acetylation (using pharmaceutical/molecular modalities to activate Sirt1) during the early stages of the disease, in addition to protecting MMP-9 activation [16, 17], will also protect

Rac1-Nox2 mediated cytosolic ROS production and mitochondrial damage, and ameliorate the progression of diabetic retinopathy.

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Highlights

Transcription of retinal *Rac1* in diabetes is regulated by NF- κ B.

Hyperacetylation of NF- κ B increases its binding at Rac1 promoter.

Overexpression of deacetylase, Sirt1, prevents diabetes-induced increase in Rac1.

Rac1, via Nox2-ROS, damages mitochondria, initiating a futile cycle of ROS.

Regulation of Sirt1 should ameliorate development of diabetic retinopathy.

Figure 1.

Diabetes increases binding of p65 at *Rac1* promoter. Retinal microvessels from diabetic rats were analyzed for (A) p65 binding at *Rac1* promoter by immuno-precipitating the protein-DNA complex from crosslinked retinal microvessels with p65 antibody, followed by amplification of the *Rac1* promoter regions by SYBR green q-PCR. IgG as antibody was used as a control (indicated as ^). (B) *Rac1* mRNA levels were measured by q-PCR using β -actin as housekeeping gene, and its activity by G-LISA kit. (C) *Nox2* mRNA levels were measured by q-PCR and its activity using lucigenin as an electron acceptor. Each measurement was made in duplicate in 5-7 rats/group, and values are represented as mean $\pm$ SD. The numbers obtained from normal rats are considered as 1 for p65 binding and 100% for gene transcripts and activity assays. Norm=Normal and Diab=Diabetes, * $p < 0.05$ vs normal

Figure 2.

Sirt1 overexpression in mice prevents diabetes-induced increase in p53 binding at *Rac1* promoter and protects mitochondrial homeostasis. (A) Binding of p53 at *Rac1* promoter was determined in the crosslinked retina by immuno-precipitating protein-DNA complex with p53 antibody (or, IgG as control), and (B) acetylation of p53 was quantified by immunoprecipitating acetyl lysine, followed by western blotting for p53 subunit of NF- κ B. Gene transcripts of (C) *Rac1* and (D) *Nox2* were quantified by real time q-PCR using 18S as a housekeeping gene. (E) To confirm *Sirt1* overexpression in the retina, its expression (protein and mRNA) was compared between normal C57BL/6J and *Sirt1* overexpressing mice. The values are represented as mean $\pm$ SD of 4-6 mice in each group. WT-N and WT-D= C57BL/6J mice normal and diabetic respectively, and *Sirt1*-N and *Sirt1*-D=*Sirt1* overexpressing mice normal and diabetic respectively. * $p < 0.05$ vs WT-N and # $p < 0.05$ vs WT-D

Figure 3.

Retinal mtDNA damage and its transcription are protected in diabetic mice overexpressing *Sirt1*. (A) Damage to mtDNA was evaluated by extended length PCR, and the ratio of long to short and relative amplification was quantified by normalizing the intensity of the long product to the short product. (B) Gene transcripts of *Cytb* and *ND6* were quantified by SYBR green based q-PCR using 18S as the housekeeping gene. The values are mean $\pm$ SD of 5-7 mice in each group. WT-N and WT-D= C57BL/6J mice normal and diabetic respectively, and *Sirt*-N and *Sirt*-D=*Sirt1* overexpressing mice normal and diabetic respectively. *p<0.05 vs WT-N and #p<0.05 vs WT-D

Figure 4.

Inhibition of Sirt1 in diabetes increases the binding of acetylated p65 subunit of NF- κ B (Ac-p65) at *Rac1* promoter. Rac1 activation, via Nox2, increases cytosolic ROS (CytROS), and ROS damage mitochondria increasing mitochondrial ROS (mtROS). Mitochondrial damage initiates the apoptotic machinery, and accelerated apoptosis of retinal capillary cells leads to the development of diabetic retinopathy. Further, damaged mtDNA attenuates transcription of mtDNA, and compromises the function of the electron transport chain (ETC) system, fueling into the futile cycle of ROS, and the retinopathy continues to progress.

Table I

Primer sequence

Rat	
<i>Rac1</i> promoter, p65 binding site	Forward 5'-GTCCAGCACTGGGATTCAAGA-3' Reverse 5'-AGCAGTAGAATCCTCAGGTACA-3'
<i>Rac1</i>	Forward 5'-TGCGTTCCCTGGAGAGTACA-3' Reverse 5'-AACACGTCTGTTGCGGGTA-3'
<i>Nox2</i>	Forward 5'-TGCCAGTGTGTCGGAATCTC-3' Reverse 5'-TGTGAATGGCCGTGTGAAGT-3'
<i>β-actin</i>	Forward 5'-CCTCTATGCCAACACAGTGC-3' Reverse 5'-CATCGTACTCCTGCTTGCTG-3'
Mouse	
<i>Rac1</i> promoter, p65 binding site	Forward 5'-CGGAACCCCGTGGTCAATAA-3' Reverse 5'-CCCACAAGACGACAGGGAAA-3'
<i>Rac1</i>	Forward 5'-CACGACCAATGCATTTCTGG-3' Reverse 5'-AAGAACAGTCTGTCTGCGG-3'
<i>Nox2</i>	Forward 5'-TATTGTGGGAGACTGGACGG-3' Reverse 5'-CCAAAGGGTCCATCAACTGC-3'
<i>mtDNA</i> (long)	Forward 5'-AAAATCCCCGAAACAATGACCACCCC-3' Reverse 5'-GGCAATTAAGAGTGGGATGGAGCCAA-3'
<i>mtDNA</i> (short)	Forward 5'-CCTCCCATTCAATTATCGCCGCCCTTGC-3' Reverse 5'-AAAATCCCCGAAACAATGACCACCCC-3'
<i>Cytb</i>	Forward 5'-ACCCGCCCCATCCAACATCTCAT-3' Reverse 5'-TTGAGGCTCCGTTTGCGTGT-3'
<i>ND6</i>	Forward 5'-CCGAAACAAAGATCACCCAG-3' Reverse 5'-GAAGGAGGATTGGGGTAGC-3'
<i>18S</i>	Forward 5'-GCCCTGTAATTGGAATGAGTCCACTT-3' Reverse 5'-CTCCCAAGATCCAACACTACGAGCTTT-3'