

Glucosamine-induced ER Stress Accelerates Atherogenesis: A Potential Link between Diabetes and Cardiovascular Disease

Vi T Dang, Daniel R Beriault, Arlinda Deng, Yuanyuan Shi and Geoff H Werstuck*

Thrombosis and Atherosclerosis Research Institute, McMaster University, Canada

*Corresponding author: Geoff H Werstuck, Thrombosis and Atherosclerosis Research Institute, McMaster University, Hamilton, Canada, Tel: 9055212100; Fax: 9055771427 E-mail: Geoff.Werstuck@taari.ca

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Abstract

Background: Cardiovascular disease is the leading cause of death worldwide and is responsible for three out of four deaths in diabetic individuals. Our lack of understanding of the molecular mechanisms linking diabetes and atherosclerosis impedes the development of effective treatment strategies. Hyperglycemia and glucosamine-supplementation have been shown to induce endoplasmic reticulum (ER) stress and activate the unfolded protein response (UPR) in murine models of atherosclerosis. We hypothesize that diabetes/hyperglycemia promotes atherosclerosis by a mechanism involving glucosamine-induced ER stress/UPR activation and that attenuation of ER stress, using the chemical chaperone 4-phenylbutyric acid (4PBA), will slow the accelerated development of atherosclerosis.

Methods: Hyperglycemia was induced in female Apolipoprotein E-deficient (ApoE^{-/-}) mice by multiple low-dose streptozotocin injections or by the introduction of the Ins2+/Akita mutation. Glucosamine-supplementation was achieved by adding different concentrations of glucosamine (0.625-5% w/v) to the drinking water of ApoE^{-/-} mice. Subsets of mice from each group were also treated with 4PBA. The development of atherosclerosis was evaluated based on atherosclerotic lesion area and volume at the aortic sinus. Levels of protein O-linked N-acetylglucosamine (O-GlcNAc) and ER stress markers were determined in atherosclerotic lesions using immunohistochemistry and immunofluorescence staining.

Results: Hyperglycemic and glucosamine-supplemented mouse models showed similar increases in O-GlcNAc and ER stress/UPR activation levels in atherosclerotic lesions. Lesion area was not significantly different between the three models of accelerated atherosclerosis. Glucosamine supplementation at $\geq 2.5\%$ (w/v) significantly increased lesional O-GlcNAc, UPR activation and atherosclerotic lesion area/volume, independent of changes in any measured metabolic parameters. 4PBA mitigated ER stress and attenuated accelerated atherosclerosis in both hyperglycemic and glucosamine-supplemented mouse models.

Conclusion: These findings suggest that hyperglycemia promotes accelerated atherosclerosis by a mechanism involving glucosamine-induced ER stress. Accelerated atherosclerosis can be attenuated in hyperglycemic ApoE^{-/-} mice by reducing ER stress levels.

Keywords: Atherosclerosis; Hyperglycemia; Glucosamine; ER stress

Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide [1]. Risk factors of CVD include obesity [2], dyslipidemia [3], smoking [4], hypertension [5] and diabetes mellitus [6]. Diabetes is a debilitating disease that affects millions of individuals and is increasing in prevalence at a dramatic rate [7,8]. Although CVD is responsible for approximately 75% of deaths in individuals with diabetes [9], the development of therapeutic strategies to prevent and/or treat CVD has been impeded due to the lack of understanding of the underlying biochemical mechanisms linking diabetes to CVD. Atherosclerosis is one of the major underlying causes of CVD, and expanding our knowledge of how diabetes promotes atherosclerosis, can facilitate the development of treatment strategies to slow or prevent the development of atherosclerosis.

All forms of diabetes are characterized, and clinically defined, by increased blood glucose levels, known as hyperglycemia. In an attempt to understand the pro-atherogenic effects of hyperglycemia, most of the research has been focussed on determining the pathways affected by the increase in glucose metabolism and the oxidative stress associated with the hyperglycemia [10,11].

Although a number of pre-clinical studies supported the causative role of oxidative stress [12,13], antioxidant treatments have not demonstrated beneficial effects in reducing CVD risk in diabetic population in any of the large clinical trials in which they were tested [14,15]. This observation suggests that there are other causative mechanisms behind hyperglycemia-promoted atherosclerosis that may act independently, or work in parallel to, oxidative stress. Impaired endoplasmic reticulum (ER) function has been associated with the development of atherosclerosis in humans and in mouse models [16,17].

