Advanced glycation end products induce a prothrombotic phenotype in mice via interaction with platelet CD36

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Short title: Thrombosis, CD36 and advanced glycation end products
Abstract

Diabetes mellitus has been associated with platelet hyper-reactivity, which plays a central role in hyperglycemia related prothrombotic phenotype. The mechanisms responsible for this phenomenon are not established. Here, we investigated the role of CD36, a class B scavenger receptor in this process. Using both in vitro and in vivo mouse models, we demonstrated direct and specific interactions of platelet CD36 with advanced glycation end products (AGE) generated under hyperglycemic conditions. AGE bound to platelet CD36 in a specific and dose dependent manner, and binding was inhibited by a high affinity CD36 ligand NO2LDL. CD36 null platelets did not bind AGE. Using diet and drug induced mouse diabetes models we showed that cd36 null mice had delayed time to formation of occlusive thrombi in a FeCl3-induced carotid artery injury model compared with wild type. Cd36 null mice had similar level of hyperglycemia and similar level of plasma AGE compared with WT mice under this condition, but WT mice had more AGE incorporated into thrombi. Mechanistic studies revealed that CD36-dependent JNK2 activation is involved in this pro-thrombotic pathway. Thus, this study couples vascular complications in diabetes mellitus with AGE-CD36-mediated platelet signaling and hyper-reactivity.
Introduction

Diabetes mellitus is associated with an increased risk of pathological arterial thrombosis, including myocardial infarction and stroke.\textsuperscript{1-3} Factors underlying this risk include accelerated atherosclerosis, endothelial dysfunction, and platelet hyper-reactivity, but the mechanisms linking diabetes to platelet hyper-reactivity are not well understood.\textsuperscript{4,5} Our lab and others have shown that platelets express receptors, including scavenger receptors and toll-like receptors, that recognize endogenous and exogenous "danger signals", so called Danger Associated Molecular Patterns (DAMPs).\textsuperscript{6, 7} We hypothesized that endogenous DAMPs generated in hyperlipidemia, diabetes, obesity and other chronic inflammatory conditions could interact with platelets through these receptors to mediate platelet activation and induce a pro-thrombotic state. Indeed, in mouse models of hyperlipidemia and oxidant stress we showed that oxidized LDL, a lipid-based DAMP, induced a pro-thrombotic state via interactions with the platelet type 2 scavenger receptor CD36.\textsuperscript{8-10} Similarly, oxidized phospholipids on the surface of cell-derived microparticles were shown to interact with platelet CD36 and promote thrombosis after oxidant injury to the arterial wall.\textsuperscript{11}

Chronic hyperglycemia is a common feature of diabetes and is an important initiator of vascular complications, many of which have been related to vascular and inflammatory cell interactions with advanced glycation end products (AGE).\textsuperscript{12,13} These heterogeneous long-lived protein adducts are produced through non-enzymatic chemical reaction between sugars and the amino groups of proteins.\textsuperscript{14} Circulating levels of AGE, detected most commonly as hemoglobin A1C, are clinically important biomarkers used for monitoring diabetes therapy. On a pathophysiological level,
cellular interactions with AGE induce biological responses that have been directly linked to the development of diabetic vascular complications, and that are mediated by specific cell surface receptors. The best studied AGE receptor is the Ig superfamily member RAGE (Receptor for AGE), although limited studies suggest that scavenger receptors such as SR-A, SR-BI and CD36 may also serve this function.\textsuperscript{15}

Platelets from patients with diabetes have been reported to be hyper-reactive and this has been related to AGE accumulation,\textsuperscript{8,16-18} but no evidence exists directly linking AGE to platelet function and no mechanisms have been defined. In this manuscript we tested the hypothesis that AGEs are DAMPs that interact specifically with platelet receptors to induce platelet hyper-reactivity and a prothrombotic state. We found that although RAGE, SR-A and SR-BI immuno-reactivity could be detected in platelets, the binding of AGE to platelets was mediated primarily by CD36, and that AGE-induced CD36 signaling resulted in enhanced platelet reactivity in vitro and enhanced arterial thrombosis in vivo. The latter was shown using mouse models of both type 1 and type 2 diabetes as well as direct injection of AGE into normal mice.

