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Posttranslational Modification of Mitochondrial Transcription Factor A in Impaired Mitochondria Biogenesis: Implications in Diabetic Retinopathy and Metabolic Memory Phenomenon

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Abstract

Mitochondrial transcription factor A (TFAM) is one of the key regulators of the transcription of mtDNA. In diabetes, despite increase in gene transcripts of TFAM, its protein levels in the mitochondria are decreased and mitochondria copy numbers become subnormal. The aim of this study is to investigate the mechanism(s) responsible for decreased mitochondrial TFAM in diabetes. Using retinal endothelial cells, we have investigated the effect of overexpression of cytosolic chaperone, Hsp70, and TFAM on glucose-induced decrease in mitochondrial TFAM levels, and the transcription of mtDNA-encoded genes, NADH dehydrogenase subunit 6 (*ND6*) and cytochrome b (*Cytb*). To investigate the role of posttranslational modifications in subnormal mitochondrial TFAM, ubiquitination of TFAM was accessed, and the results were confirmed in the retina from streptozotocin-induced diabetic rats. While overexpression of Hsp70 failed to prevent glucose-induced decrease in mitochondrial TFAM and transcripts of *ND6* and *Cytb*, overexpression of TFAM ameliorated decrease in its mitochondrial protein levels and transcriptional activity. TFAM was ubiquitinated by high glucose, and PYR-41, an inhibitor of ubiquitination, prevented TFAM ubiquitination and restored the transcriptional activity. Similarly, TFAM was ubiquitinated in the retina from diabetic rats, and it continued to be modified after reinstitution of normal glycemia. Our results clearly imply that the ubiquitination of TFAM impedes its transport to the mitochondria resulting in subnormal mtDNA transcription and mitochondria dysfunction, and inhibition of ubiquitination restores mitochondrial homeostasis. Reversal of hyperglycemia does not provide any benefit to TFAM ubiquitination. Thus, strategies targeting posttranslational modification could provide an avenue to preserve mitochondrial homeostasis, and inhibit the development/progression of diabetic retinopathy.

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6. Author Disclosure and contribution
Authors declare that they do not have any competing financial interests. JMS researched data and wrote the manuscript, MM researched data, and RAK wrote and edited the manuscript. RAK is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Keywords

diabetic retinopathy; mitochondria; mitochondrial transcription factor A; mtDNA; posttranslational modifications; ubiquitination

1. Introduction

In diabetes, retinal mitochondria become dysfunctional, their biogenesis is compromised, DNA (mtDNA) is damaged, and the proteins encoded by mtDNA are decreased (Madsen-Bouterse, Mohammad et al. 2010; Santos and Kowluru 2011; Santos, Mohammad et al. 2011; Santos, Tewari et al. 2011). Since mtDNA encodes proteins which are important in the electron transport chain, this transport system becomes dysfunctional resulting in increased superoxide levels, and activation of the apoptosis machinery (Madsen-Bouterse, Zhong et al. 2010; Madsen-Bouterse, Mohammad et al. 2010; Santos, Tewari et al. 2011). Apoptosis of retinal cells, including capillary cells, is accelerated, and capillaries begin to degenerate resulting in the histopathology which is the hallmark of diabetic retinopathy (Kern, Tang et al. 2000; Frank 2004).

Mitochondria are equipped with a small circular DNA which transcribes only 13 proteins (Scarpulla 2006; Scarpulla 2008), and the majority of proteins required for mitochondria biogenesis and ATP generation are encoded by nuclear DNA. Nuclear encoded proteins required for mitochondria homeostasis are synthesized in the cytosol and transported to the mitochondria via chaperons, such as heat shock protein 70 (Hsp70), and into the mitochondria via mitochondrial membrane transporters (Scarpulla 2006; Scarpulla 2008; Schmidt, Pfanner et al. 2010). In diabetes, the association of cytosolic Hsp70 with mitochondrial transcription factor A (TFAM) is decreased, and that with matrix metalloproteinase-9 (MMP-9), a protein associated with damage of mitochondria and capillary cell apoptosis, is increased in the retina (Scarpulla 2008; Kowluru, Mohammad et al. 2011; Santos and Kowluru 2011; Santos, Tewari et al. 2011).

TFAM is one of the key factors with many important roles; it binds to the regulatory area of the mtDNA, the displacement loop (D-loop), to initiate the transcription of the proteins encoded by mtDNA (Scarpulla 2006; Scarpulla 2008). In diabetes, although the gene expression of TFAM is increased in the retina, its total protein levels remain unchanged, but mitochondrial protein levels are decreased (Santos and Kowluru 2011; Santos, Tewari et al. 2011). In addition, diabetes also compromises mitochondria membrane transport systems in the retina (Zhong and Kowluru 2011). Diabetic environment favors posttranslational modifications, and these modifications are considered to play important role in its complications (Goldberg, Whiteside et al. 2006; Reddy and Natarajan 2011; Zhong and Kowluru 2011; Song, Peng et al. 2013; Zhong and Kowluru 2013). Posttranslational modifications, in addition to increasing the functional diversity of the proteome and facilitating proteolytic cleavage of the entire proteins, can also alter the localization and interaction of proteins with other cellular molecules (Piantadosi and Suliman 2006; Bergink and Jentsch 2009; Madsen-Bouterse, Mohammad et al. 2010). Ubiquitination of mitochondrial proteins is shown to be vital for the maintenance of mitochondrial

homeostasis by removing the dysfunctional mitochondria (Neutzner, Benard et al. 2008). The role of posttranslational modification in the impaired translocation of TFAM into the mitochondria in diabetes remains to be investigated.

Epidemiologic and experimental studies have demonstrated that prior exposure to hyperglycemia has long-lasting consequences, and diabetic retinopathy continues to progress even after the hyperglycemic insult is terminated, suggesting a 'metabolic memory' phenomenon (Engerman and Kern 1987; Diabetes Control and Complications Trial Research Group 1993; Diabetes Control and Complications Trial Research Group 1998; Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group 2000; Kowluru 2003). Mitochondria remain dysfunctional, and mtDNA continues to be damaged with impaired biogenesis and subnormal protein levels of TFAM in the mitochondria even after re-institution of normal glycemic control in diabetic rats (Madsen-Bouterse, Mohammad et al. 2010; Santos and Kowluru 2011). Furthermore, we have shown that the retinal proteins continue to be posttranslationally modified (Kanwar and Kowluru 2009; Madsen-Bouterse, Mohammad et al. 2010). The role of posttranslational modification of TFAM in the metabolic memory phenomenon is not understood.

The aim of this study is to investigate the mechanism(s) responsible for decreased TFAM protein levels in the retinal mitochondria in diabetes. Using retinal endothelial cells, the cells associated with histopathology of diabetic retinopathy, we have investigated the effect of posttranslational modifications of TFAM on mtDNA transcription, and confirmed the *in vitro* results in the retina from rodent model of diabetic retinopathy. The role of posttranslational modification of TFAM in the metabolic memory phenomenon was also investigated, both *in vivo* and *in vitro* models of diabetic retinopathy.

2. Methods

2.1 Retinal endothelial cells

Retinal endothelial cells isolated from bovine eyes (BRECs) were cultured on polystyrene culture plates coated with 0.1% gelatin in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air, as routinely performed in our laboratory (Kowluru and Abbas 2003; Madsen-Bouterse, Mohammad et al. 2010; Santos and Kowluru 2011; Tewari, Zhong et al. 2012). The cells from 4^{th}-6^{th} passage were incubated in Dulbecco's modified Eagle medium (DMEM) containing 2% heat-inactivated fetal bovine serum, 10% Nu serum, 50μg/ml heparin, 1μg/ml endothelial growth factor, and antibiotic/antimycotic, supplemented with 5 or 20mM glucose for 4 days. To evaluate the effect of inhibition of ubiquitination on mitochondrial transcription, the cells were pre-incubated with 5μM PYR-41 (Sigma Aldrich, St. Louis, MO) for 4 hours (Guan and Ricciardi 2012) before incubating in 5mM glucose or 20mM glucose for 4 days. Each experiment included an osmotic control in which the cells were incubated with 20mM mannitol instead of 20mM glucose.

