

Epac1 deacetylates HMGB1 through increased IGFBP-3 and SIRT1 levels in the retinal vasculature

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Purpose: Inflammation is a key component of retinal disease. We previously reported that exchange protein for cAMP 1 (Epac1) reduced inflammatory mediators, including total levels of high mobility group box 1 (HMGB1) in retinal endothelial cells (RECs) and the mouse retina. The goal of this study was to determine intermediate pathways that allow Epac1 to reduce HMGB1, which could lead to novel targets for therapeutics.

Methods: We used endothelial cell-specific conditional knockout mice for Epac1 and RECs to investigate whether Epac1 requires activation of insulin like growth factor binding protein 3 (IGFBP-3) and sirtuin 1 (SIRT1) to reduce acetylated HMGB1 levels with immunoprecipitation, western blot, and enzyme-linked immunosorbent assay (ELISA).

Results: Data showed that high glucose reduced IGFBP-3 and SIRT1 levels, and increased acetylation of HMGB1 in RECs. An Epac1 agonist reduced acetylated HMGB1 levels in high glucose. The Epac1 agonist could not reduce HMGB1 or SIRT1 levels when IGFBP-3 siRNA was used. The agonist also could not reduce HMGB1 when SIRT1 siRNA was used. The mouse retina showed that loss of Epac1 increases acetylated HMGB1 levels and reduces IGFBP-3 and SIRT1 levels.

Conclusions: Taken together, the data suggest that Epac1 activates IGFBP-3 to increase SIRT1, leading to a significant reduction in acetylated HMGB1. These findings provide novel therapeutic targets for reducing key inflammatory cascades in the retina.

The role of inflammation in diabetes is becoming increasingly key to diabetic complications, particularly in the retina. We have shown that the β -adrenergic receptor agonist, Compound 49b, is highly effective in reducing tumor necrosis factor alpha (TNF- α) through activation of insulin-like growth factor binding protein 3 (IGFBP-3) in retinal endothelial cells (RECs) [1,2]. As Compound 49b must be delivered locally, we initiated work on pathways that lie downstream from β -adrenergic receptor agonists through cAMP. Exchange protein for cAMP (Epac) can serve as an alternative pathway for β -adrenergic receptor/cAMP activation of downstream pathways [3]. Epac1 and Epac2 have been localized in the retina [4], are expressed by bovine retinal endothelial cells, and have been shown to play a role in leukostasis. We recently demonstrated that Epac1 is a potential key signaling protein in β -adrenergic receptor regulation of IGFBP-3 to protect the retina against leukostasis and inflammatory mediators [5]. In this work, data indicated that only Epac1, not Epac2, has actions on human retinal endothelial cell regulation of inflammatory mediators. Additionally, we recently reported that Epac1 reduces blocked inflammasome activation, leading

to reduced interleukin-1 beta (IL-1 β) and cleaved caspase 1 levels [6].

Since Epac1 can block inflammatory pathways, inhibit retinal leukostasis, and reduce total high mobility group box 1 (HMGB1) levels in REC [6], the next step was to investigate the cellular pathways by which Epac1 regulated HMGB1 levels. HMGB1 is a member of the HMG super family and is known to bind to toll-like receptor 4 (TLR4) and the receptor for advanced glycation end products (RAGE) to mediate inflammation when reaching the cytoplasm [7]. HMGB1 is expressed in the retina on multiple cell types, including retinal pigmented epithelium (RPE) [8], microglia [9], retinal endothelial cells (REC) [10], 661W photoreceptor cells [11], and retinal ganglion cells [12]. We had previously reported that IGFBP-3 could inhibit TNF- α levels in REC [2], so a key question was whether Epac1 required IGFBP-3 actions to reduce HMGB1 levels. In addition to IGFBP-3, we investigated whether sirtuin 1 (SIRT1) may be key to decreasing HMGB1 levels in the retina. SIRT1 has been localized in retinal neurons [13], the retinal vasculature [14], and the rat lens [15]. We chose to focus on SIRT1 as studies have reported reduced SIRT1 in diabetic patients and cells grown in high glucose. Additionally, the retinal vasculature of human diabetic donors had significant decreases in SIRT1 mRNA and activity [16]. SIRT1 can promote the deacetylation of HMGB1, leading to reduced cytoplasmic translocation [17].