CD36, which was first described as platelet glycoprotein IV, is constitutively expressed at high levels on platelets and modulates platelet function by ligand-dependent triggering of a signaling pathway that involves specific Src family kinases, JNK family MAP kinase,\textsuperscript{10} and vav family guanine nucleotide exchange factors.\textsuperscript{8} Importantly, recent genetic studies revealed that platelet CD36 expression levels vary widely among individuals and that this variation is correlated with platelet responsiveness to oxidized LDL and associated with inheritance of specific CD36 gene polymorphisms.\textsuperscript{7} CD36 polymorphisms have also been associated with risk of acute
myocardial infarction (AMI) or stroke,\textsuperscript{19-21} suggesting that CD36 interaction with disease-specific DAMPs may be an important contributor to the pathogenesis of arterial thrombotic events. The present study provides novel insights on the etiology of thrombotic complications in diabetic patients and defines the CD36 pathway as a potential target for development of novel anti-thrombotic therapeutic strategies.
Material and Methods

Materials. AGE–BSA and BSA were from Cell Biolabs. EZ-Link Sulfo-NHS-Biotinylation kit was from Thermo Scientific. \( \text{NO}_2^+ \text{LDL} \) and control \( \text{NO}_2 \text{LDL} \) were generated using the MPO-hydrogen peroxide-nitrite system described previously.\(^7\) RAGE blocking antibody (AF1179) and its Control goal IgG were from R&D system.\(^{22}\) Streptozotocin (STZ) was from Sigma. RAGE immunoblotting antibody was from abcam (ab30381), OSR-48 (AGE-receptor 1), galectin-3 (AGE receptor 3), SR-BI, SR-A and Actin antibodies were from Satan Cruz. Phosphorylated JNK2 and total JNK2 antibodies were from Cell Signaling. MBP protein was from New England Biolabs.

Carotid artery thrombosis model. All procedures on animals were approved by the Cleveland Clinic Institutional Animal Care and Use Committees (IACUC). Mice were housed in a facility fully accredited by AALAC and in accordance with all federal and local regulations. C57Bl/6 or \( cd36 \) null mice backcrossed 10 times into a C57BL/6 background were subjected to common carotid artery injury by application of 10% \( \text{FeCl}_3 \) for 1 min as previously described.\(^{10,11,20}\) Rhodamine 6G (100\( \mu \)l; 0.5mg/ml) was injected directly into the right jugular vein to label platelets and thrombus formation was observed in real time using intravital fluorescence microscopy and video image capture. Time to complete cessation of blood flow was determined by visual inspection and end points were set as cessation of blood flow for more than 30 sec or observation of 30 min. In some studies, 50\( \mu \)g AGE-BSA dissolved in 50\( \mu \)l saline was injected through the right jugular vein 20 min prior to vessel injury.
Diabetes models. Type I diabetes was induced in 10 - 12 week old mice by 5 daily intraperitoneal injections of STZ (50mg/kg). Blood glucose levels were measured by FreeStyle glucometer (Abbott). To induce type 2 diabetes, mice were maintained on a high fat, high fructose diabetogenic diet (DBD) (Bio-Serv S3282; 35.5% lard, 32.7% sucrose and maltose dextrin) for 8 wks, beginning at 4 wks of age after weaning. Control mice were fed either normal chow (Harlan Teklad 2918; 5% fat) or a “western” diet (WD) (Harlan Teklad 88137, 42% calories from milk fat). The latter induces equivalent hyperlipidemia to the DBD diet but without hyperglycemia.

Platelet preparation and aggregometry assays. Mice were anesthetized with ketamine (90 mg/kg) and xylazine (15 mg/kg). Whole blood (600 μl) was collected from the inferior vena cava in 100μl 0.109M sodium citrate and then diluted in 500μl Ca²⁺/Mg²⁺ free modified Tyrode’s buffer. Diluted platelet rich plasma (PRP) was separated by centrifuging at 100g for 10 min at 22°C. Diluted platelet poor plasma (PPP) was prepared by further centrifugation at 800g for 2 min. Platelets were counted using a hemocytometer and concentrations adjusted to 2 x 10⁸/ml with PPP. CaCl₂ and MgCl₂ (both 1mM final concentration) were added immediately before platelet aggregation studies. Platelet aggregation in response to 1μM ADP was assessed at 37°C in a dual channel Type 500 VS aggregometer (Chrono-log, Havertown, PA) with stirring at 1200 rpm. For human studies, PRP was prepared from normal donors and aggregometry assessed as previously described.⁷

Immunohistochemical staining. After carotid artery thrombosis assays, mice were sacrificed and the thrombosed carotid arteries were harvested and embedded in OCT
and frozen on dry ice. Cross sections (5 µm) were prepared and stained with antibodies to phospho-JNK2 or AGE using a DAB + substrate chromogen system (DAKO). Images were scored based on staining intensity as described previously. 

