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TITLE: Control of Atherosclerosis Regression by PRMT2 in Diabetes

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14. ABSTRACT: Diabetics have more heart disease than their non-diabetic counterparts, even though drugs like Lipitor are equally effective in both groups at lowering the blood levels of harmful cholesterol. We propose to understand why comparable reductions in harmful cholesterol are less effective in diabetics. We have identified an enzyme called PRMT2, which regulates the abundance of a cellular cholesterol transporter that helps to prevent cells from accumulating in arteries and forming a plaque. We have shown that the level of PRMT2, while high in healthy cells, is very low in cells from diabetics when blood sugar levels are elevated. Because PRMT2 isn't around in cells under diabetic conditions, we predict that more cells accumulate in the artery, and exacerbating heart disease in diabetics. To test this we will determine what happens to the growth of a plaque when we eliminate PRMT2 with and without diabetes in mouse models of heart disease. We expect that plaques will grow larger in the absence of PRMT2. To better understand how PRMT2 suppresses plaque growth, we will also identify proteins that are modified by PRMT2 and determine if these proteins participate in plaque formation. Given that we also don't understand why PRMT2 levels decrease in diabetes, we will identify the factors that regulate PRMT2 levels. That knowledge might enable us to develop ways to restore the normal level of PRMT2 in diabetes, and prevent the cells from contributing to plaque formation.					
15. SUBJECT TERMS PRMT2, atherosclerosis regression, diabetes, macrophages, asymmetric dimethyl arginine substrates, regulation of gene expression					
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TABLE OF CONTENTS:	page number
1. Introduction.....	1
2. Keywords.....	1
3. Accomplishments.....	1
4. Impact.....	5
5. Changes/Problems.....	5
6. Products.....	5
7. Participants & Other Collaborating Organizations.....	5
8. Special Reporting Requirements.....	6
9. Appendices.....	None

1. INTRODUCTION:

High plasma cholesterol and diabetes are major risk factors for atherosclerosis. People with diabetes are 2-4 times more likely to suffer from coronary heart disease (CHD). Given that 1 in 4 veterans who receive care from VA hospitals has diabetes testifies to the importance of this problem for military personnel. In human diabetics, treatment with cholesterol-lowering drugs such as statins fails to fully reduce the risk for atherosclerosis. Understanding the factors that are altered as a result of impaired glucose homeostasis is a significant area of cardiovascular research that has important ramifications for the health and well-being of the military workforce. Established coronary lesions of adulthood begin in childhood. Over time macrophages accumulate cholesterol, which promotes their differentiation into foam cells that become trapped in the artery, contributing to the growth of atherosclerotic plaques. Owing to a diminished capacity of cholesterol-engorged macrophages to migrate, they accumulate and fail to resolve inflammation, which leads to the plaque becoming unstable and rupturing, resulting in heart attacks and stroke. Accordingly, an important clinical goal to reduce CHD risk is to promote the regression of atherosclerosis by eliciting macrophage cholesterol efflux and macrophage migration from plaques.

Work from our labs has shown that regression of atherosclerosis is mediated in part by the Liver X Receptor (LXR) family of nuclear receptors through the induction of genes involved in cholesterol efflux. Given that we also found that regression of atherosclerosis is impaired in the context of diabetes, we proposed that changes in glucose levels modulate LXR-dependent gene expression and reduce the expression of LXR target genes like the cholesterol transporter protein *ABCA1*, and that this molecular mechanism might account for the increased rate of atherosclerosis in diabetics. We showed that LXR-mediated *ABCA1* expression and ABCA1-dependent cholesterol efflux is compromised in macrophages exposed to high, compared to normal, levels of blood glucose. Moreover, we identified the protein arginine methyltransferase 2 (PRMT2), cellular levels of which are reduced in high versus normal glucose, as a factor that mediates this effect. Macrophages devoid of PRMT2 showed reduced LXR-dependent induction of *ABCA1* and reduced ABCA1-mediated cholesterol efflux, thus mimicking the effect of high glucose. Expression of *PRMT2* was lower in monocytes from diabetic mice. Our studies revealed PRMT2 as a glucose-sensitive factor that plays a role in the induction of *ABCA1* by LXR and affects cholesterol efflux. Thus, PRMT2 might be critical in mediating the increased incidence of atherosclerosis in diabetics. The goal of this proposal is to understand how PRMT2 deficiency promotes atherosclerosis during diabetes.

2. **KEYWORDS:** PRMT2, atherosclerosis regression, diabetes, macrophages, asymmetric dimethyl arginine, regulation of gene expression

3. ACCOMPLISHMENTS:

- What were the major goals and objectives of the project?

