Aldose Reductase drives hyperacetylation of Egr-1 in hyperglycemia and consequent upregulation of proinflammatory and prothrombotic signals

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**ABSTRACT**

Sustained increases in glucose flux via the aldose reductase (AR) pathway have been linked to diabetic vascular complications. Previous studies revealed that glucose flux via AR mediates endothelial dysfunction and leads to lesional haemorrhage in diabetic (DM) human AR (hAR) expressing mice in an apoE-/− background. Our studies revealed sustained activation of Egr-1 with subsequent induction of its downstream target genes tissue factor (TF) and Vascular Cell Adhesion Molecule1 (VCAM1) in DM apoE-/− hAR mice aortas and in high glucose-treated primary murine aortic endothelial cells expressing hAR. Furthermore, we observed that flux via AR impaired NAD⁺ homeostasis and reduced activity of NAD⁺ dependent deacetylase sirt-1 leading to acetylation and prolonged expression of Egr-1 in hyperglycemic conditions. In conclusion, our data demonstrate a novel mechanism by which glucose flux via AR triggers activation, acetylation and prolonged expression of Egr1 leading to proinflammatory and prothrombotic responses in diabetic atherosclerosis.
INTRODUCTION

Post-translational modification (PTM) of histones via deacetylation, mediated by a family of histone deacetylases (HDACs), was initially identified as a mechanism to silence gene transcription (1, 2). In addition, it is well-established that acetylation and deacetylation of non-histone proteins are common PTMs found across the cytosol, nucleus, mitochondria, and endoplasmic reticulum (3), including enzymes involved in intermediary metabolism (4, 5). These findings support a broader role for acetylation beyond the nucleus. Sirtuins are NAD+ dependent enzymes, well known to deacetylate proteins and enzymes (6), including the proteins that play important roles in metabolism (7). Sirtuins have been shown to regulate various transcription factors such as p53 (8, 9), forkhead box class O (FOXO) (10), Peroxisome Proliferator – activated receptor–γ (PPAR-γ) (11), p65 subunit of NF-κB (12, 13), and PGC1-α (14). Sirt1 has been shown to have atheroprotective effects and inhibition of its activity using pharmacological agents or genetic deletion induces arterial thrombus formation (13).

Expression of human aldose reductase (AR; hAR) in an atherosclerosis-vulnerable LDL receptor knockout mouse (Ldlr−/−) background increased atherosclerosis in diabetic mice (15). Subsequent studies revealed AR mediated defects in vasorelaxation, endothelial function and lesional hemorrhage in streptozotocin induced diabetic hAR overexpressing mice in an apoE−/−background (16). Flux of glucose via the AR pathway consumes NAD+ by the action of the sorbitol dehydrogenase (SDH) to generate fructose. As a consequence, increased flux of glucose via this pathway in hyperglycemia leads to a decrease in NAD+/NADH ratio (17). In this study, we investigated if flux via AR causes pro-inflammatory and pro-thrombotic signaling via NAD+ reduction and subsequent inhibition of Sirt-1 dependent deacetylation of Egr-1 (“immediate early response gene”). Our data demonstrate a novel mechanism linking glucose metabolism to
increased inflammatory and prothrombotic signaling in diabetic atherosclerosis via PTM of Egr-1.
RESEARCH DESIGN AND METHODS

All animal studies were performed with the approval of the Institutional Animal Care and Use Committee at New York University, NY. The hAR mice and apoE/-hAR mice, both backcrossed >10 generations into C57BL/6, were characterized and rendered diabetic with streptozotocin as described (18). Details of the treatment of diabetic mice with inhibitors of AR are described in the supplement.

Cell culture:

Murine aortic endothelial cells (MAECs) were established from mouse aortas as described previously (19), while human aortic endothelial cells (HAECs) were from commercial source (Cell Applications Inc). Studies on these cultured cells included treatment with the AR inhibitor (ARI) zopolrestat (200 µM); SDH inhibitor (SDI) CP-470,711 (200 nM); nicotinamide mononucleotide (NMN) (500 µM); or the sirtuin inhibitor, sirtinol (20 nM); DMSO or sirt activator, SRT1720 (10 µM). Endothelial cells were transfected overnight using an adenoviral vector overexpressing hAR or GFP (Vector Biolabs) in serum free medium.

Generation of Egr-1 mutants, In-vitro acetylation and de-acetylation assays:

The mutant Egr-1 was generated as described ((20). Briefly, an EcoRV-SmaI full-length, flag-tagged Egr1 from pCMVFLAG-Egr1 was inserted into the EcoRV site in pcDNA3 to generate pcDNA3-Egr1. Egr1 has acetylation sites from 422-425 corresponding to KDKK and the acetylation site mutations of (KDKK) of Egr1 expressing pcDNA3 constructs were made using primers: EGRM1 (atccatattaagacagCggacaagaagcgacagaaagtgtggtg), EGRM2 (atccatattaagacagCGggacGCGGCAcagagaaaaagtgtggtg), and EGRM3 (atccatattaagacagaagacGCCGCGGCAcagagaaaaagtgtggtg), using site directed mutagenesis according
to the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA). The mutants generated were sequenced and confirmed for the appropriate mutations and further transformed in *E. coli* and purified using Ni column. The purified Egr-1 was used as substrate for in-vitro acetylation studies.

The in-vitro acetylation studies were performed as described (9). Briefly, 1 ug of the purified Egr-1 protein was added to the 30 ul assay mixture consisting of 50 mM HEPES (pH 8.0), 10% glycerol, 1mM DTT, 1 mM PMSF, 10 mM sodium butyrate, 0.2 mCi of [14C]-acetyl-CoA (Perkin Elmer, 4mCi/mmol), cold acetylcoA (10 mM) and 100 ng of recombinant p300 protein (Activ Motif, USA) for 1 hr at 30°C. For electrophoretic mobility shift assays, the reaction mixture was subjected to SDS-PAGE gels and either developed by auto-radiography or by western blot using acetyl lysine antibody (Millipore Inc., USA).