**Immunoblotting assay.** Platelets were isolated by serial centrifugation and lysed in cell lysis buffer containing (in mM): 20 Tris-HCl, pH 7.0, 2 EGTA, 5 EDTA, 30 NaF, 60 β-glycerophosphate, 20 Na₄P₂O₇·10H₂O, 1 Na₃VO₄, and 1% Triton X-100 as well as proteinase cocktail (R&D). 20 µg of protein was analyzed by immunoblot using standard techniques with antibodies to indicated proteins. Blots were stripped and re-probed with anti-actin antibody as a loading control. Band intensities were determined using ImageJ software v1.37 from NIH and then normalized to non-phosphorylated forms of the targeted protein or actin.

**Flow Cytometry AGE-platelet binding assay.** Washed platelets were incubated with 5% human serum albumin for 30 min at 22°C. Platelets were then treated with different concentration of biotin-labeled AGE-BSA or BSA as controls for 1-2hr followed by 5 µg/ml of avidin-AlexaFluor488 conjugate (Invitrogen) for 30min before analysis by flow cytometry. In some cases, platelets were first incubated with 10µg/ml RAGE blocking antibody or control IgG 45 min before treated with biotin-labeled AGE-BSA; and all other incubation time was shortened as indicated.

**CD36 fusion protein binding to AGE.** A recombinant CD36-Maltose Binding Protein (MBP) fusion protein containing the N-terminal extracellular domain of CD36 (aa29-22) was expressed in bacterial cells and purified to homogeneity by affinity chromatography.
The fusion protein or MBP control at 25μg/ml were coated in PBS at 4°C overnight in wells of a 96 well plate. Wells were then incubated with different concentrations of AGE-BSA for 2 hours at 22°C. After washing, the amount of bound AGE-BSA was detected using an AGE ELISA Kit (Cell Biolabs, San Diego, CA).

**Plasma Metabolic Parameters.** Tail vein blood from overnight fasted mice was collected between 9:00am and 10:00am into EDTA containing tubes and centrifuged at 2000g for 10 min to isolate plasma. Plasma was aliquoted and stored at -80°C until assayed. Total cholesterol and non-esterified fatty acids were assayed using colorimetric kits (Wako). AGEs was analyzed by ELISA (Cell Biolabs, San Diego, CA). Non-starving blood glucose levels were measured by FreeStyle glucometer (Abbott).

**Statistical analysis.** Each experiment was repeated at least 3 times, values were expressed as mean ± SE. Statistical significance was evaluated by one-way ANOVA or unpaired t tests as appropriate using Prism 5.0 (GraphPad Software).
**Results:**

**AGE binds to platelets via CD36.** To determine whether AGE could bind specifically to murine platelets, we developed a flow cytometry based binding assay using biotinylated AGE-BSA and avidin conjugated Alexa fluor-488. As shown in Figure 1A (upper panels), biotin-AGE-BSA bound to platelets from C57Bl/6 wild type (wt) mice in a concentration dependent manner reaching a plateau at ~75μg/ml. Binding of biotinylated “native” BSA, used as a control, was significantly lower than biotin-AGE-BSA and was not concentration dependent, consistent with a non-specific effect. Because of the relatively high background binding of BSA the signal for binding at the lowest concentration of AGE-BSA tested (5μg/ml) was not distinguishable from that of the control. We next explored the possibility that CD36 could be mediating the specific binding of AGE to platelets. As shown in Figure 1A (lower panels), no specific binding of AGE-BSA was seen to platelets from cd36 null mice. The degree of binding was similar to that of native BSA and was not concentration-dependent.

To determine if platelet activation influences AGE-BSA binding we examined thrombin-stimulated platelets and found a modest increase in AGE-BSA immunofluorescence in both wild type and cd36 null platelets compared to resting platelets. No significant differences were seen when comparing the increase in wild type to that seen in cd36 null (data not shown), suggesting that platelet activation results in exposure of additional CD36-independent binding sites for AGE.