The ER is responsible for the folding, modification and trafficking of approximately one-third of all proteins produced in a typical eukaryotic cell [18]. When the influx of newly synthesized proteins exceeds the ER processing capacity, unfolded or misfolded proteins can accumulate and induce ER stress. The unfolded protein response (UPR) is a multifaceted, cellular self-defence mechanism that alleviates ER stress by attenuating de novo protein synthesis, increasing protein folding capacity and facilitating the degradation of irreversibly misfolded proteins [18,19]. Chronic ER stress/UPR activation has been associated with various pro-atherogenic processes including endothelial cell apoptosis, macrophage-foam cell inflammation and lipid accumulation [20].

The objective of this study is to investigate the role of glucosamine-induced ER stress/UPR activation in diabetic atherosclerosis. Previous research in our lab has shown that glucosamine-supplementation accelerates atherosclerosis in ApoE^{-/-} mice [21,22]. We hypothesize that the concentration of glucosamine required to promote ER stress would be similar to that required to promote atherosclerosis. Furthermore, we hypothesize that reducing ER stress levels, using the chemical chaperon 4-phenylbutyric acid (4PBA), will attenuate the accelerated development of atherosclerosis in both hyperglycemic and glucosamine-supplemented mouse models.

Materials and Methods

Animal models

ApoE^{-/-} mice were crossed with Ins2^{+/+}/Akita mice to produce ApoE^{-/-}:Ins2^{+/+}/Akita offspring (n=4/group) [23]. Five-week-old female ApoE^{-/-} mice were randomly divided into three groups (n=12/group): control, 5% glucosamine-supplemented and STZ-injected groups. Multiple low-doses (40 mg/kg/day for ten days) of STZ (Sigma-Aldrich) were injected intraperitoneally to induce hyperglycemia.

A different set of five-week-old female ApoE^{-/-} mice were given regular drinking water (n=10), or water supplemented with 0.625% (w/v) (n=4), 1.25% (n=4), 2.5% (n=6) or 5% (n=9) glucosamine (w/v, Sigma-Aldrich) in drinking water. Subsets of control, 5% glucosamine-supplemented ApoE^{-/-} and ApoE^{-/-}:Ins2^{+/+}/Akita mice (n=4-5/group) were treated with 20 mM 4PBA (Scandinavian Formulas Inc., PA) in drinking water.

This level of 4PBA has previously been shown to attenuate ER stress in mice [24]. All mice were given unrestricted access to water and standard chow diet (TD92078; Harlan Teklad, Madison, WI) and were maintained on a 12-hour light/dark cycle throughout the study. Triglycerides and cholesterol were measured using Infinity reagents (Thermo Scientific). All mice were sacrificed at 15 weeks of age, plasma and tissues were collected for analysis. All procedures were approved by McMaster University Animal Research Ethics Board.

Histochemistry

Formalin-fixed hearts were cut transversely and embedded in paraffin. Aortas including aortic root were sectioned, as previously described [25]. Serial sections (5 µm) were stained with hematoxylin and eosin (Sigma-Aldrich) [25], or Masson's Trichrome (Sigma-Aldrich) to visualize atherosclerotic lesion area/volume.

Masson's Trichrome staining was performed based on Sigma-Aldrich's instruction in which nuclei, collagen and muscle/erythrocytes are stained in black, blue and red, respectively. Stained sections were

imaged using a Leitz LABORLUX S microscope connected to a DP71 Olympus camera. Lesion area was quantified using Image J (1.48v) software. Lesion volume was computed as area under the curve of lesion area [25].

Immunohistochemistry and immunofluorescence

Paraffin imbedded serial sections were stained with the primary antibodies against KDEL (StressGen, Canada) for GRP78/GRP94, CTD110.6 (Convance Inc., CA) or RL2 (Affinity Bioreagent) for O-GlcNAc, or GADD153 (Santa Cruz, CA) for CHOP, as previously described [25]. Negative controls were stained with IgG (Sigma-Aldrich) instead of primary antibodies to correct for non-specific staining. Stained sections were imaged using a Leitz LABORLUX S microscope connected to a DP71 Olympus camera. Positively stained area was quantified using Image J (1.48v) software.

Plasma 4PBA quantification

Plasma was extracted using an ice-cold mixture of 1:1 methanol:ethanol (v/v). L-phenylalanine-d8 (20 µM, Cambridge Isotope Laboratories, MA) was used as the internal standard. An Agilent 1200 RR series liquid chromatography system (Agilent Technologies Inc., CA) coupled to a Bruker micrOTOF II (Bruker Daltonics, MA) mass analyzer equipped with an electrospray ionization (ESI) source (Agilent Technologies Inc.) was used to quantify plasma 4PBA concentration. A volume of 2 µL of plasma extract was injected per run into a 2.1 x 50 mm Halo C8 column.