The major goals of the project are:

- 1) determine the role PRMT2 plays in the impaired regression of atherosclerosis in diabetes
- 2) determine the substrates of PRMT2 in macrophages in normal and high glucose
- 3) determine the molecular regulation of PRMT2

- What was accomplished under these goals? Major activities for this reporting period:

Specific Aim 1: To determine the role PRMT2 plays in the impaired regression of atherosclerosis in diabetes

Major Task 1: Bone Marrow transplant from *PMRT2*^{-/-} into *LDLR*^{-/-} mice (completed)

Subtask 1: Generate *PMRT2*^{-/-} and WT mice for bone marrow transplant into *LDLR*^{-/-} mice

Subtask 2: Perform bone marrow transplants of *PMRT2*^{-/-} into *LDLR*^{-/-} mice and enter mice into

study protocols (**completed**)

We have completed the breeding of the *PRMT2*^{-/-} mice as donors for the bone marrow transplant. We also performed bone marrow transplant from littermate WT and *PRMT2*^{-/-} mice into atherosclerosis prone *LDLR*^{-/-} mice using the following scheme (Fig.1). After bone marrow transplant, mice were allowed to recover for 4 weeks, then placed on a high fat, high cholesterol “western diet” for 20 weeks to promote the formation of atherosclerotic plaques. At 19 weeks, half the mice were made diabetic using STZ, and the other half were not, and injected with citrate buffer as a control. To induce regression, mice were treated with an anti-sense oligonucleotide (ASO) against the *APOB* gene to reduce LDL cholesterol levels and promote regression. Mice were placed on chow diet for 3 weeks, plaques harvested and plaque area

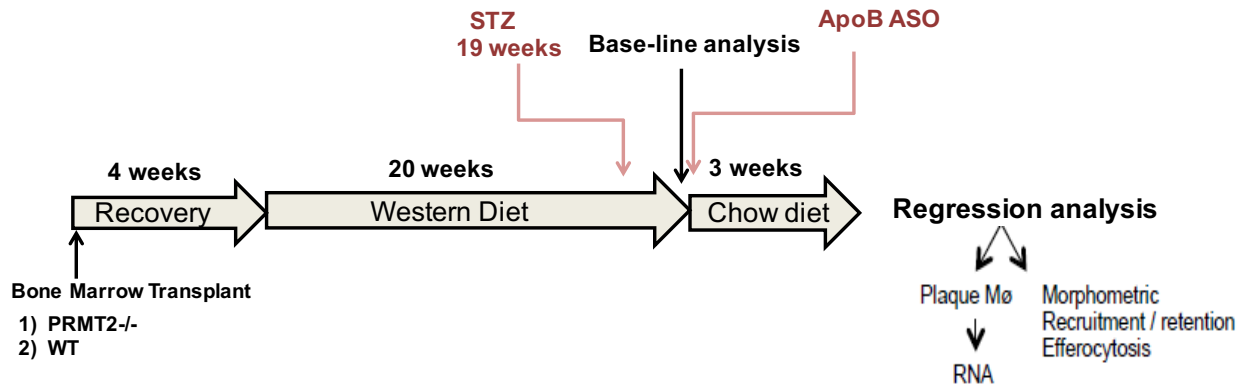


Figure 1. Experimental design for the regression of atherosclerosis studies. *LDLR*^{-/-} mice were irradiated to kill off the endogenous bone marrow and bone marrow transplant were performed from *PRMT2*^{-/-} or litter mate wild type controls. Mice were allowed to recover for 4 weeks and placed on a western diet for 20 weeks. A group of animals was sacrificed after 20 weeks of western diet feeding and used as the baseline group. Two other groups of animals received citrate buffer or STZ to induce diabetes at 19 weeks. At 20 weeks, regression and regression/STZ groups will received *APOB* anti-sense oligonucleotide (ASO) and were switched to a chow diet to lower LDL cholesterol. Animals were sacrificed 3 weeks after the *APOB* ASO injection and the indicated parameters analyzed.

measured.