We also found that pre-incubation of platelets with 100 μg/ml NO$_2$–LDL, a specific ligand for CD36, inhibited binding of biotin-AGE-BSA to resting platelets by up to 50% (Figure 1B), whereas the control NO$_2$–LDL has no effect. These results support the
conclusion that platelet-AGE interactions are mediated by CD36. The lack of full inhibition by saturating concentrations of NO$_2$-LDL suggests that the recognition site for AGE may not completely overlap that for oxidized phospholipids.

To demonstrate a direct interaction between CD36 and AGE in cell-free conditions, we used a solid phase immunologic binding assay in which ELISA plate wells were coated with a recombinant CD36-MBP fusion protein containing a large section of the CD36 N-terminal extracellular domain. As shown in Figure 1C, AGE bound to the CD36-MBP fusion protein in a concentration–dependent, saturable manner with maximal binding seen at 2-5µg/ml. AGE binding to MBP control peptide was not concentration dependent and was significantly lower than that to CD36-MBP.

To determine the potential role of other AGE receptors in platelet activation, we analyzed platelet expression levels of candidate molecules, including RAGE. Although RAGE is widely expressed on vascular cells, its presence in platelets has not been established. Using an immunoblot assay we detected immuno-reactive RAGE in platelet lysates; no differences in expression levels were seen when comparing cd36 null platelets to wt (Figure 2A). Similarly, AGE receptor 1(AGE-R1, OST-48), SR-BI and SR-A, all of which have been reported to bind to AGE, were detected in mouse platelets with no differences in expression seen in cd36 null vs wt cells (Figure 2A). AGE-R3 was not detected in either wt or cd36 null platelets (not shown). These data demonstrate that the loss of AGE binding capacity in cd36 null platelet was not due to unexpected loss of other AGE receptors. To assess the role of RAGE in platelet-AGE binding we pre-incubated wt platelets with 10 µg/ml of a well-characterized blocking monoclonal antibody to RAGE.$^{22}$ As shown in Figure 2B, blocking RAGE had no
significant effect on biotin-AGE-BSA binding. These results strongly suggest that platelet-AGE binding is mediated primarily by CD36.

**AGE enhances platelet reactivity ex vivo in a CD36-dependent manner.** Having shown that AGE bind to platelets via CD36, we tested whether this interaction could influence platelet activation by assessing platelet aggregation in response to low dose of adenosine diphosphate (ADP), a physiologically relevant agonist. As shown in Figure 3A, pretreatment of murine PRP with AGE-BSA significantly increased the extent of platelet aggregation in a concentration-dependent manner. This response was significantly diminished in platelets from cd36 null mice (Figure 3B), even at the highest AGE-BSA concentration tested. Native BSA had no statistically significant impact on aggregation responses in either wt or cd36 null platelets, demonstrating specificity. As shown in Figure 3C, AGE-BSA also significantly enhanced ADP-induced aggregation in human PRP.

**Hyperglycemia enhances carotid artery thrombus formation in vivo in a CD36-dependent manner.** To test the in vivo relevance of these findings we used a FeCl₃ induced carotid injury model of arterial thrombosis in wt or cd36 null mice rendered chronically hyperglycemic either by diet induced insulin resistance or drug-induced pancreatic islet destruction, models of type 2 and type 1 diabetes respectively. Both interventions significantly increased non-fasting blood glucose. Three weeks after STZ treatment non-fasting glucose levels stabilized at ~500mg/dl in both wt and cd36 null mice. On the DBD diet glucose levels stabilized at ~300mg/dl with a small, but
significant difference between the two strains (supplemental Figure 1A). Both DBD and STZ increased serum levels of cholesterol and non-esterified fatty acids (NEFA) (supplemental Figure 1B), with no differences between wt and cd36 null animals. In these studies we also used chow fed and western diet (WD) fed mice as controls. The latter produced hypercholesterolemia comparable to that seen with the DBD diet and STZ, but without hyperglycemia.