Statistical analysis

One-way ANOVA followed by Tukey's HSD test was used to compare results between multiple groups. Data were presented as the mean ± standard deviation. For all experiments, p-value of <0.05 was considered statistically significant, *p<0.05, **p<0.01, ***p<0.001.

Results

Hyperglycemia and glucosamine-supplementation induce similar increases in vascular O-GlcNAc, ER stress and accelerated atherosclerosis in ApoE^{-/-} mice

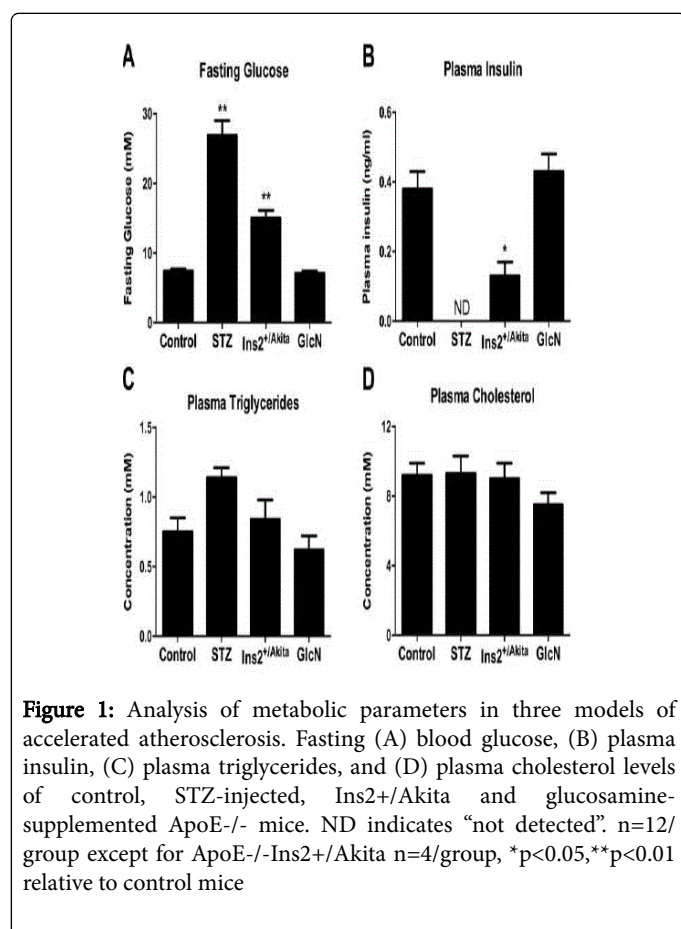
Hyperglycemia was induced in female ApoE^{-/-} mice by multiple low-dose STZ injections, or by the introduction of the Ins2Akita mutation. A separate group of ApoE^{-/-} mice were supplemented with glucosamine (5% w/v) in the drinking water. At 10 weeks of age, STZ-injected mice were severely hyperglycemic (FBG=26.9 ± 2.1 mM), Ins2^{+/+}/Akita mice were moderately hyperglycemic (15.0 ± 1.1 mM) and glucosamine-supplemented mice were normoglycemic (7.1 ± 0.3 mM), relative to ApoE^{-/-} controls (7.4 ± 0.3 mM) (Figure 1A).

Plasma insulin levels of both hyperglycemic models were significantly lower than that of the control or glucosamine-supplemented ApoE^{-/-} mice (Figure 1B). Up to 15 weeks of age, none of the mice showed any significant changes in plasma cholesterol or triglycerides (Figure 1C and 1D).

Consistent with previous findings, both hyperglycemia and glucosamine-supplementation significantly accelerated atherogenesis at the aortic sinus, relative to controls (Figure 2) [21-23]. Atherosclerotic lesion area was not significantly different between the three models of accelerated atherosclerosis. Protein O-linked N-

acetylglucosamine (O-GlcNAc) levels have been directly correlated to intracellular levels of glucosamine [26].

We found that both hyperglycemic mouse models and the glucosamine-supplemented mouse model had similar elevations in the levels of lesional O-GlcNAc, relative to ApoE^{-/-} controls (Figure 2). Glucosamine is a potent ER stress inducer in cultured vascular cells [22]. Both hyperglycemic and the glucosamine-supplemented mouse models showed similar increases in the levels of UPR proteins GRP78/94 and CHOP within the atherosclerotic lesions. Together these data are consistent with our hypothesis that hyperglycemia promotes atherosclerosis by a mechanism involving glucosamine-induced ER stress.