Major Task 2: Analysis of aortic arches from regression cohort +/- PMRT2, +/- diabetes

Subtask 1: Sac mice, collect serum, harvest, fix and embed aortic tissues (**in progress**)

The *PRMT2*^{-/-} bone marrow recipients had similar body weight, cholesterol and glucose levels compared to their WT bone marrow recipient counterparts (Fig 2A-C; see 19 week columns). As expected, the

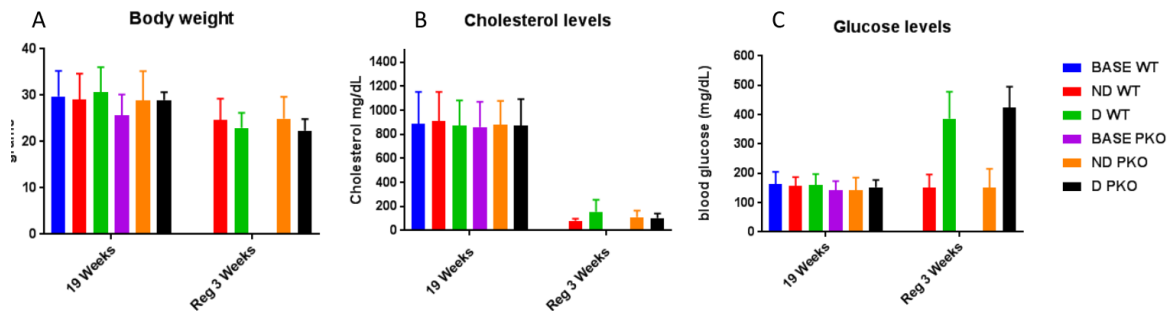


Figure 2. Parameters of the *PMRT2*^{-/-} and WT bone marrow transplant mice. Mice weights, total cholesterol and blood glucose levels were determined in wild type and *PRMT2*^{-/-} cohorts. Base= baseline; ND= non-diabetic; D= diabetic; WT= wild type; PKO= *PRMT2* knock out (*PRMT2*^{-/-} bone marrow). The bars above “19 weeks” are the measurements taken after 19 weeks on western diet (progression phase). The bars above the “Reg 3 weeks” are measurements taken at the end of the 3-week regression period.

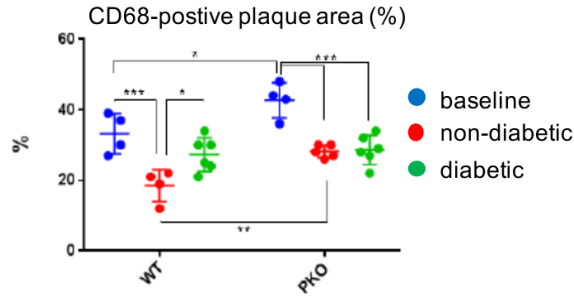


Figure 3. PRMT2 expression affects plaque macrophage content in diabetic mice. Aortic roots from baseline and regression groups from WT and PRMT2^{-/-} (PKO) without or with diabetes were sectioned, fixed, and stained for CD68. The percent area occupied by CD68⁺ cells of the plaque area was quantified using ImagePro Plus Software. Data (mean ± SEM) (n≥8) were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. P < 0.05 values were considered to be significant. *P < 0.05, **P < 0.005, ***P < 0.0005.

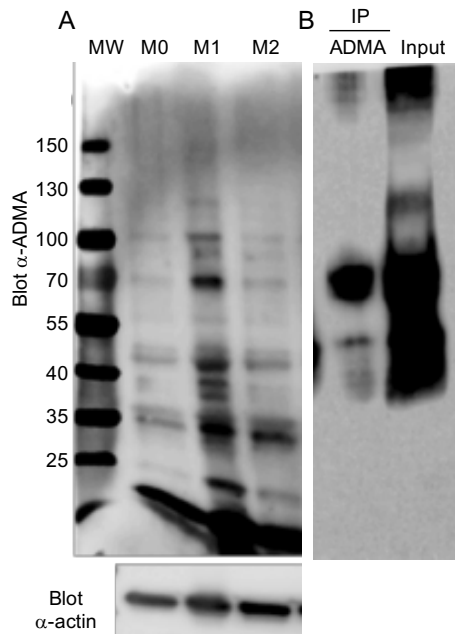


Figure 4. Asymmetric arginine dimethylation (ADMA) substrate capture A) Western blot of whole cell lysates from BMDMs in the M0, M1 or M2 state cultured in low glucose blotted with an ADMA-specific antibody, and actin as a loading control. B) Immunoprecipitation of ADMA-modified proteins and blotted with an ADMA specific antibody.

glucose levels were dramatically higher in STZ-treated mice than in control mice (Fig 2C; see green and black bars, Reg 3 weeks). Similar reductions in cholesterol were observed in normal and diabetic mice upon reversal of hyperlipidemia (Fig 2B; see Reg 3 weeks).