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SIRT1 deacetylation of HMGB1 should lead to decreased inflammatory responses [16].

To address these questions, we used primary REC treated with an Epacl agonist and SIRT1 or IGFBP-3 siRNA, as well as endothelial cell specific knockout mice for Epacl.

METHODS

Mice: For these studies, we used 3-month-old Epacl floxed mice (B6;129S2-Rapgef3^{tm1Geno/J} mice, Stock No. 018,389) and B6 FVB-Tg (cdh5-cre)7Mlia/J Cre mice (Stock No 006137). We crossed these mice to generate Epacl CreLox mice where Epacl was eliminated from endothelial cells [5]. All animal procedures meet the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Research, were approved by the Institutional Animal Care and Use Committee of Wayne State University, and conform to National Institutes of Health (NIH) guidelines.

Retinal endothelial cells: Primary human RECs were purchased from Cell Systems Corporation (CSC, Kirkland, WA). Cells were grown in Cell Systems medium (normal glucose (5 mM glucose) or high glucose (25 mM glucose)) supplemented with microvascular growth factors (MVGS), 10 µg/ml gentamycin, and 0.25 µg/ml amphotericin B (Invitrogen, Carlsbad, CA) on attachment factor coated dishes. Cells were grown in high glucose a minimum of 3 days, with most dishes in culture for up to 5 days. Additional groups received normal glucose medium with 20 mM mannitol as an osmotic control for up to 5 days in culture. Cells were quiesced by incubating in high or normal glucose medium without MVGS for 24 h before experimental use. All cells before passage 5 were used.

Some RECs were treated with an Epacl agonist (8-CPT-2Me-cAMP; Tocris Bioscience, Minneapolis, MN) at 10 µM for 2 h. This particular Epacl agonist has been used at various doses and time points by several groups [18,19]. We have used this agent in the past on RECs [5,6]. Additional RECs were transfected with IGFBP3 siRNA (5 nM, L-004777-00-0005; Dharmacon, Lafayette, CO), SIRT1 siRNA (5 nM, L-004777-00-0005; Dharmacon), or scrambled siRNA for 24 h before the Epacl agonist treatment. Transfection was performed using GenMute (SignaGen Laboratories, Rockville MD), following the manufacturer's instructions. SIRT1 and IGFBP-3 western blotting were conducted to confirm successful knockdown.

Immunoprecipitation: After rinsing with PBS (137 mM sodium chloride, 11.9 mM phosphates, 2.7 mM potassium chloride), the cells were lysed with freeze thawing in lysis

buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin, Lysis Buffer 10X; Cell Signaling Technology, Danvers, MA) containing the protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor; Fisher Scientific, Pittsburgh, PA) for 20 min on ice. The cells were transferred into 1.5 µl Eppendorf tubes and cleared by centrifugation at for 20 min at 4 °C. The cells containing an equal amount of protein from control and each treatment were incubated with HMGB1 antibodies (ab227168; Abcam, Cambridge, MA) at 1:30 concentration overnight at 4 °C and rocking with Protein A/G PLUS-Agarose beads. The beads were washed three times with lysis buffer and once with PBS. The immunocomplexes were released by heating in Laemmli sample buffer and analyzed with western blotting for the acetyl-lysine antibodies at a 1:500 concentration.

Western blotting: Whole retinal lysates from mice or cell culture lysates were collected in lysis buffer containing protease and phosphatase inhibitors. Equal amounts of protein were separated onto a precast Tris-glycine gel (Invitrogen) and blotted on nitrocellulose membrane. After blocking in TBST (10 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, and 0.1% Tween-20) and 5% (w/v) bovine serum albumin (BSA), the membranes were treated with acetyl-lysine (1:500, Cat #80178) and IGFBP-3 (1:500, Cat# 76,001; Abcam) or GAPDH and beta actin (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies overnight followed by incubation with secondary antibodies (anti-rabbit-horseradish peroxidase; Promega, Madison, WI) for 0.5–1.0 h. Antigen-antibody complexes were detected with a chemiluminescence reagent kit (Thermo Scientific, Pittsburgh, PA), and data were acquired using an Azure C500 (Azure Biosystems, Dublin, CA). Western blot data were assessed using Image Studio Lite software. A representative blot for each treatment group is shown.