Determining the glucosamine threshold level required to accelerate atherosclerosis

If ER stress is necessary to accelerate the development of atherosclerosis, then the concentration of glucosamine required to promote ER stress will be similar to that required to promote atherosclerosis. To determine this threshold level of glucosamine, five-week-old female ApoE^{-/-} mice were supplemented with 0, 0.625, 1.25, 2.5 or 5% (w/v) glucosamine in drinking water for 10 weeks. At 15 weeks of age, all mice were sacrificed and analysed. Glucosamine-supplementation did not alter any metabolic parameters including body weight, fasting glucose concentration, plasma cholesterol and triglycerides levels in ApoE^{-/-} mice (Figure 3).

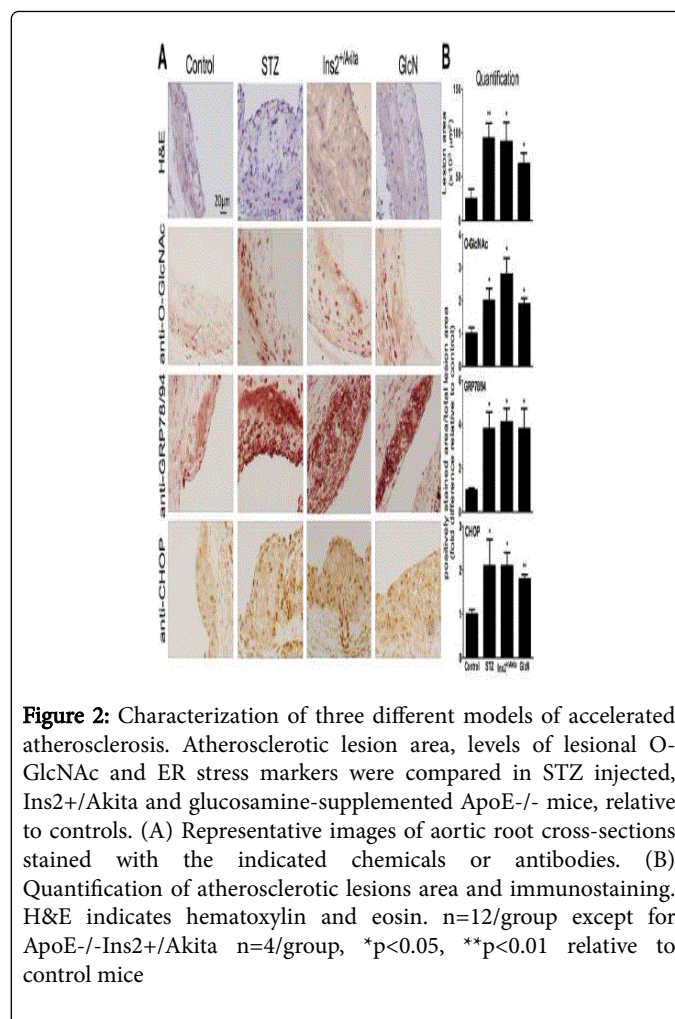


Figure 2: Characterization of three different models of accelerated atherosclerosis. Atherosclerotic lesion area, levels of lesional O-GlcNAc and ER stress markers were compared in STZ injected, Ins2^{+/Akita} and glucosamine-supplemented ApoE^{-/-} mice, relative to controls. (A) Representative images of aortic root cross-sections stained with the indicated chemicals or antibodies. (B) Quantification of atherosclerotic lesions area and immunostaining. H&E indicates hematoxylin and eosin. n=12/group except for ApoE^{-/-}Ins2^{+/Akita} n=4/group, *p<0.05, **p<0.01 relative to control mice

Supplementation at 0.625 and 1.25% (w/v) did not significantly alter lesional protein linked O-GlcNAc (Figure 4A and Suppl. Figure 1) or UPR protein levels (Figure 4B-4C and Suppl. Figures 2, 3), nor did they affect atherosclerotic area or volume at the aortic sinus. Glucosamine-supplementation at 2.5 and 5% significantly increased lesional protein linked O-GlcNAc and also activated the UPR, which is indicative of ER stress in the atherosclerotic lesions (Figure 4). Furthermore, significantly larger atherosclerotic lesion area and volume were observed at the aortic sinus in mice supplemented with $\geq 2.5\%$ glucosamine, relative to mice receiving $< 2.5\%$ glucosamine (Figure 5). These results are consistent with the hypothesis that glucosamine-induced ER stress promotes atherosclerosis.