For the in-vitro de-acetylation studies (21), the assay mixture containing the acetylated Egr-1 was subjected to a de-acetylation reaction containing an assay buffer (50 mMTris-HCl, pH 8.0, 4 mM MgCl2, 0.2 mM DTT), 300 ng of recombinant sirt-1 protein and 50 uM NAD+. The assay mixture was incubated for 1 hr at 30°C and was subjected to SDS-PAGE gel, visualized either through auto-radiography or western blot by acetyl lysine antibody.

**Chromatin Immunoprecipitation (ChIP):**

ChIP was performed using the ChIP-IT kit (Activ Motif, USA). Briefly, MAECs were cross linked with 1% formaldehyde in PBS for 15 min at room temperature. Samples were processed as per instructions provided by the manufacturer to obtain chromatin. The chromatin was initially
pre-cleared using protein-G beads and subsequently treated with Egr1 antibody (Santa Cruz Biotechnology, USA) at a concentration of 1:200 for immunoprecipitation overnight. The immunoprecipitated ChIP product was washed, reverse crosslinked, treated with proteinase K and eluted to get the ChIP enriched DNA. Simultaneously, IgG was used as a negative control to check the efficiency of the ChIP experiment. 25 ng of ChIP enriched DNA was used to perform PCR for the tissue factor (TF) promoter using the following primers-Forward “5’ CATCCCTTGCAGGGTCCCGGAGTT 3’, Reverse-“5’ GGGGTGCGGGAGCTCGCAGTC 3’”. The PCR products were run on a 2% agarose gel and the amplicons were visualized using UV light.

Standard methods for studies involving RNA silencing, western blotting, immunoprecipitation, quantitative real-time PCR; immunohistochemistry and transient transfection of MAEC or HAECs with hAR or GFP were followed (16). Assays for murine plasma TF (MyBiosource,), NAD+/NADH measurements(Biovision,USA), luciferase reporter, Sirt-1(Sigma Aldrich, USA) and nicotinamide phosphoribosyl transferase (NAMPT) (Cyclex Co. Ltd., USA)) activities were performed as per manufacturer’s instructions.

Statistical analysis:

All data are reported as mean ± SD unless otherwise noted. Data were analyzed by one way ANOVA using commercially available software (SPSS, version 18, USA). Probability values ≤ 0.05 were considered statistically significant.
RESULTS

Expression of Egr1 in diabetic apoE-/-hAR mice aorta:

We previously reported that transgenic expression of human-relevant levels of aldose reductase (AR) accelerated vascular dysfunction, atherogenesis, lesional hemorrhage, and inflammation in diabetic apoE-/-hAR mice (16). In this study, we sought to test the hypothesis that expression of hAR in these mice upregulated Egr-1, a key transcription factor linked to regulation of inflammation and prothrombotic responses. Aortas collected from 14 week old diabetic apoE-/-hAR mice demonstrated a three-fold higher gene expression of Egr1 compared to non-diabetic apoE-/-hAR aortas (p<0.05) (Figure 1A). Similarly, there was a significant rise in Egr-1 protein expression in diabetic apoE-/-hAR aorta compared to the non-diabetic apoE-/-hAR group (p<0.05) (Figure 1B). Aortas obtained from non-diabetic WT and hAR overexpressing mice at comparable age showed no significant changes in the expression level of Egr1 (supplement fig S1a) Expression of Egr-1 in the aorta was observed from 10 weeks of diabetes (Supplement fig S1b).

Immunohistochemistry of apoE-/-hAR aortic root revealed significant localization of Egr1 to the endothelial lining of the aorta in the 14 weeks diabetic group compared to the non-diabetic group (Figure 1C). Quantitation of Egr1 and AR signals in the endothelial lining indicate that expression of Egr-1, AR, colocalized signal is increased in diabetic vs. non-diabetic apoE-/-hAR mice (fig 1D, 1E, 1F). We previously demonstrated the role of ARI, zopolrestat, in reducing atherosclerotic lesions in diabetic apoE/-hAR mouse aortas. We sought to determine if administration of AR inhibitors (ARIs) modulated expression and action of Egr1 in the vasculature. Expression of Egr1 protein was reduced in the diabetic apoE-/-hAR mice aorta treated with zopolrestat vs. vehicle (Figure 1G; p<0.05).
High glucose-induced Egr1 expression in MAECs: effect of hAR.

Primary MAECs cultured from the aortas of hAR mice treated with high glucose (25 mM D-glucose) for 24 hrs showed a significant increase in nuclear Egr1 protein expression compared to cells cultured in physiologic levels of glucose (5.5 mM D-glucose) (Figure 2A; p<0.05). Reduction of hAR expression by siRNA (Figure 2A) or activity by ARI zopolrestat (Figure 2B), showed a significant decrease (p<0.05) in expression of nuclear Egr1.