As shown in Figure 4, induction of hyperglycemia in wt mice in both the STZ and DBD models was associated with significant shortening of carotid thrombosis times, with mean occlusion times of 467±29.1 and 448.1±11.8 seconds respectively, compared to 717.7±27.9 seconds in the chow fed controls (p < 0.05). Similar to what we reported previously,9,11 cd36 deletion had no impact on occlusion times in chow fed or WD fed mice at this dose of FeCl₃. However, absence of CD36 rescued the pro-thrombotic phenotype in both diabetes models, with mean occlusion times not significantly different from those in WD fed animals (p = 0.39 and 0.49 respectively).

**AGE accumulation in thrombi in diabetic mice is attenuated by cd36 deletion.** We hypothesized that circulating AGE levels would increase in mice subjected to diet or STZ-induced diabetes and that these would function as ligands for platelet CD36 to promote thrombosis. In the absence of CD36 we predicted that AGE would not bind platelets and therefore would not accumulate within the thrombi formed after FeCl₃ induced carotid injury. Plasma levels of AGE, quantified by ELISA, were elevated ~2 fold in DBD mice and ~12 fold in STZ mice when compared to the chow group, but there were no differences comparing wt to cd36 null animals (Figure 5A), suggesting
that CD36 has no effect on AGE generation. Immunohistochemical staining with polyclonal anti-AGE antibody of frozen sections prepared from dissected carotid artery thrombi showed that AGE was readily detectable in the thrombi from diabetic mice (Figure 5B). Semi-quantitative scoring of antibody staining intensity showed that cd36 deletion significantly decreased AGE staining scores (Figure 5B and C). Interestingly, AGE immunoreactivity was also seen in the vessel wall of the diabetic mice (Figure 5B), but CD36 deficiency did not affect the staining intensity. These data are consistent with CD36 function as the predominant AGE receptor on platelets, but not on vascular endothelium and smooth muscle, where presumably RAGE predominates.

**CD36-dependent platelet signaling is induced by AGE in vitro and hyperglycemia in vivo.** CD36-mediated platelet activation in response to oxLDL or cell-derived microparticles is mediated by a signaling pathway that requires activation of the MAP kinase JNK. To show that this same pathway can be activated by AGE, we treated platelets with AGE-BSA and demonstrated markedly increased JNK2 phosphorylation in wt platelets but not cd36 null platelets (Figure 6A). Native BSA had no effect on JNK2 phosphorylation. We then assessed JNK2 phosphorylation status in circulating platelets isolated from both wt and cd36 null mice after induction of diabetes with DBD or STZ and found that both treatments increased basal platelet JNK2 phosphorylation levels by 2-3 fold compared to control mice (p < 0.05). This response was not seen in platelets from cd36 null mice (Figure 6B). These data suggest that the CD36 signaling pathway is activated in “resting” platelets in the diabetic mice. Immunohistochemical staining for p-JNK2 in dissected carotid artery thrombi demonstrated that thrombi from diabetic
CD36 null mice had 20-50% lower staining intensity scores than those from *wt* animals (Figure 6C).

**Systemic infusion of AGE accelerates thrombosis in a CD36 dependent manner.**

To demonstrate a direct effect of AGE on thrombus formation *in vivo* and to eliminate potential confounding effects from metabolic disturbances in the diabetic models unrelated to AGE, we injected 50µg of AGE-BSA or native BSA via the jugular vein into *wt* and CD36 null mice 20 minutes prior to subjecting them to FeCl₃ induced carotid injury. Injection of this amount of AGE-BSA into either mouse strain resulted in final plasma AGE concentrations of ~25µg/ml, similar to levels seen in DBD mice (Figure 7A). AGE-BSA injection shortened carotid artery occlusion times in *wt* mice (*p < 0.0001*, vs BSA-*wt*) (Figure 7B). The degree of shortening was similar to that seen in the diabetic mice. Deletion of CD36 significantly protected mice from the pro-thrombotic effect of AGE-BSA injection (*p < 0.0001*, vs BSA-CD36⁻/⁻), although the protection was not complete.
Discussion

Cardiovascular disease is the leading cause of morbidity and mortality in patients with DM. Diabetes accelerates the atherosclerotic process and patients with DM also have a higher risk of thrombotic and recurrent ischemic events than non-DM patients. Several factors contribute to the diabetic pro-thrombotic state, including impaired pro-coagulant/anticoagulant balance, endothelial dysfunction, and platelet hyperreactivity. Mechanisms believed to contribute to the “diabetic platelet” phenotype include hyperglycemia; impaired insulin signaling; and abnormalities associated with disordered metabolism, such as obesity, dyslipidemia, and inflammation. Although the mechanisms by which hyperglycemia causes platelet hyperreactivity are not well understood, studies have implicated decreased platelet membrane fluidity, osmotic effects, protein kinase C activation, and advanced glycation of circulating proteins and LDL. The biochemical process of advanced glycation, which is accelerated in DM as a result of chronic hyperglycemia and increased oxidative stress, has been associated with platelet hyper-reactivity, but mechanisms have not been defined.