To investigate the effect of overexpression of cytosolic Hsp70, and that of increasing overall TFAM, on glucose-induced decrease in mtDNA transcription, these proteins were

overexpressed using 2μg/ml *HSPA1A* (gene that encodes the cytosolic HSP70) or GFP-tagged *TFAM* plasmid with TurboFectin 8.0 from OriGene Technologies (Rockville, MD). After transfection, cells were rinsed with DMEM, and incubated in 5mM or 20mM glucose media for 4 days. In parallel, incubation with only the transfection reagent was carried out (Mock). The transfection efficiency was verified by microscopy by assessing the red fluorescence with mouse monoclonal antibody Hsp70 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by Image J software quantification, or by quantifying green fluorescence of GFP-tagged TFAM. Cells were washed with PBS mounted with Vecta Shield containing DAPI (Vector Laboratories Burlingame, CA) and examined under a Zeiss ApoTome using 40X magnification (Carl Zeiss Inc.) (Tewari, Santos et al. 2012; Santos and Kowluru 2013).

To examine the effect of reversal of high glucose insult on posttranslational modifications of TFAM, the cells from 4^{th}-6^{th} passage were incubated in 20mM glucose for 4 days followed by 5mM glucose for 4 additional days (20-5). Parallel controls included cells incubated in continuous 5mM glucose or in 20mM glucose for the entire duration of the experiment. The cells received fresh media every 48 hours. In the 20-5 group, at the end of the initial 4 days of 20mM glucose, the cells were rinsed with DMEM before changing to 5mM glucose medium (Zhong and Kowluru 2011; Zhong and Kowluru 2013).

2.2 Rats

Wistar rats (male, body weight 200g) were randomly assigned into two groups: normal or streptozotocin-induced diabetic (55 mg/kg body weight). Diabetic rats were either allowed to remain in poor glycemic control for 8 months (PC); in PC for 4 months, followed by good glycemic control for 4 additional months (Rev) or in good glycemic control for 8 months (GC).The rats in poor glycemic control received 1-2 IU insulin 4-5 times a week to prevent ketosis and weight loss and the rats in which good glycemic control received insulin twice daily (5-7 IU/day) to maintain a steady gain in body weight and blood glucose values below 150 mg/dl. These procedures are routinely performed in our laboratory (Kowluru 2003; Santos and Kowluru 2011; Zhong and Kowluru 2011; Santos and Kowluru 2013). Each group had 10 or more rats, and at the end of the desired experimental duration, the animals were euthanized by CO_2 inhalation, and the retina were immediately isolated. Treatment of animals conformed to the Association for Research in Vision and Ophthalmology's Resolution on Treatment of Animals in Research (National Institutes of Health) and the Institutional guidelines.

2.3 Sample preparation

Mitochondria and cytosol were isolated from retina using mitochondria isolation kit from Invitrogen (Carlsbad, CA, USA), according to the vendor's protocols. As reported previously, mitochondria prepared by this method are largely devoid of contaminations (Madsen-Bouterse, Mohammad et al. 2010; Santos, Tewari et al. 2011; Tewari, Santos et al. 2012). To isolate the cytosol fraction, the homogenate was centrifuged at 105,000×*g* for 90 minutes. Protein was estimated by the bicinchoninic acid protein assay (Sigma-Aldrich, St Louis, MO).

2.4 Protein expression

Protein (20-60 g cytosol, mitochondrial) was separated on an 8–19% SDS–PAGE, transferred to a nitrocellulose membrane, and blocked with 5% non-fat milk for 1 hour. The membranes were incubated with antibodies against TFAM (Santa Cruz Biotechnology, Santa Cruz-CA) or Hsp70 (Abcam, Cambridge, MA) overnight at 4°C. β-actin (Sigma-Aldrich) was used as a loading control for cytosolic fraction, and Cox IV (Santa Cruz Biotechnology) for the mitochondria fraction.

2.5 Gene expression

Total RNA was extracted from BRECs with Trizol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). mRNA levels of *Hsp70* and mtDNA encoded protein *NADH dehydrogenase 1 and 6 (ND1 and ND6*) were quantified by real-time quantitative RT-PCR (qPCR) using the SYBR green assay using the conditions routinely employed in our laboratory (Santos and Kowluru 2011; Santos, Tewari et al. 2011; Santos, Tewari et al. 2013). Due to some concerns about proper amplification, gene transcripts of *Cytochrome oxidase b (Cytb)* were quantified by conventional qPCR using the conditions previously reported by us (Madsen-Bouterse, Mohammad et al. 2010). Primer list is presented in Table I. Relative amplification was quantified by normalizing the gene-specific amplification to that of *β-actin* in each sample. Changes in mRNA abundance were calculated using the $\Delta\Delta C_t$, and for *Cytb* the products were analyzed on an agarose gel and the ratio *Cytb* and *β-actin* was calculated as reported by us previously (Madsen-Bouterse, Mohammad et al. 2010).

2.6 Ubiquitination of TFAM

Ubiquitination of TFAM was performed by 2-3 independent techniques. For immunoprecipitation, ~150μg protein (BRECs or retina) was incubated overnight at 4°C with 1μg of ubiquitin or TFAM antibody, followed by 1 hour with 20μl Protein A and G Plus agarose immunoprecipitation beads reagent (pre-washed and suspended in the lysis buffer). The beads were washed 4 times with lysis buffer, and the proteins were separated by SDS-PAGE. The membrane was immunoblotted with anti-TFAM or ubiquitin respectively (Santa Cruz Biotechnology) and developed by chemiluminescence (Kowluru, Mohammad et al. 2011; Santos and Kowluru 2011).

Ubiquitination of TFAM was also assessed by immunofluorescence microscopy. The cells grown on 12-mm-diameter coverslips coated with 0.1% gelatin were incubated with 5 or 20mM glucose for 4 days. At the end of the treatment, coverslips were rinsed with PBS and the cells were fixed with cold methanol (-20^{o}C) for 15 minutes. They were blocked in 5% BSA for 1 hour and incubated with rabbit anti-TFAM antibody and mouse anti-ubiquitin antibody (Santa Cruz Biotechnology) overnight. After washing the cells with PBS, they were incubated with anti-mouse (Texas Red conjugated; Molecular Probes) and anti-rabbit (FITC green conjugate; Sigma) secondary antibodies for 1 hour in the dark. Cells were washed with PBS mounted with Vecta Shield mounting solution (Vector Laboratories) and examined under a Zeiss ApoTome using 40X magnification (Madsen-Bouterse, Zhong et al. 2010; Tewari, Santos et al. 2012; Tewari, Zhong et al. 2012; Santos and Kowluru 2013).

2.7 Statistics analysis

All of the data presented here was statistically analyzed using Sigma Stat software. The Shapiro-Wilk test was performed to test for normal distribution. For results with normal distribution, T-test was performed for analysis of two groups, and ANOVA followed by Bonferroni test for variables with more than two groups. Data that did not present normal distribution was analyzed by Mann-Whitney U for variable containing two groups and Kruskal-Wallis test followed by Dunn's for more than two groups. Data are expressed as means ± standard deviation, and p value <0.05 was considered statistically significant.

3. Results

3.1 Retinal endothelial cells

3.1.1 Role of Hsp70 in decreased mitochondrial protein levels of TFAM, and in mtDNA transcription—Our previous work has shown that despite increased gene expression of TFAM and no change in its total protein levels in hyperglycemic milieu, TFAM levels in the mitochondria are decreased (Santos and Kowluru 2011; Santos, Tewari et al. 2011). Cytosolic Hsp70 is a chaperone which carries mitochondrial proteins from the cytosol to the mitochondria surface. To investigate its role in transporting TFAM from cytosol to the mitochondria, association of Hsp70 with TFAM was determined by co-immunoprecipitating either Hsp70 or TFAM followed by western blotting for TFAM or Hsp70 respectively. Figures 1a and 1b show that the amount of TFAM associated with Hsp70 was less in the cells exposed to high glucose compared to the cells exposed to normal glucose. Furthermore, while high glucose significantly decreased *Hsp70* mRNA levels, 20mM mannitol had no effect (Figure 1c). Consistent with the decrease in *Hsp70* mRNA levels, its cytosolic protein levels were also decreased by ~35% in the cells exposed to high glucose (Figure 1d).