To determine the effect of PRMT2 on atherosclerosis regression, we measured plaque area and total macrophage content by quantifying the percent of the macrophage marker CD68 (Fig 3). The preliminary results show the following: PRMT2^{-/-} baseline mice had higher macrophage contents in their plaques compared to WT mice. After 3 weeks of reduction in plasma cholesterol levels, we observed reduced macrophage content in the normoglycemic mice expressing WT and PRMT2^{-/-} indicative of atherosclerosis regression. As expected, regression in WT mice under diabetic conditions mice was impaired. By contrast, PRMT2^{-/-} mice did not alter plaque macrophage content under diabetic versus non-diabetic conditions: in other words, regression was independent

of diabetes in PRMT2^{-/-} mice (Fig 3). This is consistent with PRMT2 expression being low under hyperglycemia. The remaining mice in each group will be similarly analyzed.

Specific Aim 2: To determine the substrates of PRMT2 in macrophages in normal and high glucose that affects LXR transcriptional activity.

Major Task 1: Conduct studies to assess the PRMT2 substrate capture by immunoprecipitation (IP) using asymmetric arginine dimethylation antibody. **(in progress)**

PRMT2 is responsible for asymmetric arginine dimethylation (ADMA) of as yet unidentified substrates that regulate gene expression. To gain mechanistic insights into the pathway and processes controlled by PRMT2, we will characterize PRMT2 substrates by proteomic analyses of macrophages cultured under normal glucose concentrations from WT and PRMT2^{-/-} BMDMs in the M1 state where ADMA levels are high compared to macrophages in the M0 or M2 states (Fig 4A). We will use affinity purification with an antibody specific to asymmetric arginine dimethylation to identify PRMT2 substrates. As proof of concept, we performed an IP on lysates from WT M1 macrophages. We were able to see an enrichment of asymmetric arginine dimethylated proteins in the IP fraction (Fig 4B). We will continue to optimize the IP of asymmetric arginine dimethylation polypeptides and then scale up for identification of asymmetric arginine dimethylated by mass spectrometry.

Major Task 1: Mapping PRMT2 cis regulatory elements by reporter gene assays based on ENCODE data;

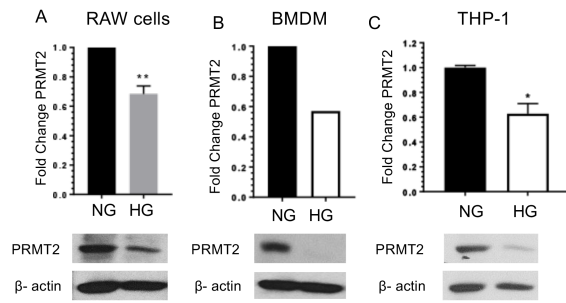


Figure 5. Reduction of steady state PRMT2 mRNA and protein in high glucose. A) RAW, B) BMDMs, C), THP1 cells were cultured in normal glucose (NG) and high glucose (HG) for 1 week, and PRMT2 expression levels were measured by qPCR and normalized to cyclophilin. Protein extract were prepared and blotted for PMRT2, and beta-actin as a loading control.

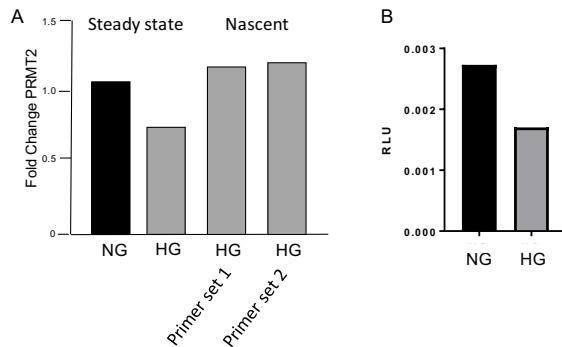


Figure 6. Post transcriptional regulation of PRMT2 mRNA. A) Steady state and nascent RNA of PRMT2 was measured by qPCR from BMDMs cultured in normal glucose (NG) and high glucose (HG). Whereas the PRMT2 steady state mRNA level is reduced in cells cultured in high glucose compared to normal glucose, the nascent RNA is not. This is true for two independent primer sets that measures nascent PRMT2 RNA. This indicates that the reduction of PRMT2 mRNA is not at the level of transcription initiation. B) PRMT2-3' UTR-luciferase reporter construct was transfected into 293 cells in normal and high glucose and activity measured and presented as relative luciferase units (RLU). Note the reduction in luciferase reporter activity in high glucose, suggesting that the decrease in PRMT2 mRNA is via the 3' UTR.