AGE exert biological effects mainly by engaging specific receptors, among which RAGE is mostly extensively studied. RAGE is minimally expressed in normal tissue and vasculature but is upregulated on endothelial cells, smooth muscle cells, mononuclear phagocytes, and lymphocytes when AGE accumulate. A key mediator of RAGE signaling is nuclear factor-κB, which translocates to the nucleus where it increases transcription of key pro-inflammatory and pro-thrombotic genes. Platelets, however, do not have nuclei and thus the role of this signaling pathway is not clear. Our data show that although platelets express RAGE, treatment of platelets with a RAGE
blocking antibody did not impact specific binding of AGE-BSA to the platelet surface, suggesting it does not play a significant role in AGE-mediated platelet responses. The family of AGE receptors known as AGE-R1, -R2, and -R3 are also not likely to be involved since they function mainly as clearance receptors\textsuperscript{38,39} and are not known to induce signal transduction.

We therefore focused our studies on CD36, which in a limited number of studies has been suggested to function as a potential AGE receptor.\textsuperscript{40,41} Using direct AGE binding assays, platelet function assays, and \textit{in vivo} models of arterial thrombosis with \textit{wt} and \textit{cd36} deficient platelets, we found that AGE bound to recombinant CD36-MBP fusion protein in vitro and that platelet CD36 serves as a specific pro-thrombotic receptor for AGE. AGE-BSA bound to wild type but not \textit{cd36} null platelets in a concentration-dependent manner, and binding was partially inhibited by NO\textsubscript{2}\textsuperscript{+}LDL, a specific CD36 ligand. In addition AGE-BSA dose-dependently increased low-dose ADP induced platelet aggregation. These data are consistent with an earlier report showing that platelet aggregation in response to the weak agonist serotonin was also increased in response to AGE.\textsuperscript{16} Importantly, our data show that the enhancing effect of AGE on platelet activation was CD36-dependent. The lack of complete inhibition of the AGE effect by NO\textsubscript{2}\textsuperscript{+}LDL and the slightly greater (p=0.14) aggregation response of \textit{cd36} null platelets compared to \textit{wt} when treated with AGE-BSA make it impossible to rule out a minor role for additional AGE receptors on platelets. It is possible that our AGE-BSA binding assay was not sensitive enough to detect low level binding to the other platelet AGE receptors on \textit{cd36} null platelets.

The potential clinical relevance of our findings is supported by \textit{in vivo} evidence
using well-established mouse models of arterial injury and thrombosis.\textsuperscript{10,11,20} Using diabetes models that produced levels of circulating endogenous AGE similar to those reported in human diabetic patients as well as a model of direct infusion of AGE-BSA we found that CD36 deficiency “rescued” the pro-thrombotic phenotype associated with these interventions without impacting the circulating AGE levels. Despite similar circulating AGE levels, significantly less AGE accumulated in carotid thrombi from \textit{cd36} null mice compared to those in wild type animals, as assessed by semi-quantitative immunohistochemistry. The pro-thrombotic phenotype in these models was dramatic, with shortening of thrombosis times by \textasciitilde40\%. The rescue by \textit{cd36} deletion was nearly complete in the diabetes models, but only partial in the direct AGE infusion model. The latter may be related to acute effects of AGE, such as AGE-induced oxidative stress and decreased nitric oxide formation, mediated by RAGE signaling on vascular cells other than platelets.\textsuperscript{18,31,42}