ELISA: A SIRT1 enzyme-linked immunosorbent assay (ELISA; Abcam) was performed according to the manufacturer's instructions using a 50 µg protein concentration for all groups. Samples were prepared as described for western blotting.

Statistical analyses: All the experiments were repeated a minimum of three times, and the data are presented as mean ± standard error of the mean (SEM). Data were analyzed with a non-parametric Kruskal–Wallis 1-way ANOVA, followed by a Dunn's test. A p value of less than 0.05 was considered statistically significant. For western blotting, one representative blot is shown. One-way ANOVA with the Student–Newman–Keuls post-hoc test was used for the

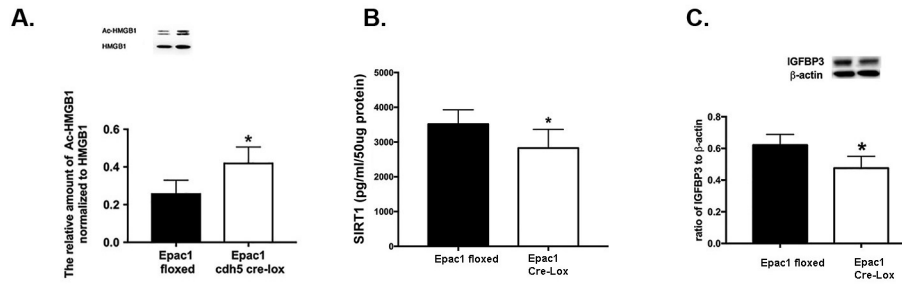


Figure 1. Protein data from Epac1 floxed and endothelial cell-specific Epac1 knockout mice. **A:** Acetylation of HMGB1. **B:** SIRT1 enzyme-linked immunosorbent assay (ELISA). **C:** IGFBP-3 levels. * $p < 0.05$ versus floxed mice. $n = 5$ mice (two eyes per mouse pooled). Panels **A** and **C** show representative blots from the mice. Data are mean \pm standard error of the mean (SEM).

animal work. A p value of less than 0.05 was considered statistically significant.

RESULTS

Loss of Epac1 increased acetylation of HMGB1, while decreasing SIRT1 and IGFBP-3: As we previously reported that Epac1 could regulate total HMGB1 levels [6], we wanted to investigate whether Epac1 alters the acetylation of HMGB1, as well as two potential regulators of HMGB1 levels. Figure 1 shows that HMGB1 is acetylated in the retina from Epac1 Cre-Lox mice compared to their floxed littermates. Loss of Epac1 decreased the SIRT1 (Figure 1B) and IGFBP-3 (Figure 1C) levels.

An Epac1 agonist reduced acetylation of HMGB1 of RECs grown in high glucose: To investigate potential mechanisms involved in Epac1 actions on HMGB1, we grew RECs in normal or high glucose and treated them with the Epac1 agonist. Mannitol was used as an osmotic control. Figure 2A shows that high glucose statistically significantly reduced the SIRT1 levels, which increased following treatment with the Epac1 agonist. We also found that high glucose increased

acetylation of HMGB1, which was statistically significantly reduced by the Epac1 agonist (Figure 2B).

Epac1 requires IGFBP-3 to deacetylate HMGB1 and increased SIRT1 levels: We previously reported IGFBP-3 regulates inflammatory and apoptotic pathways [1,20]. We wanted to ascertain whether Epac1 requires IGFBP-3 to reduce HMGB1 levels. Figure 3A shows that high glucose increased acetylation of HMGB1, which was reduced by the Epac1 agonist in RECs. However, the Epac1 agonist could not reduce acetylation of HMGB1 in RECs treated with IGFBP-3 siRNA. Figure 3B demonstrates that the Epac1 agonist could counter the high glucose-induced decrease in SIRT1. The agonist was not able to increase SIRT1, when IGFBP-3 siRNA was used. Figure 3C is a control to demonstrate successful knockout of IGFBP-3 using siRNA. The Epac1 agonist had no effect on SIRT1 or IGFBP-3 in RECs grown in normal glucose.