To further evaluate the role of cytosolic Hsp70 in the translocation of TFAM, cells overexpressing *Hsp70* were used. Overexpression of *Hsp70* did not ameliorate glucose-induced decrease in mitochondrial TFAM levels, the values obtained from *Hsp70* transfected and untransfected cells exposed to high glucose were similar (Figure 2a). Since the transcription of mtDNA is dependent on TFAM protein levels in the mitochondria, and the gene expression of mtDNA-encoded proteins, e.g., *Cytb*, *ND6*, represents an indirect marker of the mitochondrial accumulation of TFAM, the effect of *Hsp70* overexpression on mtDNA transcription was evaluated. As shown in figure (2b & c) overexpression of *Hsp70* had no beneficial effect on glucose-induced decrease in mtDNA-encoded *ND6* and *Cytb;* their mRNA levels remained 30-50% lower compared to the cells incubated in normal glucose. Consistent with this, the transfection reagent alone did not increase high glucose-induced decrease in *ND6* mRNA levels (Figure 2b). Figures 2d&e are included to show the transfection efficiency of *Hsp70*. Quantification of the Hsp70 fluorescence using Image J software shows ~50% more Hsp70 in the cells transfected with *Hsp70* plasmids compared to the cells incubated with the transfection regent alone.

3.1.2 Effect of overexpression of TFAM on mtDNA biogenesis—TFAM plays an important role in mitochondria biogenesis, and its levels are decreased in the retinal

mitochondria in diabetes (Scarpulla 2008; Santos and Kowluru 2011; Santos, Tewari et al. 2011). To investigate the effect of TFAM overexpression on the mitochondria biogenesis, the cells were transfected with the GFP-tagged *TFAM* plasmids. Overexpression of *TFAM* prevented glucose-induced decrease in its levels in the mitochondria (Figure 3a), and also restored mtDNA transcription, as shown by increased expression of mtDNA-encoded *ND6* and *Cytb* compared to untransfected cells exposed to high glucose (Figures 3b & c). In contrast, when the cells were incubated with the transfection reagent alone, without any plasmids, the expression of *ND6* remained subnormal. Cells overexpressing *TFAM*, incubated in normal or high glucose had similar mtDNA transcription, as evidenced by similar mRNA levels of *ND6* (Figure 3b). Figure 3d is included to show that the cells transfected with GFP-tagged *TFAM* plasmids had high levels of TFAM-GFP.

3.1.3 Ubiquitination of TFAM—Ubiquitination, a post translational modification which is initiated by ubiquitin-activating enzyme UBA1 (E1), covalently attaches ubiquitin to the proteins labeling them for destruction (Rosenbaum, Fredrickson et al. 2011). To investigate if increase in ubiquitination has any effect on the decreased TFAM protein levels in the mitochondria, ubiquitination of TFAM was quantified by immunoprecipitation, and confirmed by fluorescence microscopy. The expression of TFAM in the ubiquitin immunoprecipitate was increased in the cells exposed to high glucose compared to normal glucose (Figures 4a). Ubiquitination is a complex process in which proteins can be mono, multi-mono or poly ubiquitinated, and the polyubiquitination could be homotypic or heterotypic (Komander 2009). The presence of a high molecular weight band, instead of ~25KD band, suggests that high glucose polyubiquinated TFAM. In support others have shown tetra-ubiquitination of TFAM in sperm extracts during porcine gametogenesis (Antelman, Manandhar et al. 2008). Figure 4b presents the quantification of the ubiquitinated TFAM. TFAM ubiquitination was confirmed by fluorescence microscopy; figure 4c shows that the cells incubated in high glucose had significantly high ubiquitin levels compared to the cells incubated in normal glucose as evidenced by increased red staining. When TFAM (green) was co-localized with ubiquitin (red), increased yellowish-orange staining was observed in high glucose conditions compared to normal glucose.

To confirm the role of ubiquitination of TFAM in its impaired transport into the mitochondria, the effect of an irreversible inhibitor of E1, PYR-41 (Guan and Ricciardi 2012), on mitochondria transcription was evaluated. As shown in Figure 5a, PYR-41 inhibited glucose-induced ubiquitination of TFAM and the values obtained from the PYR-41 treated cells incubated in 5mM or 20mM glucose were not significantly different from each other (Figure 5b). Furthermore, PYR-41 also prevented glucose-induced decrease in the transcription of mtDNA- encoded proteins *ND1* and *ND6* (Figure 5c & d), suggesting amelioration of glucose-induced increase in mitochondrial ROS levels.

3.2 Rat Retina

3.2.1 Effect of diabetes on ubiquitination of TFAM—Consistent with the results from endothelial cells, diabetes decreased the expression of Hsp70 in the retina; its mRNA levels were decreased by ~50% and protein expression by 30% in diabetic rats compared to normal rats (Figure 6a & b). In the same retina, ubiquitination of TFAM, as estimated by

immunoprecipitating either ubiquitin, or TFAM, followed by western blotting for TFAM or ubiquitin respectively (Figures 7a-c), was increased by over 70% in the retina from diabetic rats compared to the values from normal rat retina.

3.2.2 Reversal of hyperglycemia and ubiquitination—Reinstitution of good control in diabetic rats, which has followed poor control, fails to reverse mtDNA damage in the retina and mtDNA-encoded proteins remain subnormal with decreased protein levels of TFAM in the mitochondria (Madsen-Bouterse, Mohammad et al. 2010; Santos and Kowluru 2011). To investigate the mechanism responsible for decreased TFAM in the mitochondria, the effect of reversal of hyperglycemia on the posttranslational modification of TFAM was investigated. In addition to providing no beneficial effect of re-institution of good glycemic control on the Hsp70 expression (Figure 6), retinal proteins continued to be ubiquitinated. As shown in figure 7, TFAM remained polyubiquitinated, and the values of TFAM ubiquitination in the retina from rats in PC and Rev groups were significantly higher compared to those from normal group. However, in contrast, when good glycemic control was initiated soon after induction of diabetes, expression of Hsp70 and ubiquitination of retinal TFAM was similar to that observed in the age-matched normal rat retina (Figures 6&7).

The role of the posttranslational modification of TFAM in the metabolic memory was further confirmed in retinal endothelial cells. Gene expression of *Hsp70* remained subnormal even after 4 days of normal glucose that followed 4 days of high glucose (Figure 1c). Consistent with our previous reports (Madsen-Bouterse, Zhong et al. 2010), the gene transcripts of mtDNA-encoded *ND6* and *Cytb* continued to be subnormal (Figure 2b&c). In the same cell preparations, reversal of high glucose insult had no beneficial effect on the ubiquitination of TFAM, as confirmed by both immunoprecipitating ubiquitin or TFAM, followed by western blotting for TFAM or ubiquitin respectively (Figures 4a,b&d).

4. Discussion

Apoptosis of capillary cells precedes the development of histopathology that is considered the hallmark of diabetic retinopathy, and mitochondria dysfunction plays a pivotal role in the accelerated apoptosis of retinal capillary cells (Kern, Tang et al. 2000; Kowluru and Abbas 2003; Madsen-Bouterse, Zhong et al. 2010; Madsen-Bouterse, Mohammad et al. 2010; Santos, Tewari et al. 2011; Tewari, Santos et al. 2012; Santos and Kowluru 2013). Mitochondrial ROS are increased in the retina in diabetes impairing mtDNA biogenesis, and mitochondria copy numbers are decreased (Kanwar, Chan et al. 2007; Madsen-Bouterse, Mohammad et al. 2010; Santos, Tewari et al. 2011). TFAM is an essential transcriptional factor in the biogenesis of mitochondria, and in diabetes its mRNA levels are increased in the retina, but its mitochondrial levels are decreased (Scarpulla 2008; Santos and Kowluru 2011; Santos, Tewari et al. 2011). Here, we show that TFAM is posttranslationally modified, and the chaperone which carries TFAM to the mitochondria surface, Hsp70, appears to play less significant role, as its overexpression does not alleviate hyperglycemia-induced decrease in mitochondrial protein levels of TFAM. However, pharmacological inhibition of ubiquitination of TFAM helps restore normal mtDNA transcription and mitochondrial homeostasis. Furthermore, reversal of hyperglycemia by normal glycemia

does not attenuate TFAM ubiquitination, and it continues to be polyubiquitinated suggesting the role of TFAM ubiquitination in the metabolic memory phenomenon associated with the continued progression of diabetic retinopathy.