Subtask 1: Test PRMT2 promoter luciferase construct for glucose regulated expression. **(in progress)**

We have shown that the steady state PRMT2 mRNA and protein levels are reduced in high as compared to low glucose in the murine macrophage RAW cell line, in mouse BMDMs and in human macrophage cell line THP-1 (Fig 5). However, we have recently found that PRMT2 nascent RNA, a surrogate for newly transcribed RNA and measured by using primer pairs that span an intron-exon junction, is not reduced when cells are cultured in high glucose (Fig 6A). This suggests that the effect of glucose is not at the level of transcription initiation, but rather regulated post-transcriptionally. Consistent with this idea the PRMT2 promoter linked to a luciferase reporter gene did not show a change in luciferase activity in cells cultured in low and high glucose. Thus, the PRMT2 promoter alone is not sufficient to confer glucose regulation on PRMT2 expression (shown in the previous progress report).

Given that the glucose-dependent regulation of PRMT2 appears to be post-transcriptional, we examined whether the PRMT2 mRNA 3'UTR was a potential target of regulation. To do this, we used a luciferase reporter construct fused to the 3' UTR of PRMT2 compared to a reporter without the PRMT2 3' UTR. We found a reduction in expression of the luciferase reporter in high compared to normal glucose for the PRMT2 3' UTR construct compared to control (Fig 6B), suggesting that the high glucose-dependent decrease in PRMT2 mRNA is controlled via its 3' UTR.

• **What opportunities for training and professional development did the project provide?**

Nothing to Report.

• **How were the results disseminated to communities of interest?**

We will disseminate our findings through publications in peer reviewed journals, although at this point we have nothing to report.

• **What do you plan to do during the next reporting period to accomplish the goals and objectives?**

Our goals for the next reporting period are to increase the number of mice in the regression study, and to examine macrophage trafficking and changes in gene expression by RNA-seq from laser captured micro

to examine macrophage trafficking and changes in

dissected CD68+ macrophages. If the trends we observed in the regression study above continues in the replicate study, and depending on the outcome and from the bulk RNA seq study from laser captured micro dissected CD68+ macrophages from the plaque, we will then perform single cell RNA seq to determine the impact of PRMT2 on cell type specific gene expression.

We will continue to optimize and scale the IP capture of asymmetrically dimethylated substrates, in order to identify the asymmetrically dimethylated substrates of PRMT2 by mass spec from WT and *PRMT2*^{-/-} bone marrow derived macrophages.

We will also continue to elucidate the mechanism whereby expression of PRMT2 is controlled by high glucose, focusing on the post-transcriptional regulatory mechanisms that target the 3' UTR of PRMT2, such as glucose sensitive micro RNAs.

4. IMPACT:

Our work will determine the impact of PRMT2 in atherosclerosis upon diabetes. We also predict that our proteomic analyses of PRMT2 substrates will be useful in elucidating the mechanism of PRMT2 function in macrophages, and in identifying possible interventions and biomarkers. Finally, understanding the regulation of PRMT2 will enable us to develop ways to restore the normal level of PRMT2 in diabetes, and prevent the cells from contributing to plaque formation.

What was the impact on technology transfer?

None at this point

What was the impact on society beyond science and technology?

None at this point

5. CHANGES/PROBLEMS:

We will modify aim 3 as the glucose dependent regulation appears to be not at the level of transcription initiation but rather post-transcriptionally via the PRMT2 3 UTR. Also, because of the almost one year delay in working out the mouse protocol details, we are still behind our original time table, but as summarized above, we have made significant gains and we expect to maintain steady progress.

6. PRODUCTS:

Nothing to Report

7: PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

PIs

Name: Michael Garabedian, PhD.

Project Role: PI.

Nearest person month worked: 3

Contribution to Project:

Dr. Garabedian helped design and analyze the experiments involving the identification of the PRMT2 substrates and determining the regulation of PRMT2 expression by high glucose.

Funding Support: CDMRP/NIH

Name: Edward Fisher, MD, PhD

Project Role: Partner PI.

Nearest person month worked: 2

Contribution to Project:

Dr. Fisher helped design experiments involving the role of PRMT2 in the regression of atherosclerosis and diabetes.

Funding Support: CDMRP/NIH

Post docs

Prashanth Thevkar Nagesh, PhD

Project Role: Post doc

Nearest person month worked: 9

Contribution to Project:

Dr. Nagesh is performing the experiments involving the PRMT2 substrates identification and the PRMT2 gene regulation studies.

Funding Support: CDMRP/NIH

Beyza Vurusaner Aktas, PhD

Project Role: Post doc

Nearest person month worked: 9

Contribution to Project:

Dr. Aktas is performing the experiments involving the impact of the loss of PRMT2 expression in the regression of atherosclerosis in diabetes.

Funding Support: CDMRP/NIH

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8 SPECIAL REPORTING REQUIREMENTS: COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.

An independent report will be submitted by Dr. Fisher, the partnering PI.

9 APPENDICES: none