MAECs subjected to high glucose revealed a time dependent upregulation of Egr1 mRNA expression. The wild-type (WT) cells showed an increased expression of Egr1 mRNA at 30 min that peaked at 2 hrs (5 fold compared to “0 hr”, p<0.05) and then tapered down to levels comparable to the “0 hr” time point. In contrast, the cells expressing hAR showed a significant rise in Egr-1 expression at 2 hrs (compared to control cells at similar time point, p<0.05) that was sustained until 8 hrs and then subsequently tapered down by 24 hrs; yet, was fivefold higher compared to the WT cells (Figure 2C; p<0.05). A similar observation was made when the cells were probed for Egr1 protein expression at these time points (Figure 2D). To further validate that these changes in Egr1 expression were due to transcriptional regulation, we tested Egr1 promoter activity. Egr1 promoter luciferase reporter activity was significantly greater in hAR overexpressing cells (9.75 ± 1.76 RLU) compared to the control WT cells grown in high glucose (3.54 ± 1.46 RLU), p<0.05 (Figure 2E). To determine if flux via AR and SDH, the latter the second enzyme in the pathway which consumes NAD+, is required for Egr-1 regulation in high glucose, we incubated MAECs in high glucose alone or in the presence of ARI or SDI. A significant increase in total Egr-1 protein expression was noted compared to the low glucose (5.5
mM)-treated cells; consistent with key roles for AR and SDH, MAECs treated with either ARI or SDI demonstrated significantly reduced expression of Egr-1 in the presence of high glucose vs. untreated cells (Figure 2F).

**Acetylation of Egr-1 is required for its sustained expression:**

Since stabilization and expression of Egr-1 occurs through its acetylation by CBP/p300 at a CBP/p300 consensus sequence (KDKK) (20), we asked if the difference in expression patterns of Egr1 in high glucose in WT vs. hAR MAECs was dependent, at least in part, on differences in post-translational modification, that is, acetylation. To test this, we performed immunoprecipitation experiments with antibody recognizing acetyl-lysine followed by Western blotting with antibody to Egr-1 in high or low glucose-treated MAECs. Upon 24 hrs treatment with high glucose, significantly greater degrees of acetylated Egr-1 protein were immunoprecipitated from hARtg vs. control MAECs (Figure 3A). Time course experiments revealed that the first evidence of acetylated Egr-1 protein in high glucose occurred after 2 hrs incubation in MAECs (Figure 3B). Consistent with key roles for AR and SDH in mediating acetylation of Egr-1, treatment of MAECs with ARI or SDI resulted in decreased acetylated Egr-1 immunoprecipitated with antibody recognizing acetyl-lysine from high glucose-treated cells (Figure 3A). We next sought to test if analogous findings were evident in human aortic endothelial cells (HAECs). HAECs treated with high glucose for 24 hrs showed significantly more immunoprecipitated acetylated Egr-1 expression vs. the low glucose condition. Presence of ARI or SDI in high glucose medium markedly reduced acetylated Egr-1 expression to the levels observed in low glucose treated HAECs (Figure 3C). Consistent with in vitro cell culture data, significant acetylation of Egr-1 was observed in streptozotocin treated apoE-/−hAR mice aorta
compared to non-diabetic controls in an AR-dependent manner, as treatment of diabetic apoE/-
hAR mice with ARI reduced acetylation of Egr1 in the aorta vs. vehicle treatment (Figure 3D).

*Polyol flux through AR decreases NAMPT activity, NAD+ synthesis and sirt-1 activity:*

Flux of glucose via the AR pathway has been demonstrated to cause reductions in the ratio of NAD+/NADH. We hypothesized that reductions in the NAD+/NADH ratio, due to AR flux, decreases Sirt-1 activity, which, in-turn, would expose Egr-1 for acetylation by the histone acetyltransferases (HATs) such as CBP/p300. To address this question, we assessed expression of sirt-1 protein in MAECs from hAR overexpressing mice. Time dependent exposure of the cells to high glucose revealed a significant decrease in the total (Figure 4A) and nuclear (Figure 4B) expression of sirt-1 by 24 hrs. Prolonging the incubation time to 48 hours under high glucose did not lead to additional decreases in sirt1 expression of MAECs from hAR overexpressing mice (data not shown). Treatment with an ARI restored levels of sirt-1 to those observed in the low glucose-treated cells. Similarly, treatment of MAECs with either resveratrol or NMN, that improves the sirt-1 activity, resulted in increased expression of sirt-1 in the nucleus (Figure 4B). We next measured sirt-1 activity; those cells treated with high glucose showed a significant 40% decrease in sirt-1 activity compared to low glucose treated cells (p<0.05, Figure 4C). However, the sirt-1 activity was restored by almost three fold (compared to high glucose treatment) when the cells were exposed to NMN (an activator of NAD synthesis and subsequently sirt-1 activity), and was improved when cells were treated with either ARI or SDI (p<0.05, Figure 4C). Aortic tissues isolated from 14 weeks diabetic apoE/-hAR mice with streptozotocin treatment showed a 45% decrease in sirt-1 activity compared to the non-diabetic apoE/-hAR aorta (p<0.05, Figure 4D). In vivo, treatment of the diabetic mice with ARI for the entire duration of diabetes (14
weeks) normalized the sirt-1 activity to levels comparable to those of the non-diabetic apoE/-hAR mice (Figure 4D).

To determine whether the availability of NAD+ was limiting or if its synthesis was impacted, we measured the expression of NAMPT, the rate limiting enzyme in the NAD+ salvage pathway. MAECs treated with high glucose for the indicated time points showed a significant reduction in NAMPT protein expression by 24 hrs (p<0.05, Figure 4E). In HAECs, the NAMPT activity was 60% less in these cells treated with high glucose in comparison to the low glucose treated cells (p<0.05, Figure 4F). Pharmacological inhibition of AR resulted in a significant improvement in NAMPT activity which was comparable to that of normal glucose treated HAECs (Figure 4F). Diabetic apoE/-hAR mice demonstrated a 55% decrease in NAMPT activity in the aorta compared to non-diabetic apoE/-hAR mice, which was fully restored by treatment of the diabetic mice with ARI for 14 weeks (Figure 4G).