TFAM binds to the mtDNA to regulate transcription and mtDNA, but to reach to the mitochondria, it requires a complex mechanism (Ohgaki, Kanki et al. 2007; Malarkey, Bestwick et al. 2012). Cytosolic Hsp70 is one of the key chaperones which transports most of the proteins to the mitochondria membrane (Atalay, Oksala et al. 2009). We have shown that the amount of Hsp70 associated with MMP-9 is significantly increased in the retina, while that with TFAM is decreased (Kowluru, Mohammad et al. 2011; Santos and Kowluru 2011; Santos, Tewari et al. 2011). In addition to the decreased mitochondrial Hsp70 (Kowluru, Mohammad et al. 2011), here our data show that the levels of cytosolic Hsp70 are also decreased, suggesting subnormal chaperon activity. However, when cytosolic Hsp70 is overexpressed, it fails to ameliorate glucose-induced impaired mtDNA biogenesis, clearly implying that cytosolic Hsp70 might not be the major factor responsible for chaperoning TFAM to the mitochondria.

Using multiple independent methods, including copy number and transcription, mtDNA density and mtDNA replication, we have shown that retinal mitochondria biogenesis is decreased in diabetes. However, despite decrease in biogenesis, the gene expression of transcription factors and proteins important in mtDNA biogenesis, e.g., peroxisome proliferator-activated receptor γcoactivator-1α, nuclear regulatory factor and TFAM are increased (Santos and Kowluru 2011; Santos, Tewari et al. 2011; Santos, Tewari et al. 2012). Diabetes also downregulates mitochondrial transport system, and the association of TFAM with Tom70 and Tim44 of the mitochondria transport system is decreased (Santos and Kowluru 2013). Here we show that the overexpression of TFAM protects the cells from high glucose-induced decrease in TFAM protein levels in the mitochondria and revives its transcriptional capacity; these results suggests that posttranslational modification of TFAM could be contributing to in its decreased transport to the mitochondria. In support, diabetic environment facilitates posttranslational modifications of proteins and histones (Goldberg, Whiteside et al. 2006; Suarez, Hu et al. 2008; Kanwar and Kowluru 2009; Reddy and Natarajan 2011; Zhong and Kowluru 2011; Harcourt, Penfold et al. 2013; Song, Peng et al. 2013; Zhong and Kowluru 2013). Furthermore, in support of the role of posttranslational modification in decreased protein levels of TFAM in the mitochondria, our previous study has shown that the posttranslational modifications of glyceraldehyde dehydrogenase favor its translocation into the nucleus (Kanwar and Kowluru 2009; Madsen-Bouterse, Mohammad et al. 2010) .

Ubiquitination, an enzymatic posttranslational modification, targets proteins for destruction, and regulates protein stability, subcellular distribution, DNA-binding affinity and transcriptional activity (Neutzner, Benard et al. 2008; Bergink and Jentsch 2009). Ubiquitination of mitochondrial proteins is considered vital for the maintenance of mitochondrial homeostasis, and in removing the dysfunctional mitochondria (Neutzner, Benard et al. 2008). This proteasomepathway helps eliminate misfolded proteins that are generated under stress conditions, and is initially activated by E1 enzyme which activates ubiquitin. In diabetes, ubiquitin-proteasome pathway is increased in the kidney affecting its

insulin activity (Gao, Chen et al. 2013), and ubiquitination of sarcoplasmic reticulum calcium ATPase 2 is implicated in diabetes-induced diastolic dysfunction (Takada, Miki et al. 2012). Here, our results suggest that polyubiquitination of TFAM could be one of the mechanisms responsible for its subnormal levels in the mitochondria. Consistent with our results, others have shown that polyubiquitination of TFAM in sperm extracts during porcine gametogenesis induces mitochondria degradation (Antelman, Manandhar et al. 2008). To further support the role of ubiquitination, inhibition of E1 by PYR-41 alleviates posttranslational modification of TFAM and restores mitochondria transcription machinery. Since impaired mitochondria homeostasis is important in diabetic retinopathy (Kowluru and Abbas 2003; Madsen-Bouterse, Zhong et al. 2010; Madsen-Bouterse, Mohammad et al. 2010; Santos and Kowluru 2011; Santos, Tewari et al. 2011; Tewari, Santos et al. 2012; Tewari, Zhong et al. 2012; Santos and Kowluru 2013), our data presented here clearly implicate that the inhibition of ubiquitination has potential to prevent its development by maintaining mitochondria homeostasis and TFAM's transcriptional activity. The focus of our study was to understand the role of posttranslational modifications in impaired mtDNA biogenesis, and we cannot rule out the role of Ubiquitin-26S proteasome system in decreased levels of TFAM in the mitochondria. However, since diabetes does not decrease the total protein expression of TFAM suggesting that the degradation of TFAM in the cytosol might not be responsible for its decreased levels in the mitochondria. Furthermore, phosphorylation of TFAM is also shown to decrease its protein levels in mitochondria and reduce its subnormal transcriptional activity (Lu, Lee et al. 2013). Although the present study investigated ubiquitination of TFAM, the role of other posttranslational modifications, including phosphorylation, nitration and glycation, in its impaired transport to the mitochondria in diabetes, however, cannot be ruled out.

It is well established that the progression of diabetic retinopathy does not halt once the glycemic insult is terminated, and the previous severity and the duration of glycemic control dictates the outcome of the glycemic control that follows suggesting a metabolic memory or the legacy effect (Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group 2000; Kowluru 2003). Retinal mitochondria remain dysfunctional, and their DNA continues to be damaged and transcription impaired with subnormal levels of TFAM in the mitochondria (Santos and Kowluru 2011; Tewari, Zhong et al. 2012). The results presented here show that the TFAM remains polyubiquitinated, possibly contributing to its continued decreased mitochondrial protein levels and the transcription of mtDNA. Consistent with this, posttranslational modifications are postulated to play a critical role in the metabolic memory associated with diabetic complications, including retinopathy (Kowluru 2003; Kanwar and Kowluru 2009; Reddy and Natarajan 2011; Zhong and Kowluru 2011; Zhong and Kowluru 2013). However, if good glycemic control is initiated soon after induction of diabetes, TFAM escapes polyubiquitination, suggesting that once TFAM is posttranslationally modified, de-ubiquitination of TFAM becomes difficult even if the glycemic insult is reversed.

5. Conclusion

In conclusion, we have provided novel data suggesting that due to ubiquitination of TFAM in diabetes, its transport to the mitochondria (the site of its action) is impaired, resulting in

subnormal mtDNA biogenesis. Furthermore, reversal of hyperglycemic insult by normal glycemia does not provide any benefit and TFAM continues to be posttranslationally modified, and retinopathy continues to progress. Thus, modulation of ubiquitination by pharmacological means could have potential to maintain mitochondrial homeostasis, and inhibit/retard the development of diabetic retinopathy.

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Highlights

TFAM is one of the key regulators of mtDNA transcription.

TFAM ubiquitination in diabetes impedes its transport to the retinal mitochondria.

Decreased TFAM levels in the mitochondria attenuate retinal mtDNA biogenesis.

Preserving TFAM will protect retinal mitochondria and inhibit diabetic retinopathy.