Epac1 increased SIRT1 levels and deacetylated HMGB1: Literature suggests that SIRT1 can deacetylate HMGB1 to keep it in the nucleus [17]. Figure 4B shows that SIRT1 siRNA blocked the ability of Epac1 to deacetylate HMGB1.

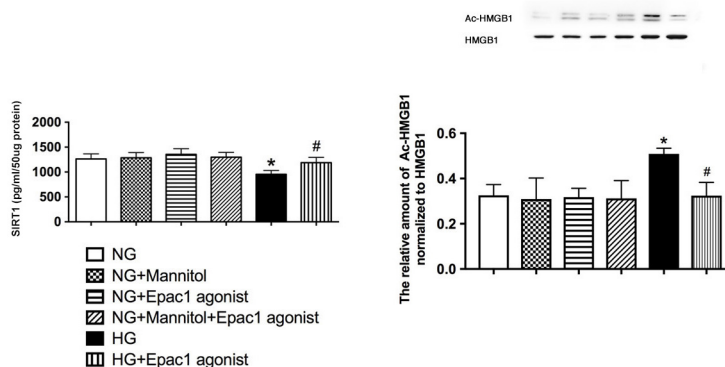


Figure 2. Retinal endothelial cells grown in NG, HG, or normal glucose plus mannitol. Cells in normal glucose (NG) and high glucose (HG) were treated with an Epac1 agonist. **A:** Enzyme-linked immunosorbent assay (ELISA) results for SIRT1. **B:** HMGB1 levels. * $p < 0.05$ versus NG, # $p < 0.05$ versus HG, $n = 4$ for all groups. Data are mean \pm standard error of the mean (SEM).

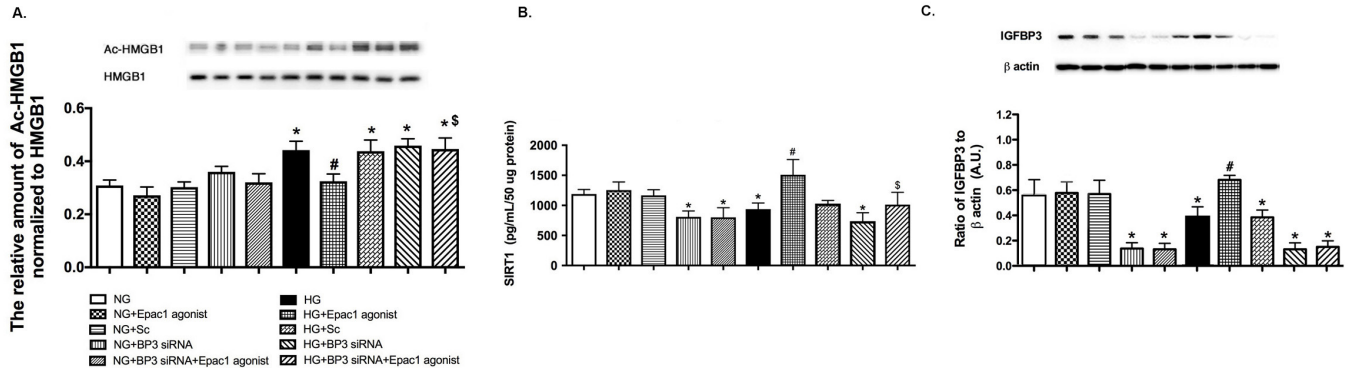


Figure 3. Retinal endothelial cells grown in NG or HG. Cells in normal glucose (NG) and high glucose (HG) were treated with an Epac1 agonist, and some cells received the Epac1 agonist after transfection with IGFBP-3 siRNA or scrambled siRNA. **A:** Acetylation of HMGB1. **B:** SIRT1 levels. **C:** IGFBP-3 to verify knockdown. * $p < 0.05$ versus NG, # $p < 0.05$ versus HG, \$ $p < 0.05$ versus HG+Epac1 agonist. $n = 4$ for all groups. Data are mean \pm standard error of the mean (SEM).

Figure 4A is a western blot to show successful knockdown of SIRT1. Taken together, these data suggest that Epac1 increases IGFBP-3 and SIRT1 to deacetylate HMGB1 levels.