4PBA mitigates ER stress and attenuates accelerated atherosclerosis

We have identified a correlation between glucosamine-induced activation of the UPR and accelerated atherosclerosis. If elevated ER stress level is necessary for the accelerated development of atherosclerosis in glucosamine-supplemented mice then we would predict that the chemical chaperone, 4PBA, which attenuates ER stress, will also diminish the accelerated development of atherosclerosis in both glucosamine-supplemented and hyperglycemic mouse models. To test this, 4PBA was supplemented in drinking water of five week old

ApoE^{-/-} controls, glucosamine-supplemented ApoE^{-/-} mice and ApoE^{-/-}Ins²⁺/Akita mice for 10 weeks.

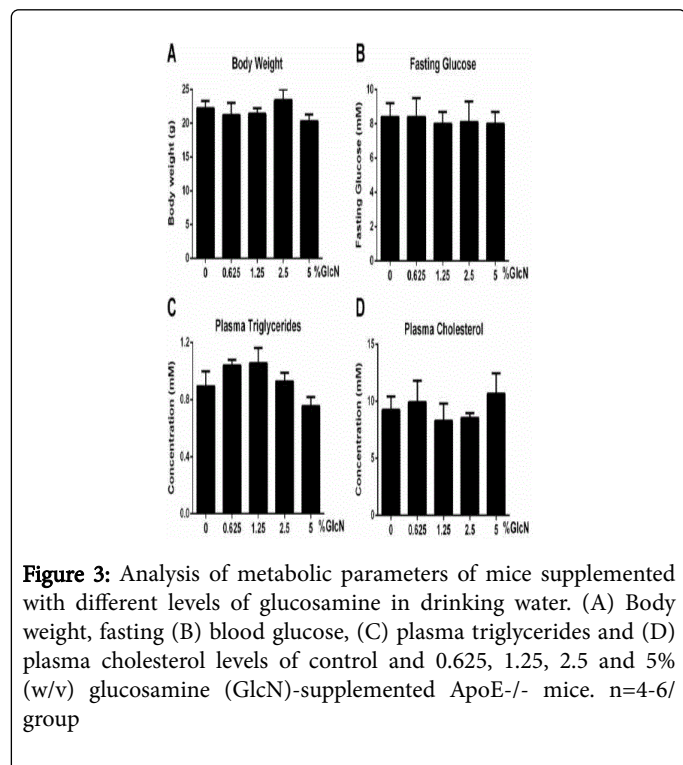


Figure 3: Analysis of metabolic parameters of mice supplemented with different levels of glucosamine in drinking water. (A) Body weight, fasting (B) blood glucose, (C) plasma triglycerides and (D) plasma cholesterol levels of control and 0.625, 1.25, 2.5 and 5% (w/v) glucosamine (GlcN)-supplemented ApoE^{-/-} mice. n=4-6/group

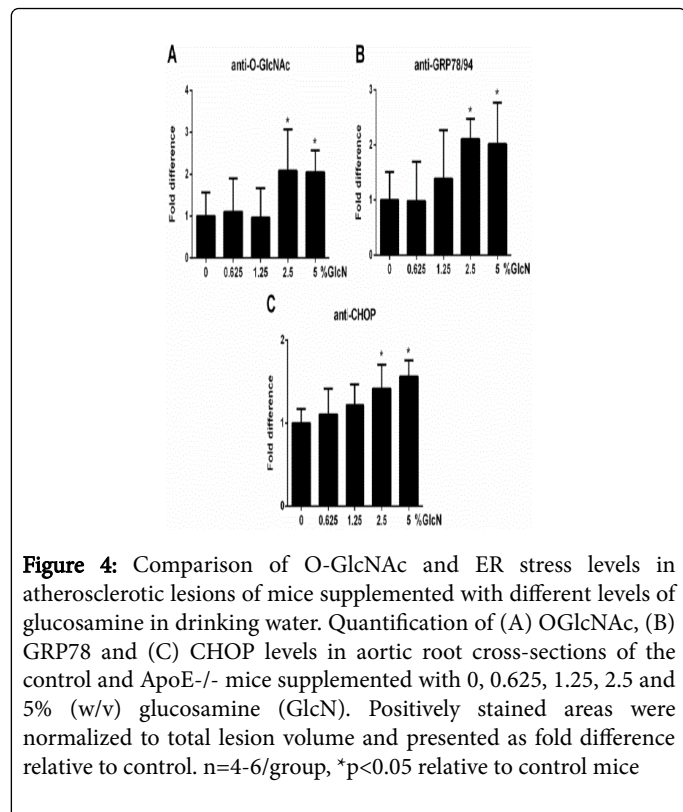


Figure 4: Comparison of O-GlcNAc and ER stress levels in atherosclerotic lesions of mice supplemented with different levels of glucosamine in drinking water. Quantification of (A) O-GlcNAc, (B) GRP78 and (C) CHOP levels in aortic root cross-sections of the control and ApoE^{-/-} mice supplemented with 0, 0.625, 1.25, 2.5 and 5% (w/v) glucosamine (GlcN). Positively stained areas were normalized to total lesion volume and presented as fold difference relative to control. n=4-6/group, *p<0.05 relative to control mice