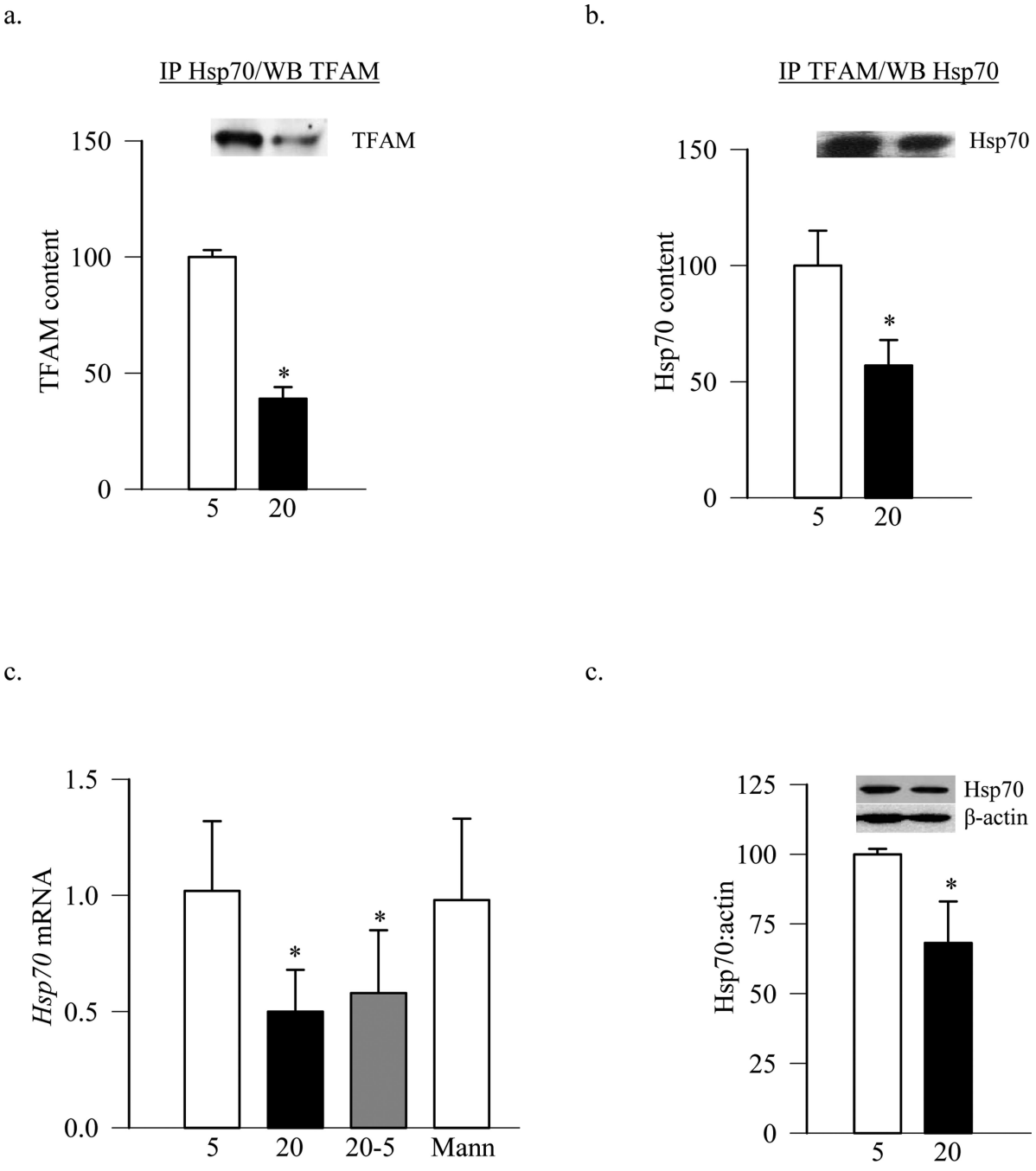

Figure 1.

Exposure of retinal endothelial cells to high glucose decreases the association of TFAM with Hsp70. Association of Hsp70 with TFAM was quantified by immunoprecipitation of (a) Hsp70 or (b) TFAM in the cells (150μg protein) incubated in 5mM or 20mM glucose, followed by western blot analysis for TFAM or Hsp70 respectively. (c) *Hsp70* mRNA level was measured by real time q-PCR using *β-actin* as internal control. (d) Cytosolic expression of Hsp70 was quantified by western blot using the expression of β-actin as a loading control. The values are presented as means ± SD of 3-4 experiments, each done in duplicate. 5 =

5mM of glucose; 20 = 20mM glucose; 20-5= 4 days in 20mM glucose followed by 4 days in 5mM glucose; Mann= 20mM of Mannitol. *p<0.05 compared to 5mM glucose.

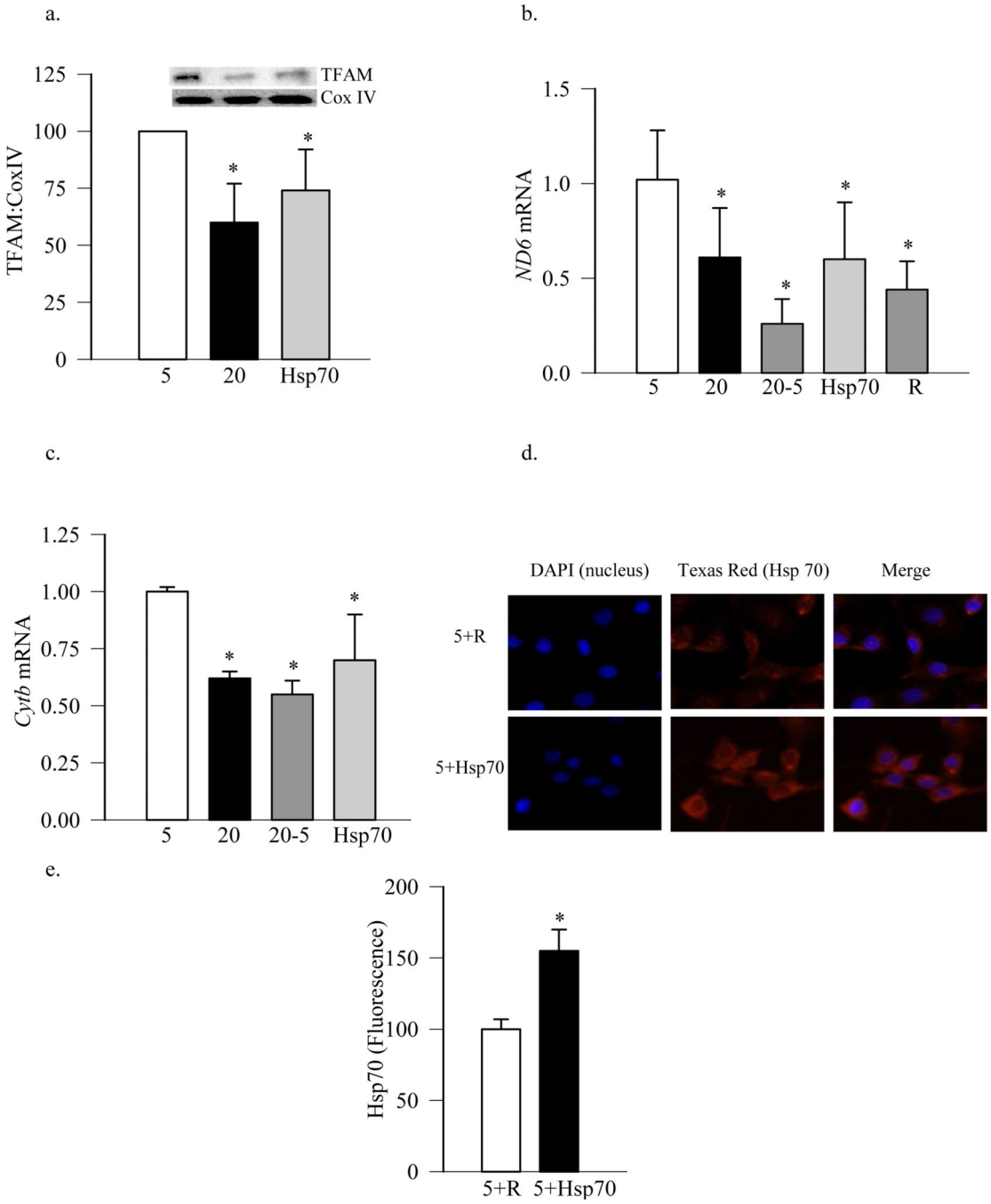

Figure 2.