Next, we measured the NAD+/NADH ratio in MAECs, as this was our chief hypothesis, that is, high glucose-mediated flux via AR and SDH would consume NAD+. NAD+ and NADH were measured as pmoles/million cells and the ratio was found to be at least three times lower in high glucose treated MAECs compared to the normal glucose treated MAECs (p<0.05, Figure 4H). We hypothesized that acetylation of Egr-1 is an end result of decreased sirt-1 activity driven by reduced NAD+/NADH ratio. To prove this, we first sought to determine whether treatment of cells with high glucose had any effect on the histone acetyltransferases (HATs); hence, we measured the total HAT activity in the MAECs over-expressing hAR. No significant differences in HAT activity were observed between the high glucose and the low glucose treated cells, thereby implying that acetylation is tightly regulated by the availability of NAD+, which directly impacts sirt-1 activity. To test this concept, we measured the degree of acetylated Egr-1 in hAR
over-expressing MAECs exposed to high glucose or to high glucose with NMN (the latter will restore NAD+). Indeed, we observed decreased acetylation of Egr-1 in MAECs treated with high glucose plus NMN (Figure 5A). Similarly, when cells in high glucose medium were treated with a sirtuin inhibitor (sirtinol), we observed increased acetylation of Egr-1, thereby confirming that sirt-1 is required for de-acyetylation of Egr-1 (Figure 5A). An additional study was performed to determine if inhibition of NAMPT, which affects NAD+ synthesis, would modulate Egr-1 acetylation. Treatment of MAECs with FK866, a specific inhibitor of NAMPT, in MAECs grown in low glucose, resulted in increased acetylation compared to untreated cells (Supplement Fig S3). Furthermore, treatment of HAECs with SRT1720 (an activator of sirt 1) or overexpression of sirt in HAECs at high glucose condition revealed reduced acetylation of Egr1 (supplement fig S4).

To determine if the consensus sequence KDKK within Egr-1, known site for acetylation by CBP/p300, is a potential site for sirt-1 action, we generated triple mutants of the Egr-1 protein; we mutated three of these K (lysine) residues to alanine (K419, K421, K422), and performed in-vitro acetylation assays in the wild type and the mutant protein using recombinant p300. The acetylated Egr-1 was then subjected to in-vitro de-acetylation using recombinant sirt-1 in the presence or absence of NAD+. Sirt-1 convincingly de-acyetylated the WT Egr-1 protein in the presence of NAD+ which was not observed in the reaction mixture without NAD+ (Figure 5B). We generated single and double mutants of the Egr-1 and noticed appreciable acetylation in the WT, single mutant but not in the double and the triple mutants (Figure 5C). Thus, this study shows clear evidence for de-acyetylation of Egr-1 by sirt-1 and indeed that NAD+ is critical for the catalytic activity of sirt-1 in de-acyetylating Egr-1.
AR mediated modulation of Egr-1 expression results in pro-inflammatory and pro-thrombotic signals in ECs:

We next sought to test the down-stream consequences of overexpression of hAR in MAECs in the presence of Egr-1 silencing. We observed that hAR MAECs grown in high glucose revealed increased expression of VCAM-1 protein compared to cells grown in low glucose. Upregulation of VCAM-1 in high glucose was dependent at least in part on Egr-1, as siRNA silencing of Egr-1 resulted in decreased expression of VCAM1 in high glucose (Figure 6A), which was comparable to that of cells treated with normal glucose conditions. Further, expression of TF, a well-known target gene of Egr1 and a prominent pro-thrombotic molecule, was significantly higher in hAR cells exposed to high glucose treatment compared to low glucose-treatment (Figure 6B, C). siRNA-mediated reduction of hAR (Figure 6B) or blockade of AR activity by ARI (Figure 6C) or silencing of Egr1 by siRNAs (Figure 6D) all resulted in a significant decrease in TF expression compared to the high glucose treated conditions. We performed ChIP experiments to study the promoter occupancy of Egr-1 in the TF gene. ChIP studies revealed increased occupancy of Egr-1 in MAECs expressing hAR vs. wild-type cells exposed to high glucose for 24 hrs (Figure 6E). Finally, we collected plasma from non-diabetic or diabetic apoE-/hAR mice and found increased TF expression in the diabetic (129.35±3.05, p<0.05) compared to the non-diabetic mice (112.37±4.47) (Figure 6F), which was significantly reduced by treatment of the diabetic mice with ARI (115.6±5.02, P<0.05) (Figure 6F).
DISCUSSION

High glucose flux via the AR pathway leads to altered metabolism of glucose and vascular perturbation (22). Our earlier studies demonstrated that hAR expression in apoE-/mice, both globally and particularly in ECs, contributes to accelerated diabetic atherosclerosis, and that treatment with an ARI reduced atherosclerosis in these diabetic mice (16). In this study we sought to identify the discrete molecular mechanisms driving the impact of AR in diabetic atherosclerosis. In this study we report that excess flux of glucose via AR: 1) compromised the NAMPT-mediated NAD+ synthesis due to excess demand over supply; 2) decreased NAD+ which affects the biological action of sirt-1 and NAD+ dependent deacetylases, which in turn triggers acetylation and prolonged expression of Egr1 leading to proinflammatory and prothrombotic responses; and that 3) a competitive inhibitor of AR, zopolrestat, normalizes the NAD+ levels by improving the NAMPT and sirt-1 activity with subsequent blockade of acetylated Egr1 expression, thereby prohibiting signals for inflammation and thrombus formation. This study is the first to definitively demonstrate, both in vivo and in primary MAECs and HAECs, that defects in NAD+ metabolism due to flux via AR have a pathological impact on endothelial activation in diabetes.