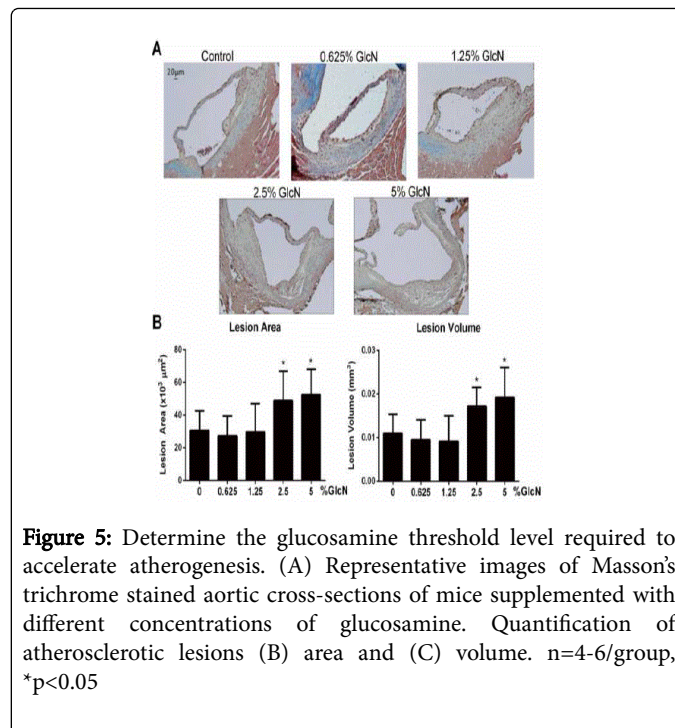


Figure 5: Determine the glucosamine threshold level required to accelerate atherogenesis. (A) Representative images of Masson's trichrome stained aortic cross-sections of mice supplemented with different concentrations of glucosamine. Quantification of atherosclerotic lesions (B) area and (C) volume. n=4-6/group, *p<0.05

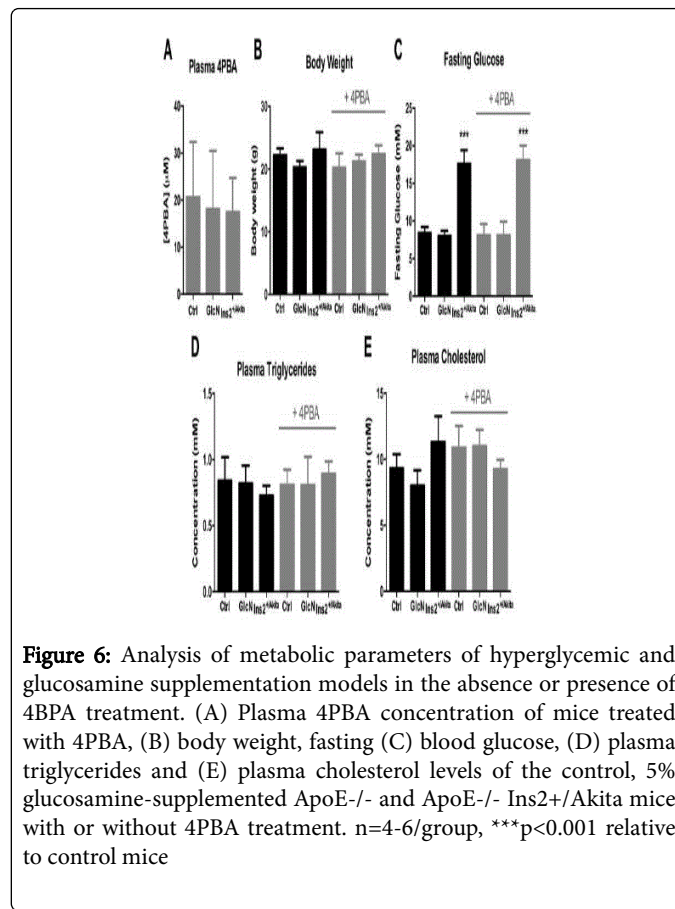


Figure 6: Analysis of metabolic parameters of hyperglycemic and glucosamine supplementation models in the absence or presence of 4PBA treatment. (A) Plasma 4PBA concentration of mice treated with 4PBA, (B) body weight, fasting (C) blood glucose, (D) plasma triglycerides and (E) plasma cholesterol levels of the control, 5% glucosamine-supplemented ApoE^{-/-} and ApoE^{-/-} Ins²⁺/Akita mice with or without 4PBA treatment. n=4-6/group, ***p<0.001 relative to control mice