Overexpression of *Hsp70* in endothelial cells does not affect TFAM levels in the mitochondria. (a) TFAM levels in the mitochondria were quantified by western blot technique using Cox IV as internal control. Gene transcripts of (b) *ND6* and (c) *Cytb* were assessed by qPCR using *β-actin* as a housekeeping gene. (d) Transfection efficiency of *Hsp70* was determined by microscopy using Texas Red conjugated secondary antibodies for Hsp70, and the cells were examined under a Zeiss ApoTome at 40X magnification. (e) The intensity of fluorescence was quantified in the cells transfected with *Hsp70* or just with the

reagent, and values obtained from the cells with the reagent were considered as 100%. Each measurement was made in duplicate using three different cell preparations. The values are presented as means ± SD. 5= 5mM glucose; 20=20mM glucose; Hsp70= cells overexpressing *Hsp70* and incubated in 20mM glucose; 20-5= cells incubated in 20mM glucose for 4 days followed by 5mM glucose for 4 additional days; R=cells transfected with reagent alone and incubated in 20mM glucose respectively; 5+R and 5+Hsp70=cells incubated with regent alone or transfected with *Hsp70* respectively, followed by incubation in 5mM glucose. *$p<0.05$ compared to 5mM glucose or R

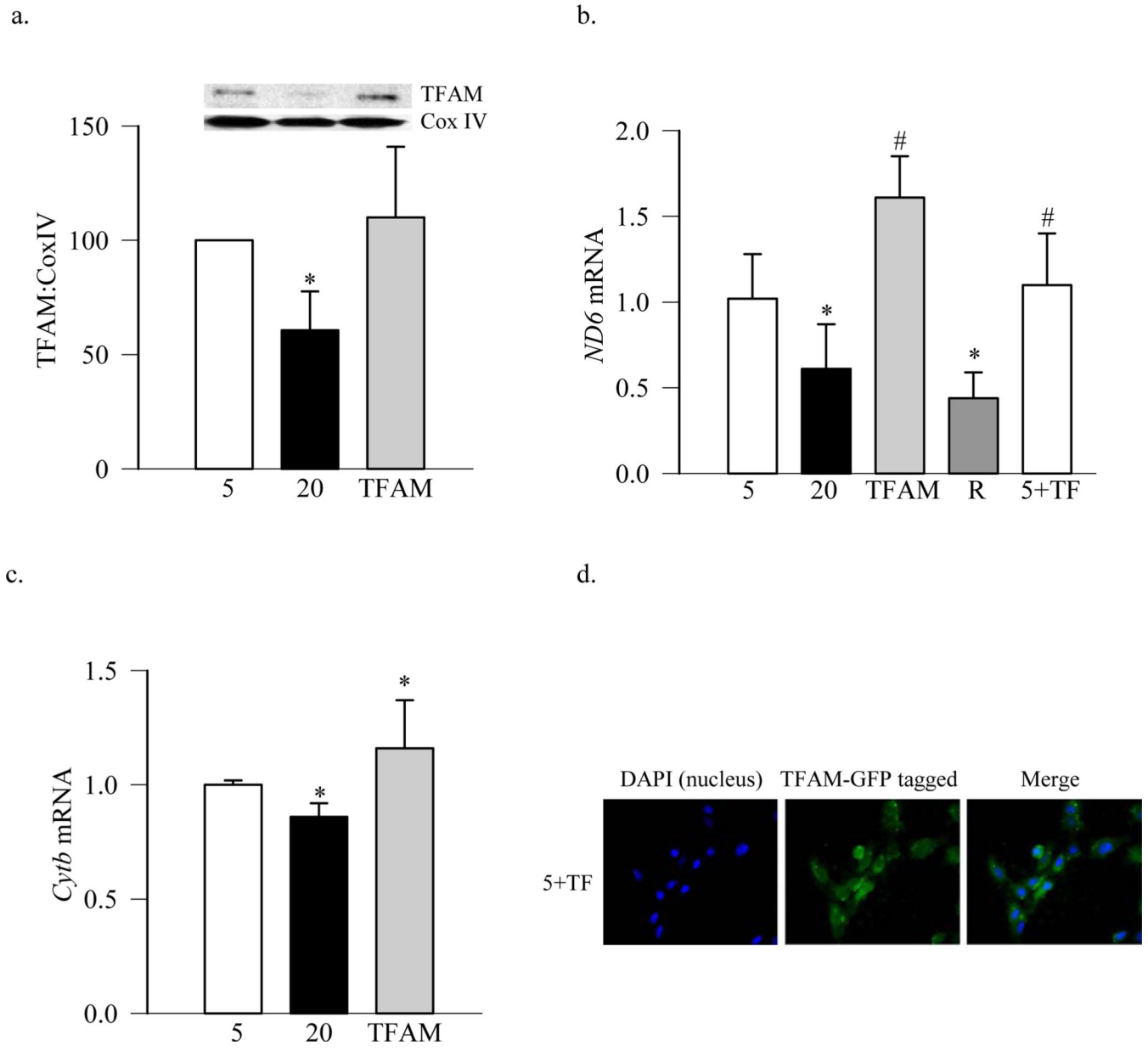

Figure 3.

Overexpression of *TFAM* ameliorates glucose-induced decrease in its mitochondrial protein levels. BRECs transfected with GFP-tagged *TFAM* plasmids were incubated in 5mM or 20mM glucose for 4 days. (a) Levels of TFAM in the mitochondria were quantified by western blot technique using Cox IV as internal control. Gene transcripts of (b) *ND6* and (c) *Cytb* were quantified by real time PCR using *β-actin* as a housekeeping gene. (d) Transfection efficiency of *TFAM* was determined by microscopy using GFP-tagged on TFAM plasmid, and the cells were examined under a Zeiss ApoTome at 40X magnification. Each measurement was made in duplicate using three different cell preparations. The values are presented as means ± SD. 5= 5mM glucose; 20=20mM glucose; TFAM and 5+TF = TFAM transfected cells incubated in 20mM and 5mM glucose respectively; R= cells

transfected with reagent alone and incubated in 20mM glucose. *p<0.05 compared to 5mM glucose, and #p<0.05 to 20mM glucose.