AR pathway flux driven changes in ratio of free cytosolic NADH to NAD+ as a critical mediator of diabetic vascular complications has been proposed (17). Excess flux of glucose through AR impacts other metabolic pathways such as glycolysis, oxidative stress, intracellular nonenzymatic glycation and protein kinase C (PKC) activation (23, 24, 25, 26, 27). Here, we draw attention to the link between AR flux and NAD+ salvage pathway. Studies have highlighted emerging roles of NAD+ as a key signaling component in transcriptional regulation, aging and other diseases (28). In addition to its function in various biological processes, NAD+ acts as
substrate for various enzymes including NAD$^+$ dependent sirtuin family of deacetylases (7, 28). In this study we show Egr-1 is an important substrate for sirt-1 in the endothelial vasculature. In vitro, at the transcript level, Egr1 upregulation is seen within 30 minutes of high glucose exposure of MAECs. However sirt activity changes are seen only at 24 hours. The initial Egr1 upregulation observed could be due to the changes at transcriptional level, whereas the sustained Egr1 upregulation is likely due to posttranslational modifications mediated by the decrease in sirt activity at 24 hrs. Inhibition of sirt-1 activity maintains Egr-1 in an acetylated state that signals prothrombotic and proinflammatory events in the vascular bed. Taken together, both NAMPT-mediated NAD$^+$ biosynthesis and sirt-1 play critical roles in regulating such processes as metabolism, stress responses, and cellular differentiation to various stimuli such as fasting and caloric restriction (28). A recent study showed decreased NAD$^+$ and NAMPT levels in multiple organs during aging, along with glucose intolerance in high fat diet fed mice (29). In this context, our study highlights the specific effect of glucose flux via AR pathway on the availability of NAD+. The effect of continuous flux through the AR pathway leads to alteration of the NAD$^+$/NADH ratio, thereby compromising the NAMPT mediated NAD$^+$ biosynthesis. Under these circumstances, the NAMPT activity is compromised either through increased demand to supply of NAD$^+$ or through a feedback inhibition of NADH on the NAMPT activity. Although in our study we observed a trend in change in NAMPT activity by 2 hrs (on exposure to excess glucose), it was only during 8 hrs and 24 hrs time point that we noticed a significant reduction in NAMPT activity. Similarly, the sirtuin activity changes were in agreement with the NAMPT activity changes. The lesser the NAMPT activity, the lesser the NAD$^+$ that was available for biological activity of sirt-1. The plausible mechanism of AR flux through changes in NAD$^+$/NADH in inactivation of sirt1 and increased acetylation was examined. The inhibition of
sirt-1 activity was reflected by the increased acetylation of Egr-1 at 24 hrs of treatment with high glucose which mediated aberrant proatherogenic signals. In our in-vitro model of MAECs exposed to NMN, we could rescue the sirt-1 activity, thereby reducing the acetylation of Egr-1. To confirm further that Egr-1 is one of the targets of sirt-1, treatment of MAECs with a specific inhibitor, sirtinol, increased the acetylation of Egr-1. Furthermore, overexpression of sirt1 or pharmacological activation of sirt1 with SRT1720 reduced the expression of acetylated Egr1 in HAECs exposed to high glucose. These findings confirm Egr-1 is a potential target for NAD+ dependent deacetylase sirt-1.

Hassan et al. reported that glomerular ECs exposed to insulin or high concentrations of D-glucose display increased expression of Egr1 protein and mRNA as well as increased promoter activity. More importantly, their report suggested that moderately increased levels of glucose were sufficient to elicit Egr1 expression irrespective of the concentration of insulin (30). Consistent with that study, we also report induction of Egr1 expression at the protein, mRNA and promoter activity levels by high glucose. We demonstrate a prolonged activation of Egr1 in AR expressing MAECs. Studies have reported insulin mediated increase in Egr1 promoter activity and cell proliferation in bovine aortic smooth muscle cells (31). However, unlike findings by us and Hassan et al, the study did not observe the effect of glucose on Egr1. The discrepancy in results could be due to various factors but the most prominent being variation in the cell type and also the genotype. McCaffrey et al. have shown increased transcripts of Egr1 and its target genes TNF, ICAM-1 and M-CSF in human atherosclerotic lesions and in mice deficient in LDL receptor fed high fat diet. Studies from human atherosclerotic lesions and the mice aorta suggest predominant expression of Egr1 in smooth muscle cells, in the areas of macrophage infiltration and in endothelial cells (32).
Egr1 belongs to a class of tumor suppressor genes like p53 (33). Promoters of Egr1 have been shown to respond to Egr1 (34). Egr1 plays many roles in response to different stimuli; a major physiological response to increased Egr1 levels by genotoxic stress in most cell types is apoptosis (20). In hyperglycemia, we observed an auto-feedback regulation of Egr1 expression. ChIP studies using Egr1 antibody showed promoter occupancy in its target gene TF. Studies in cancer cells have shown tight interrelationships between Egr1 and other classes of similar transcription factors like p53 in trans-activation and regulation of apoptosis (35). Treatment of MCF7 cells with an anti-cancer agent showed a sustained Egr1 mRNA expression for 12 hrs (20). The initial surge of Egr1 was attributed to early responses to stimulus, however, sustained expression was proposed to be an effect of stabilized p53 through the MDM2 pathway (36). Other studies have shown acetylation of Egr-1 as a determinant of its own stability. The acetylation was proposed to be mediated through p300 at the consensus KDKK region in the Egr-1 protein (20). In our hypothesis, the only possibility of polyol flux to mediate acetylation and stabilization of Egr-1 was through defective activity of sirt1 or through increased activity of p300. The latter possibility was ruled out as our total HAT activity studies showed no significant changes between the high glucose and the normal glucose treated cells (Supplement, Fig S2). Thus, to prove sirt-1 indeed could interact with Egr-1 and de-acetylate the protein, we generated mutants of the Egr-1 in which the “K” was mutated to “A”. In vitro acetylation of the mutant and wild type Egr-1 (KDKK) using p300 showed acetylation in the wild type Egr-1 and the single mutant (ADKK) but not in the double (ADAK) and triple (ADAA) mutants of Egr-1 (Figure 5C). The wild type Egr-1 when subjected to in vitro de-acetylation showed effective de-acetylation of the acetylated Egr-1. However, a limitation of this study is the inability to infer the effect of the mutants on the TF expression as any approach to express the mutants would not
account for the already appreciable endogenous expression of the Egr-1. More solid evidence for the singular role of NAD^+ availability in limiting sirt-1 activity was provided through our studies in HAECs where solely NMN treatment improved sirt-1 activity and resulted in de-acetylated Egr-1 and reduced TF expression.