Plasma 4PBA concentration was measured using liquid chromatography coupled to a mass spectrometer. The results indicate that plasma 4PBA levels were not significantly different between any

groups of mice treated with 4PBA (Figure 6A). Metabolic parameters including body weight, fasting glucose concentration, plasma cholesterol and triglycerides levels were not significantly different between mice with and without 4PBA treatment (Figure 6B-6E). Glucosamine-supplemented and hyperglycemic mice treated with 4PBA showed significant decrease in the levels of GRP78 and CHOP protein (Figure 7A, 7B and Suppl. Figures 4, 5), relative to mice without 4PBA treatment. 4PBA did not affect the level of ER stress indicators in the control mice. We found that 4PBA also reduced the levels of vascular O-GlcNAc in mice with 4PBA treatment, relative to those without 4PBA treatment, with the exception of control mice (Figure 7C and Suppl. Figure 6).

Atherosclerotic lesion area and volume were significantly decreased in glucosamine-supplemented ApoE^{-/-} mice and ApoE^{-/-}Ins2^{+/-}/Akita mice treated with 4PBA, relative to mice without 4PBA treatment (Figure 8). 4PBA did not reduce atherosclerotic lesion area/volume in the control mice. Collectively, these results indicate that reducing ER stress levels attenuates the accelerated development of atherosclerosis.

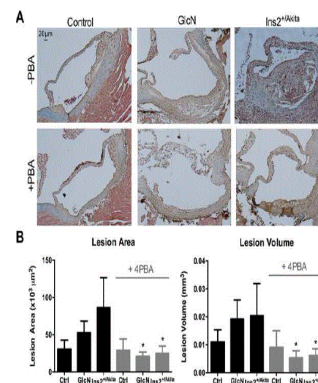
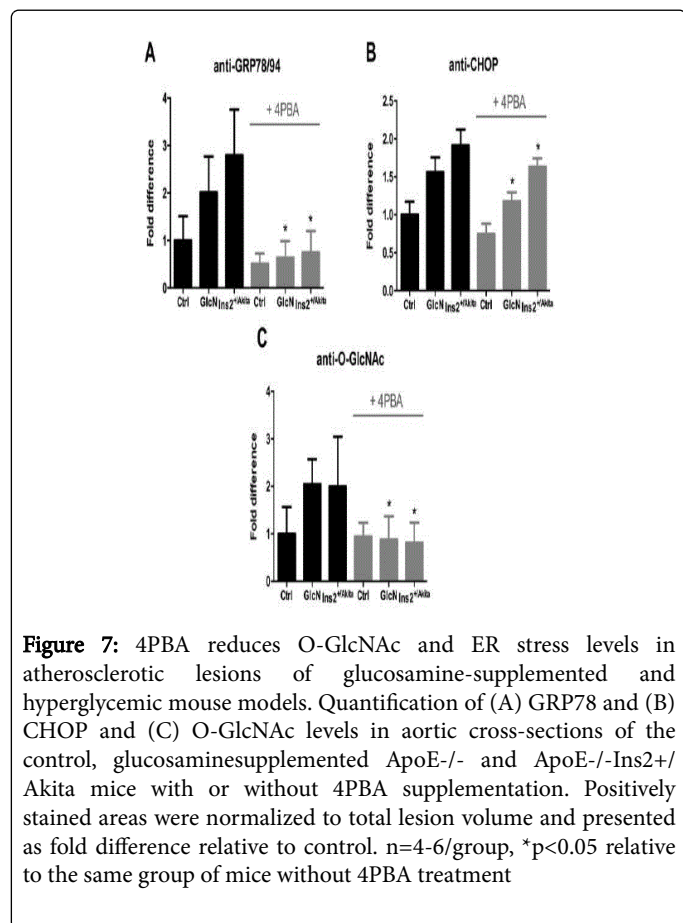