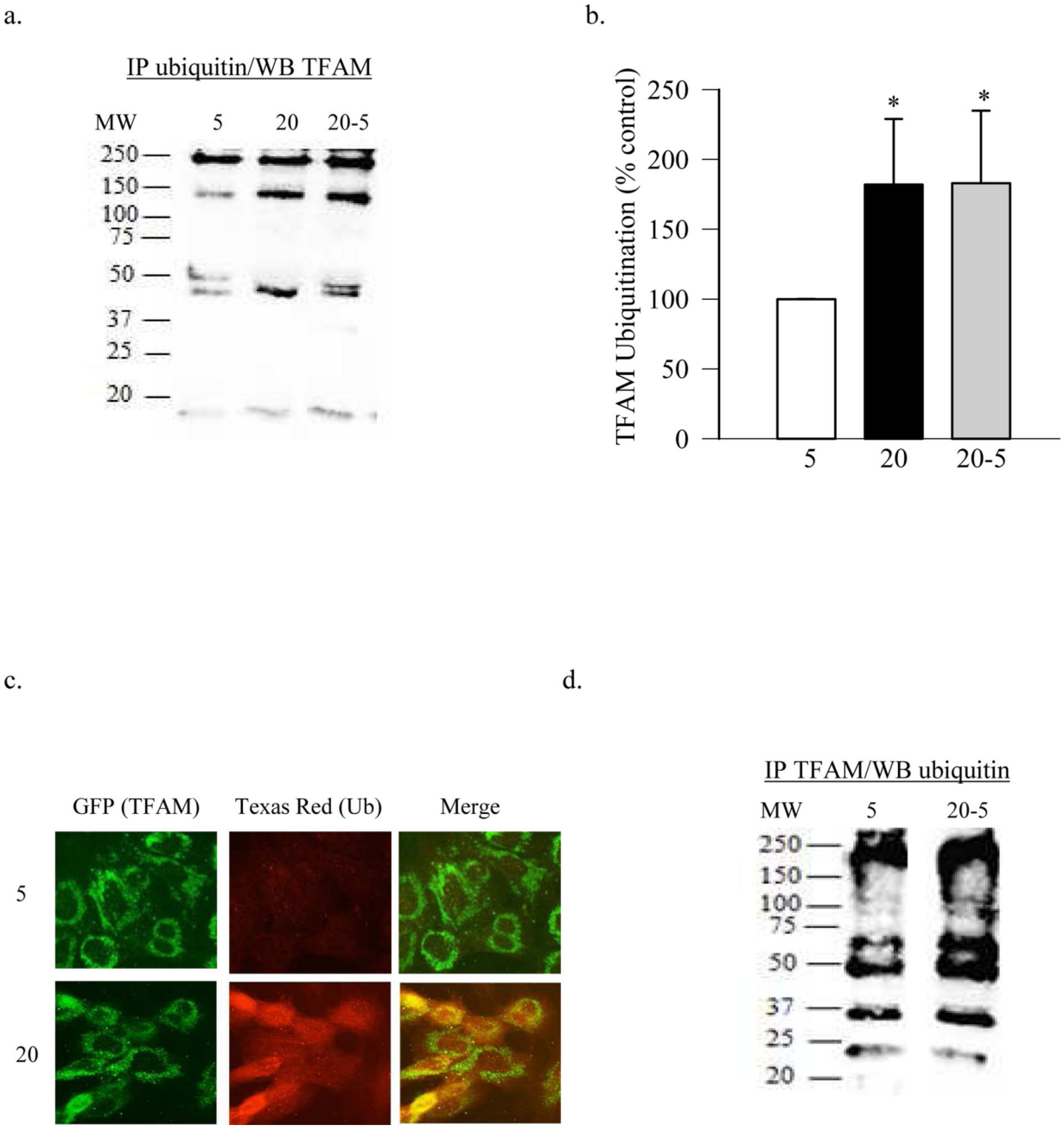

Figure 4.

High glucose ubiquitinates proteins in retinal endothelial cells. (a) Ubiquitination of TFAM was performed by immunoprecipitating ubiquitin and western blotting for TFAM, and (b) represents quantification of TFAM ubiquitination. (c) Ubiquitination was also confirmed by fluorescence microscopy using GFP-tagged TFAM and Texas Red-tagged ubiquitin antibodies. The cover slips were mounted with Vecta Shield and examined under a Zeiss ApoTome at 40X magnification. (d) Ubiquitination was quantified by immunoprecipitating TFAM, followed by western blotting for ubiquitin. MW=molecular weight marker; 5= 5mM glucose; 20=20mM glucose; 20-5= 4 days in 20mM glucose followed by 4 days in 5mM glucose.

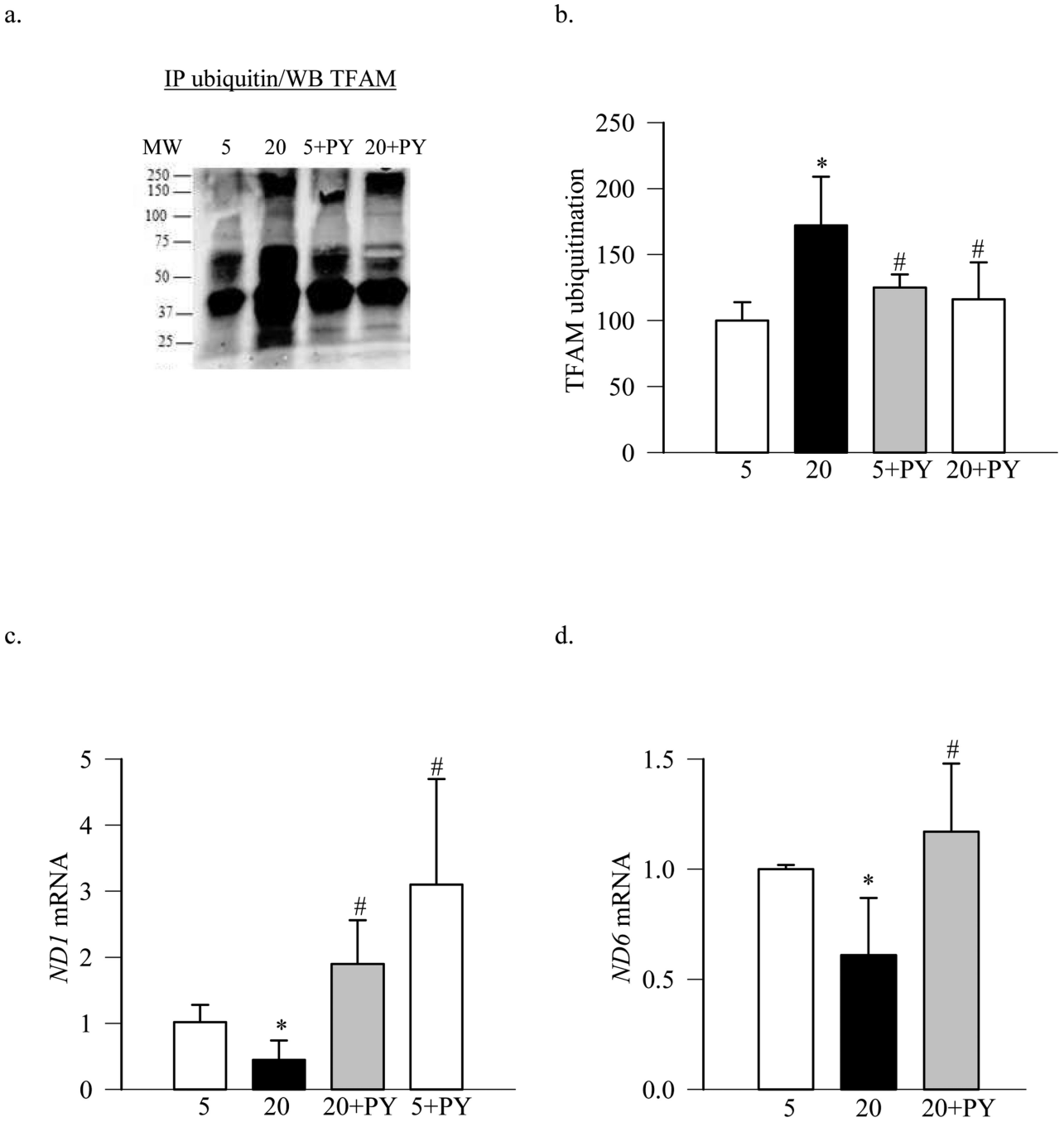

Figure 5.

Inhibition of ubiquitination attenuates high glucose-induced TFAM ubiquitination and impairment in mtDNA transcription. BRECs were pre-incubated with 5μM PYR-41 for 4 hours before incubating in 5mM and 20mM glucose media. (a) Ubiquitination of TFAM was performed by immunoprecipitating ubiquitin and western blotting for TFAM; (b) shows the quantification of ubiquitination. Gene transcripts of (c) *ND1* and (d) *ND6* were quantified by real time RT-PCR using *β-actin* as a housekeeping gene. 5= 5mM glucose; 20=20mM glucose; 20+PY and 5+PY= cells pre-incubated with PYR-41 for 4 hours followed by in

20mM and 50mM glucose media, respectively. *p<0.05 compared to 5mM glucose, and #p<0.05 to 20mM glucose.