Hyperlipidemia often accompanies hyperglycemia in patients with DM. We and others have shown that diet induced hyperlipidemia in \textit{apoe} null mouse models induces a pro-thrombotic state associated with generation of oxLDL and CD36-mediated platelet hyper-reactivity.\textsuperscript{8,9,10} To separate the potential confounding effect of hyperlipidemia in the current studies we used western diet fed C57Bl/6 mice as an additional control. This diet induced a similar level of hyperlipidemia as the DBD diet, but unlike in the \textit{apoe} null background, the lipid levels and presumably degree of oxidant stress were not enough to induce a pro-thrombotic state. These data suggest that hyperglycemia was the driving force behind the pro-thrombotic state in the DM models, a conclusion supported by the AGE-BSA infusion study.
Platelets from patients with DM are characterized by dysfunction of several signaling pathways, at the level of both receptor and downstream intracellular signaling partners. Recent work by our lab has shown that the platelet CD36 signaling pathway is mediated by the MAP kinase JNK, as well as by the Src kinases Fyn and Lyn, and the guanine nucleotide exchange factor Vav. In the current study we showed that exposure of platelets to AGE-BSA \textit{ex vivo} dramatically induced JNK phosphorylation in \textit{wt} but not \textit{cd36} null platelets and we found that circulating “resting” platelets from mice with both DBD and STZ induced DM had increased basal levels of phosphorylated JNK. This was not seen in platelets from \textit{cd36} null mice, suggesting that the CD36 signaling pathway was chronically activated by hyperglycemia. Additionally, immunohistochemical analysis confirmed increased amount of p-JNK within thrombi from diabetic wild type mice compared to \textit{cd36} null.

In summary, these studies define a novel mechanism by which diabetes and hyperglycemia trigger a CD36-mediated platelet signaling pathway leading to hyperresponsiveness to low concentrations of agonists. This could create a state in which circulating platelets are “primed” to respond to injury and thus promote enhanced thrombus formation. Targeting this pathway could lead to development of novel therapeutic strategies to prevent thrombotic complications in patients with high risk conditions, such as diabetes.
Acknowledgements

We thank Mette Johansen for providing the CD36-MBP fusion protein. This work was supported by NIH P50HL81011 (R.L.S) and NIH 5T32HL007914 (W.Z.)

Authorship

Contribution: W.Z. designed, performed, analyzed experiments and wrote the paper; W.L. performed the immunoblots shown in Figure 2 and provided input to experimental design and writing the paper; R.L.S. contributed to overall project design and wrote the paper.

Disclosure of Conflicts of Interest

None.
References


Figure legends

Figure 1. AGE bind to platelets via CD36.  A. Isolated wt or cd36 null platelets were washed and incubated at 22°C with different concentration of biotin-AGE-BSA or biotin-BSA as controls for 2 hrs, followed by avidin-Alexa Fluor 488 conjugate treatment for 1hr. Platelets were then analyzed by flow cytometry to detect bound fluorescence. The histogram shown is representative of n=3.  B. Increasing concentrations of AGE-BSA were added to wt or cd36 null platelets in the presence of either NO₂⁻LDL or NO₂⁻LDL (100μg/ml). Bound fluorescence was detected as in panel A.  C. Wells in a 96-well ELISA plate were coated with recombinant CD36-MBP fusion protein or MBP (25μg/ml in PBS) at 4°C overnight. Increasing concentrations of AGE-BSA were then added for 2hr at 22°C and bound material detected with anti-AGE using a colorimetric ELISA assay. Data are presented as mean fold change of control (± SEM); n=3.

Figure 2. RAGE and other potential AGE receptors are expressed on mouse platelets. A. Platelet lysates from wt and cd36 null mice were subjected to immuoblot assays with indicated antibodies. Membranes were stripped and re-blotted with anti-actin as a loading control. Band densities were measured and plotted as ratios to actin (n=3).  B. Isolated wt platelets were incubated with 10μg/ml RAGE blocking antibody or control IgG 45 min before treated with biotin-labeled AGE-BSA as described in Figure 1A. The histogram shown is representative of 4 and the bar graph shows mean fluorescence intensities (± SEM).
Figure 3. AGE-BSA enhances platelet aggregation in CD36-dependent manner.  

A. Platelets were incubated with increasing concentrations of AGE-BSA for 30 min and then assessed for aggregation in response to low dose ADP (1 μM). On the left are representative tracings from n=6, and on the right a bar graph showing mean amplitudes of aggregation expressed as % of light transmission using PPP control (± SEM).  

B. Platelets from wt or cd36 null mice were incubated with 100 μg/ml AGE-BSA or BSA and then analyzed as in panel A (n=4), and on the right a bar graph showing mean amplitudes of aggregation (± SEM).  