Figure 8: 4PBA attenuates accelerated atherosclerosis in diabetic and glucosamine supplemented ApoE^{-/-} mice. Representative images of Masson's trichrome stained aortic cross-sections of ApoE^{-/-} mice supplemented with glucosamine and ApoE^{-/-}Ins2^{+/-}/Akita mice in the absence or presence of 4PBA treatment. Quantification of atherosclerotic lesions (B) area and (C) volume. n=4-6/group, *p<0.05 relative to the same group of mice without 4PBA treatment

Discussion

In this study, we show that chemically (STZ injection) or genetically (Ins2^{+/-}/Akita)-induced hyperglycemia promotes accelerated atherosclerosis in ApoE^{-/-} mice in a manner that is independent of changes in plasma lipids. Similar increases in atherosclerotic lesion size were induced by supplementing ApoE^{-/-} mice with glucosamine, which is a downstream metabolite of glucose. This study is the first direct comparison of these three mouse models of accelerated atherosclerosis. Each of these models showed a similar elevation in the level of O-GlcNAc and ER stress response proteins in atherosclerotic lesions, relative to ApoE^{-/-} controls. In the titration experiments, we identified the threshold level of glucosamine required to induce ER stress is the same as that required to accelerate atherosclerosis (≥2.5% w/v in drinking water). Finally, we found that a chemical chaperone, which reduces ER stress levels in hyperglycemic and glucosamine-supplemented mice, attenuated the accelerated development of atherosclerosis. Together these findings are consistent with the mechanism in which hyperglycemia-associated atherosclerosis is driven by glucosamine-induced ER stress.

The last few decades have witnessed a dramatic, worldwide increase in the prevalence of type 1 and especially type 2 diabetes that is likely driven by multiple environmental and lifestyle changes [7,8]. CVD is the leading cause of death in both type 1 and type 2 diabetes, even after controlling for other cardiovascular risk factors including dyslipidemia, obesity and hypertension [27,28]. All forms of diabetes are clinically defined by increased blood glucose concentration and there is a progressive relationship between hyperglycemia and CVD, with CV risk rising approximately 20% for every 1.5 mM increase in fasting glucose levels [29], and for every 1% elevation in HbA1c levels [30]. Both epidemiological and pathophysiological studies have shown that hyperglycemia is an independent risk factor for CVD [6,31,32]. Hyperglycemia is known to increase flux through the HBP leading to increased production of UDP-N-acetylglucosamine (UDP-GlcNAc) –

an essential substrate for both N-linked and O-linked protein glycosylation [33,34]. Activation of the HBP has been implicated in the development of diabetic complications including glucotoxicity [35], insulin resistance [36], and cardiomyocyte dysfunction [37]. We have identified an additional consequence of enhanced HBP flux that involves the disruption of protein processing in the ER – a condition known as ER stress. Evidence from our lab and others has implicated ER stress in the development and progression of atherosclerosis in both humans and animal models [16,17]. Nonetheless, the mechanisms by which ER stress and/or UPR activation may promote the induction of pro-atherosclerotic pathways are not fully understood.

The chemical chaperone 4PBA has previously been shown to attenuate atherosclerosis in dyslipidemic mouse models [38]. However, this is the first demonstration that it can block accelerated atherogenesis associated with hyperglycemia. This observation is consistent with our hypothesis that ER stress plays a causative role in diabetes-associated atherosclerosis and further highlights the therapeutic potential of targeting the UPR as a viable strategy to treat and/or prevent atherosclerosis. The ability of glucosamine to induce ER stress is interesting because glucosamine is a popular dietary supplement used to treat joint pain associated with osteoarthritis [39]. To determine whether these supplements, when taken as directed (1500 mg/day), caused ER stress and/or activated the UPR in humans, we have previously measured markers of UPR activation in circulating peripheral blood cells isolated from fasting blood samples taken from volunteers before and after a 14 day regimen of glucosamine-supplementation. Our results suggested that this glucosamine-supplementation regimen does not result in UPR activation [40]. This is likely because this dose is insufficient to induce ER stress. Relative glucosamine concentrations in our diabetic and glucosamine-supplemented mouse models are estimated to be approximately 40 fold higher.

In conclusion, we have identified a molecular mechanism by which glucosamine-induced ER stress promotes the development of atherosclerosis. We propose that this mechanism may contribute to the accelerated atherosclerosis that is observed in mouse models of hyperglycemia and perhaps, in individuals with diabetes. Expanding the knowledge of how diabetes promotes atherosclerosis will facilitate in the development of novel, more effective therapeutic strategies to slow or stop atherogenesis.

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