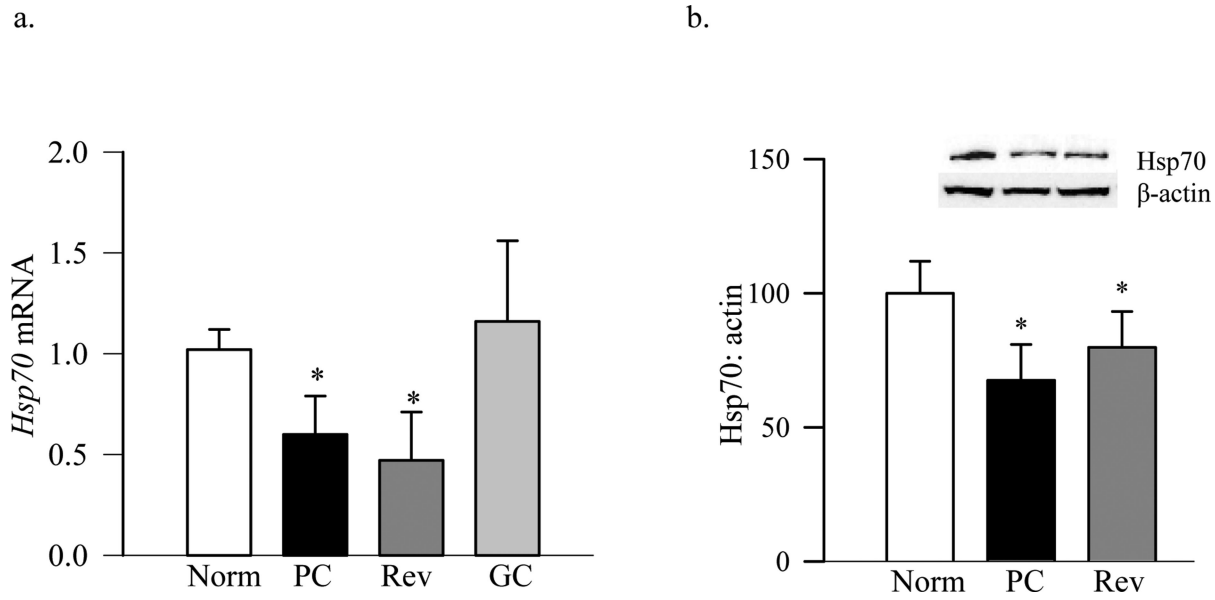

Figure 6.

Diabetes decreases Hsp70 expression in the retina. Hsp70 (a) gene expression was quantified in rat retina by qPCR and (b) protein expression by western blot technique using β-actin as a loading control. Each experiment was performed in duplicate in 5-6 rats in each group. Nor= Normal rats, PC= rats in 8 months of continuous poor glycemic control, Rev=rats in 4 months of poor glycemic control followed by 4 months of good glycemic control, GC= rats with 8 months of continuous good glycemic control. *p<0.05 vs normal

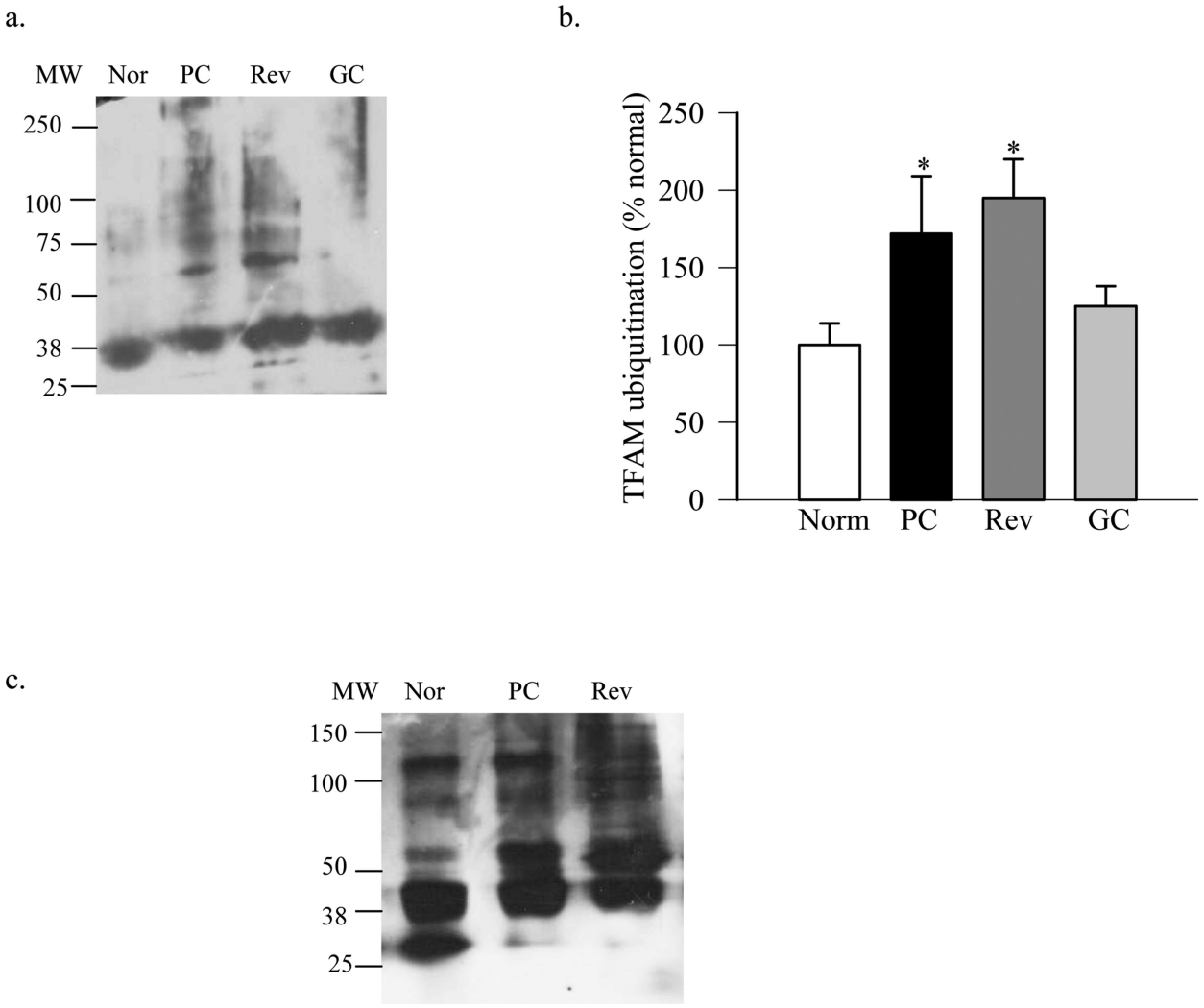

Figure 7.

Ubiquitination of retinal proteins is increased in diabetes. (a)TFAM ubiquitination was performed by immunoprecipitating ubiquitin, followed by western blot analysis for TFAM. The blots are representative from 4-5 assays, and (b) the expression of ubiquitinated TFAM was quantified. (c) Ubiquitination of TFAM was performed by immunoprecipitating TFAM, followed by western blot analysis for ubiquitin. MW=molecular weight marker, Nor= Normal rats, PC= rats in 8 months of continuous poor glycemic control, Rev=rats in 4 months of poor glycemic control followed by 4 months of good glycemic control, GC= rats with 8 months of continuous good glycemic control.

Table I

Primer for the target genes

BRECs			**Product size**
Hsp70	forward	5’-TGGACAGGACTCCCCCACTGGA-3′	227bp
	reverse	5’-TGGCGCGGGCATTTCCTCTC-3’	
ND1	forward	5’-AGGACCATTTGCCCTCTTCT-3’	297bp
	reverse	5’-GGTGGGATGCCTGATGTAAG-3’	
ND6	forward	5’-CGTGATAGGTTTTGTGGGGT-3’	250bp
	reverse	5’-GCCAGTAACAAATGCCCCTA-3’	
Cytb	forward	5’-CGATACATACACGCAAACGG-3’	298bp
	reverse	5’-AGAATCGGGTAAGGGTTGCT-3’	
β-actin	forward	5’-CCTCTATGCCAACACAGTGC-3’	205bp
	reverse	5’-CATCGTACTCCTGCTTGCTG-3’	
Rat			
Hsp70	forward	5’-TCCTCGTGGACGGTCGTCCG-3’	101bp
	Reverse	5’-CTGCACACGGAACCCGACGG-3’	
β-actin	forward	5’-CCTCTATGCCAACACAGTGC-3’	220bp
	Reverse	5’-CATCGTACTCCTGCTTGCTG-3’	