DISCUSSION

We previously reported that Compound 49b, a β -adrenergic receptor agonist, could reduce TNF- α levels in RECs [1]. As Compound 49b requires eye drop delivery, we sought to find downstream pathways from β -adrenergic receptors that may also reduce inflammatory pathways in the diabetic retina. We recently published that Epac1 statistically significantly reduces inflammatory mediators in the mouse retina, as well as in RECs [5]. We also reported that Epac1 could reduce NLR family, pyrin domain containing 3 (NLRP3) inflammasome activation, including reduced total HMGB1 levels [6]. In RECs, we showed that an Epac1 agonist statistically significantly increases SIRT1 levels, while reducing cytosolic HMGB1. The goal of this study was to determine potential pathways by which Epac1 reduced HMGB1, as they may offer new avenues for therapeutic development.

One such pathway is IGFBP-3. We previously reported that IGFBP-3 acts independently of IGF-1 and can reduce TNF- α actions in RECs [2,21]. We also showed that a mutant form of IGFBP-3 that cannot bind the IGF-1 receptor inhibited ICAM-1-mediated cellular adhesion. IGFBP-3 is reported to be neuroprotective in the retina and reduce injury-induced retinal inflammation [22]. Others have also reported that IGFBP-3 can reduce hepatic inflammation through a reduction in NF κ B and JNK actions [23]. In this study, we showed that loss of Epac1 statistically significantly reduces IGFBP-3 levels in the mouse retinal vasculature. When we treated RECs with the Epac1 agonist plus IGFBP-3 siRNA, the agonist was unable to increase SIRT1 and decrease acetylation of HMGB1, suggesting that Epac1 activates IGFBP-3 to increase SIRT1, which reduces acetylation of HMGB1.

Data also strongly suggest that SIRT1 is key to regulating HMGB1 actions. Work in a corneal wound healing model showed that IGFBP-3 increases SIRT1 to promote wound healing, despite the high glucose conditions [24]. SIRT1 is reduced in patients with diabetes and cells grown in high glucose. SIRT1 promoted the deacetylation of HMGB1,

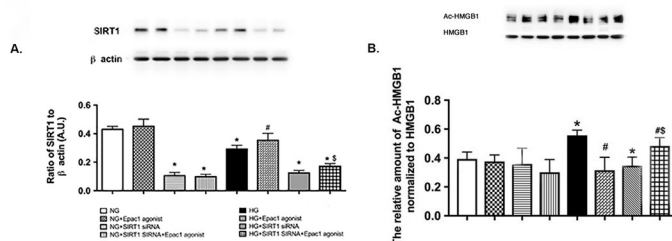


Figure 4. Retinal endothelial cells grown in NG or HG. Cells in normal glucose (NG) and high glucose (HG) were treated with an Epac1 agonist, and some cells received the Epac1 agonist after transfection with SIRT1 siRNA. **A:** SIRT1 levels. **B:** HMGB1 acetylation. * $p < 0.05$ versus NG, # $p < 0.05$ versus HG, \$ $p < 0.05$ versus HG+Epac1 agonist. $n = 4$ for all groups. Data are mean \pm standard error of the mean (SEM).

leading to reduced cytoplasmic translocation [9]. Furthermore, SIRT1 is key to reduced NF κ B, cyclooxygenase 2, and other deleterious pathways involved in diabetic retinopathy [25]. As we found that loss of Epacl reduces SIRT1 and increases HMGB1, we wanted to ascertain the role SIRT1 plays in regulation of HMGB1. High glucose increased acetylation of HMGB1 in RECs, which was blocked by the Epacl agonist. The Epacl agonist was unable to deacetylate HMGB1 when SIRT1 siRNA was used, suggesting that SIRT1 actions are directly related to Epacl's regulation of HMGB1 in the retinal vasculature. Future studies will focus on the specific signaling pathways by which Epacl can activate IGFBP-3 and SIRT1 in the retinal vasculature, as well as on whether this specific Epacl agonist is effective in vivo on the retinal vasculature.

Conclusions: Data suggest that Epacl increases IGFBP-3, leading to increased SIRT1 levels. SIRT1 blocked the HMGB1 levels. By deacetylating HMGB1, Epacl inhibits activation of the NLRP3 inflammasome, and these studies provide novel mechanisms for Epacl regulation of HMGB1. These studies provide potential new pathways for therapeutic development for vascular damage in diabetic retinopathy.

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