Glucose flux via AR transiently increased Egr-1–dependent expression of procoagulant TF. Recent reports suggest Egr1 dependent expression of TF in smooth muscle cells exposed to free hemin, thereby leading to more vascular injury (37). Egr1 contributes to CD-40 ligand induced expression of TF in human endothelial cells (38). Studies in humans showed glucose linked proinflammatory changes to increases in Egr-1, matrix metalloproteinases, and TF (39). In this context, our earlier studies have shown lesional haemorrhage in the aortic root of diabetic Tie2hAR/apoE/-/- mice. Sirt-1 is shown to have atheroprotective effects in the vasculature by mediating vasodilation via eNOS derived nitric oxide and scavenging reactive oxygen species(40). In the ECs and macrophages Sirt-1 have been shown to (a) have anti-inflammatory functions through down regulation of expression of various cytokines through the NFκB pathway (13), (b) suppress macrophage foam cell formation and to promote ABCA1 dependent reverse cholesterol transport, and (c) suppress the expression of endothelial TF and exert anti-thrombotic properties. These findings support our data though the stimulus remains different wherein we suggest inhibition of sirt-1 activity through NAD^+ depletion as a consequence of AR pathway flux result in increased acetylation of Egr-1 and thereby increased expression of TF.

While we have focused on Egr-1 acetylation as a key mechanism by which AR mediates inflammation, it is also possible that impaired Sirt-1 activity driven changes in acetylation of other transcription factors/cofactors such as FOXO 1, 3, 4, Hif-2α and NF-κB may contribute to inflammation in our experimental settings.
While we have demonstrated a mechanism by which AR leads to acetylation of Egr-1, it is possible that AR action also mediates initial increases in unacetylated Egr-1 expression prior to its acetylation. Our earlier studies have shown that aging-linked increased flux via AR generates AGEs; species which transduce endothelial injury consequent to their interaction with RAGE (41). Furthermore, our group has also shown that extracellular signaling initiated by AGE-RAGE interaction regulates Egr-1 expression (42). Hence, it is conceivable that AR may induce Egr-1 increases via the AGE-RAGE axis.

Reduced lesion size and decreased inflammatory marker levels in apoE-/-hAR+ mice were demonstrated upon treatment with ARI zopolresat (16). In cultured human umbilical vein ECs inhibition of AR reduced TNF-alpha-stimulated activation of NF-kB, and was linked to upregulation of ICAM-1 and VCAM-1 (43, 44). Gleissner et al. reported that AR gene expression and activity and ROS increases in human monocyte-derived macrophages incubated with oxLDL, and that treatment with an ARI attenuated ROS (45). In support of those beneficial effects of ARI we have provided clear evidence for improving the NAD$^+$-NADH ratio by enhancing the NAMPT, improving the sirtU1 activity. In concurrence with this we also observed reduced Egr1 expression and TF expression in the tissues from ARI treated diabetic apoE-/-hAR mice.

In conclusion, we surmise that glucose flux via AR in hyperglycemia mediates atherosclerosis, in part, by influencing NAMPT mediated NAD$^+$ biosynthesis. Alteration in NAD$^+$ levels cause inactivation of sirt-1 which leads to acetylation and sustained expression of Egr-1. This, in turn, leads to increased proinflammatory and procoagulatory markers. Blockade of AR improves the NAD$^+$ levels by rescuing the NAMPT biosynthesis pathway, improving sirt-1 activity mediated de-acetylation of Egr, and subsequent induction of Egr-1 target genes during
hyperglycemia (Figure 7). Our data provide insights into novel mechanisms linking the AR pathway to the NAD+/NAMPT/sirt-1 axis in the regulation of inflammatory genes in diabetic atherosclerosis.

**AUTHOR CONTRIBUTIONS**

S. V. - Assisted in the design, performed the experiments, analyzed data and assisted in preparation of the manuscript

D.T. - Assisted in the design, performed the experiments, analyzed data and assisted in preparation of the manuscript

R.A. - Assisted in the design, performed the experiments, analyzed data and assisted in preparation of the manuscript

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FIGURE LEGENDS

Figure 1. Expression of Egr-1 in aorta collected from non-diabetic apoE-/- and 14 weeks diabetic apoE-/-hAR mice. A) mRNA expression of Egr-1 in aorta from apoE-/- and apoE-/-hAR mice with and without diabetes. (N>=3, P<0.05). B) Protein expression of Egr-1 from diabetic and non-diabetic apoE-/- and apoE/-hAR mice aorta. (N=3, P<0.05). C) Representative immunohistochemistry of the aortic root from apoE-/-hAR mice with diabetes demonstrating colocalization of Egr-1 and AR in the endothelial cells (using anti-CD31 IgG). D) Pixel intensities of IHC sections of aorta from NDM and DM apoE/-hAR mice stained with Egr1 or (E) AR antibody. F) colocalized Egr-1 and AR were then quantitated using Axiovision LE 4.8.2 program. G) Expression of Egr-1 protein in the aortic tissues of diabetic apoE-/-hAR mice treated with ARI or vehicle. (N=3, P<0.05).

Figure 2. Expression of Egr-1 in primary aortic endothelial cells. A) Protein expression of Egr-1 in MAECs overexpressing hAR. Cells were either silenced with siRNA for human AR or treated with a scrambled siRNA and further exposed to physiological levels of glucose (5 mM) or high glucose (25 mM) for 24 hrs. Subcellular fractionation was performed to collect the nuclear fractions and probed for Egr-1 using Western blot. Results are expressed as ratio of Egr-1 to P62 (N=3, P<0.05). B) Protein expression of Egr-1 by Western blot in nuclear fractions of MAECs treated with 5.5 mM glucose or high glucose along with an ARI. MAECs treated with L-glucose were used as osmotic control. Results were expressed as ratio of Egr-1/P62 from at least 3 different cell types from the same treatments (P<0.05). C) mRNA transcript expression of Egr-1 in wild-type (WT) MAECs or MAECs overexpressing hAR treated with high glucose at various time-points. The results were expressed as fold change compared to “0” hr time-point.
(*P<0.05). D) Protein expression of Egr-1 by Western blot in MAECs over-expressing hAR treated with high glucose at various time-points. Results expressed as ratio of Egr-1/β-actin from at least 3 independent experiments (*P<0.05). E) Promoter activity of Egr-1 using a luciferase assay in MAECs isolated from WT and hAR over-expressing mice. Cells were treated with high glucose for 24 hrs and luciferase activity was measured. Results are expressed as the ratio of pcEgr-1-lucA/renilla from at least three independent experiments. F) Protein expression of Egr-1 in HAECs treated with high glucose and 5.5 mM glucose along with ARI and SDI for 24 hrs. Blot is a representative figure from at least three independent experiments.

**Figure 3.** Measurement of acetylated Egr-1 in primary MAECs, aortic tissues from apoE/-hAR mice using immunoprecipitation using anti-acetyl lysine antibody followed by Western blotting for Egr-1. A) Expression of acetylated Egr-1 in MAECs over expressing hAR in cells treated with high glucose and 5.5 mM glucose for 24 hrs along with ARI and SDI. B) Time dependent expression of acetylated Egr1 in MAECs treated with high glucose. C) Expression of acetylated Egr-1 in HAECs treated with 5.5 mM and high glucose along with ARI and SDI. D) Acetylated Egr-1 expression in aorta isolated from 14 week diabetic and non-diabetic apoE/-hAR mice and in diabetic mice treated with ARI for 14 weeks. Results are representative of at least three independent experiments. In this figure, WB indicates Western blot.

**Figure 4.** Sirt-1, NAMPT expression and activity, NAD+/NADH ratio measured in primary aortic endothelial cells and aorta tissues. A) Protein expression of sirt-1 at various time points of treatment with high glucose in MAECs over expressing hAR. Results are
expressed as ratio of sirt-1 expression to β-actin. B) Expression of sirt-1 in the nuclear fractions of MAECs treated with high and 5.5 mM glucose along with ARI, NMN and resveratrol for 24 hrs. Results are expressed as ratio of sirt-1/P62. C) Sirt-1 activity measured in MAECs treated with 5.5 mM and high glucose along with ARI, SDI and NMN. Results are expressed as relative percent activity compared to cells treated with 5.5 mM glucose. D) Sirt-1 activity in aortas isolated from apoE-/hAR mice with and without diabetes and diabetic mice treated with ARI. Results are expressed as relative sirt-1 activity compared to NDM. (N=4, P<0.05). E) Protein expression of NAMPT in MAECs over expressing hAR upon treatment with high glucose for various timepoints. F) NAMPT activity measured in MAECs over expressing hAR, upon treatment with 5.5 mM and high glucose along with ARI and SDI. Results are expressed as relative percent activity compared to the “0” hr treatment with high glucose or with 5.5 mM glucose treated cells respectively. G) NAMPT activity measured in aorta isolated from 14 week diabetic, non-diabetic, or ARI-treated apoE-/hAR mice. Results are expressed as relative percent activity compared to non diabetic mice (N=4, P<0.05). H) Measurement of NAD+/NADH ratio in MAECs over expressing hAR, treated with 5.5 mM, 25 mM, and 25 mM glucose along with ARI. All experiments are representative of at least n=3 or more replicates.

Figure 5. Expression of acetylated Egr-1 in MAECs over expressing hAR, A) treated with 5.5 mM and high glucose along with sirtinol (sirtuin inhibitor) or NMN (sirtuin activator) for 24 hrs. B) In-vitro de-acetylation assay was performed with wild type Egr-1 (preserved KDKK sequence/p300 binding site) as substrate and recombinant sirt-1 as the enzyme with and without NAD+. The figures represent acetylation of Egr-1 after the de-acetylation assay, subjected to SDS PAGE gel and Western blotting using anti-acetyl lysine antibody. C) Wild type Egr-1
(KDKK) and Egr-1 mutants (ADKK, ADAK, and ADAA) were subjected to in-vitro acetylation using recombinant p300 and the figure represents the acetylated Egr-1 after acetylation assay mixture was subjected to SDS PAGE gel and probed with anti-acetyl lysine antibody. In this figure, WB indicates Western blot.

**Figure 6. Expression of pro-inflammatory and pro-thrombotic markers in primary aortic endothelial cells and aortic tissues from apoE-/-hAR mice.** A) Expression of VCAM-1 protein in MAECs over expressing hAR treated with 5.5 mM and high glucose along with siRNA for Egr-1. The expression is represented as ratio of VCAM-1/β-actin. Expression of TF protein was measured in MAECs over expressing hAR, B) treated with siRNA for hAR or, C) treated with 5.5 mM and high glucose along with ARI or, D) treated with siRNA against Egr-1 under high glucose conditions. Results were expressed as ratio of TF/β-actin. E) ChIP was performed with anti-Egr-1 antibody in HAECs treated with high glucose to probe for promoter occupancy of Egr-1 in TF gene. The DNA after IP was subjected to PCR using specific TF primers that include the Egr-1 binding site. The amplicons are represented in the figure. The experiments are representative of at least three independent studies. F) Expression of TF in HAECs over expressing human sirt-1 further subjected to nominal and high glucose treatments. The figure shows a representation of at least three independent experiments. G) Measurement of TF by ELISA in plasma collected from apoE-/-hAR mice with or without diabetes and diabetic mice treated with ARI. The results are expressed as ng/ml plasma (N>=5, P<0.05).
Figure 7. Scheme depicting proposed mechanism by which glucose flux via AR triggers activation, acetylation and prolonged expression of Egr1 leading to proinflammatory and prothrombotic responses in diabetic atherosclerosis.
Figure 1

A

\[ \text{Egr1}/\beta\text{-actin} \]

B

\[ \text{Ratio of Egr1}/\beta\text{-actin} \]

C

CD31  AR  DAPI  Colocalization

AR-NDM apoE\(^{-}\)

AR-DM apoE\(^{-}\)

AR NDM apoE\(^{-}\)

AR-DM apoE\(^{-}\)

D

\[ \text{Egr1 signal} \]

E

\[ \text{AR signal} \]

F

\[ \text{Colocalized signal} \]

G

\[ \text{Egr1 signal} \]

\[ \text{\(\beta\)-actin} \]

DM+ AVI  Vehicle
Figure 2

A

Ratio of Egr1/PGF2

LG  HG  LG  HG
scrambled  siAR

Egr1  PGF2

B

Ratio of Egr1/PGF2

p<0.05  p<0.05

L-Glucose
- - - -
Glucose (25 mM)  + + + +
Glucose (5 mM)  + + - -
AR1  - - + +

Egr1  PGF2

C

Egr1 mRNA transcripts vs Time (hrs)

0  0.5  1  2  4  8  24

WT  AR

D

Egr1/β-actin vs Time (hrs)

0  0.5  1  2  4  8  24

hAR-MAEC  WT-MAEC

E

Ratio of p-CREB1/CreB1

WT  AR

F

Egr1/β-actin vs Time (hrs)

p<0.05  p<0.05

Glucose (25 mM)  - + + +
Glucose (5 mM)  + - - -
AR1  - - + +
SDI  - - - +

231x333mm (300 x 300 DPI)
Figure 4

A

B

C

D

E

F

G

H

Diabetes

231x333mm (300 x 300 DPI)
Figure 5

A

|                  | IP. Ac-lysine | hAR |
|------------------|--------------|-----|
|                  | WB: Egr1     |     |
| Glucose (mM)     | 5            | 25  |
|                  | 25           | 25  |
| NMN (500 μM)     | -            | -   |
|                  | -            | +   |
| Sirtinol (20 nM) | -            | -   |
|                  | -            | +   |

B

Wild type Egr1 (KDKK)

|                  |                | NAD+ (50 μM) |
|------------------|----------------|-------------|
|                  |                | +           |
|                  |                | -           |

C

Ac-Egr1

KDKK  ADEK  ADEK  ADEA

65x27mm (300 x 300 DPI)
Figure 6

A

Ratio of VCAIM1/β-actin

WT  hAR

LG  HG  si-Egr1  LG  HG  si-Egr1

p<0.05  p<0.05  p<0.05

B

hAR

scrambled  siAR

TF  β-actin

Glucose (mM)  5  25  5  25

C

TF  β-actin

Glucose (mM)  5  25  5  25

ARI (100 μM)

E

IP-Egr1  PCR-TF

Input  WT  AR  IgG

F

TF (ng/ml)

NDM  DM  DM+ARI

p<0.05  p<0.05

153x157mm (300 x 300 DPI)
Figure 7

Hyperglycemia

Glucose

Increased Polyol flux

NAD/NADH

Sirt1

Egr1

VCAM, MMPs, TF etc...

Inflammation, hemorrhage
Endothelial dysfunction, Vascular injury

Nucleus

136x104mm (300 x 300 DPI)
Figure S1. (a) Nuclear fractions of aorta samples from non diabetic WT or hAR transgenic mice were analyzed by western blot for expression of Egr-1. (b) Expression of Egr-1 in aorta collected from apoE-/-hAR treated with streptozotocin. The tissues were extracted after 8, 10, 12 and 14 weeks of treatment with streptozotocin. Results are representative of 5 mice per group.
**Figure S2.** Total HAT activity measured using assay kit (Activ Motif, USA) (Cat#56100) in MAECs. The figure represents percent total HAT activity in HAECS treated with low glucose, high glucose, high glucose along with ARI and high glucose with SDI.

**Figure S3.** Expression of acetylated Egr-1 in MAECs over expressing hAR, treated with low glucose along with FK (NAMPT inhibitor) for 4 hrs.
Figure S4. Expression of acetylated Egr-1 in HAECs treated with low glucose along with high glucose; HAEC over expressing sirt1 or GFP at high glucose and HAEC treated with DMSO or SRT1720(10uM) an activator of sirt1 for 5hrs. The figure shows a representation of at least three independent experiments.