C. PRP obtained from healthy donors was pre-incubated with 50 μM of AGE-BSA or BSA control for 30 min and then stimulated with 5 μM ADP. Left panel is representative tracings; right panel is a bar graph showing mean amplitudes of aggregation (± SEM) of 3 independent experiments.

Figure 4. Diabetes accelerates arterial thrombus formation in mice in vivo in a CD36-dependent manner.  

A. Age matched wt or cd36 null mice were maintained on chow, diabetogenic (DBD) or western (WD) diets for 8wk and then subjected to FeCl₃ induced carotid artery injury to induce thrombus formation. STZ indicates chow-fed mice treated with streptozotocin to induce pancreatic islet destruction and type 1 diabetes. Platelets were labeled in vivo by injection of rhodamine 6G and thrombi were imaged by fluorescence videomicroscopy. Repersentative images obtained at timed points are shown.  

B. Time to occlusive thrombus formation was assessed using 6-8 mice per group.
Figure 5. AGE levels are increased in plasma and within thrombi in diabetic mice.  

A. Plasma concentrations of AGEs in diet and STZ-treated wt or cd36 null mice were measured by ELASA. Data are expressed as mean ± SEM (n=6-8 per group).  

B. Carotid thrombi from wt or cd36 null mice induced as in figure 3 were frozen, sectioned and analyzed for presence of AGE by immunohistochemistry using anti-AGE IgG and a DAB + substrate chromogen detection system. Brown color indicates immunoreactivity. Representative images are shown and the red numbers indicate the intensity score of that image.  

C. Mean AGE staining scores (± SEM) of 5 sections from each thrombus (n = 5 per group).

Figure 6. JNK2 phosphorylation levels are increased in platelets and thrombi in diabetic mice.  

A. Washed platelets from wt or cd36 null mice were treated with AGE-BSA or BSA control (50 μg/ml) for 15 min. Lysates were then assessed by immunoblot using anti-phospho-JNK2 antibody. An antibody to total JNK2 was used as a loading control. Blot is representative of n=4.  

B. Platelets from chow, DBD and STZ treated wt or cd36 null mice were analyzed by immunoblot as in A. A representative immunoblot from n=4 is shown. The bar graph shows the ratio of phospho-JNK2 (p-JNK2) to total JNK2.  

C. Representative images of thrombosed carotid arteries from chow, DBD and
STZ treated wt or cd36 null mice stained with anti-p-JNK. Bar graph shows staining intensity scores for p-JNK (n=5 mice per group).

**Figure 7. Deletion of cd36 rescues accelerated thrombus formation induced by AGE injection in vivo.**  
**A.** AGE-BSA or BSA was injected directly into wt or cd36 null mice through the jugular vein and allowed to circulate for 20 min. Plasma levels of AGEs were then assessed by ELISA. Graph shows mean ± SEM.  
**B.** Mice treated as in Panel A were subjected to FeCl₃ induced thrombosis as in Figure 3. Representative images of intra-carotid thrombi at indicated time points are above and the bar graph shows the mean times to occlusive thrombosis (n>6 for all groups).
Figure 1

A

WT

AGE-BSA

Number of Events

Fluorescence

BSA

CD36/−

AGE-BSA

Number of Events

Fluorescence

BSA

Red: platelet only
Cyan: 5 μg/ml
Brown: 20 μg/ml
Light green: 40 μg/ml
Dark green: 50 μg/ml

indicated AGE-BSA and BSA

B

Mean Fluorescence Intensity

AGE (μg/ml)

0 25 50 75 100

C

Fold change of control

MBP

CD36-MBP

AGE (μg/ml) 0 0.5 1 2 3 4 5 10
Figure 2

A

RAGE
OSR48
(SAGE-R1)
SR-BI
SR-A
Actin

B

Anti-RAGE antibody
Control Ig G

AGE-BSA (μg/ml)

Red: 0
Cyan: 5
Brown: 20
Light green: 40

Number of Events

Fluorescence

Mean Fluorescence

(RBG515-A)

AGE-BSA (μg/ml)

0 5 20 40
Figure 3

A

B

C
Figure 4

A

<table>
<thead>
<tr>
<th>Chow</th>
<th>DBD</th>
<th>STZ</th>
<th>WD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>2 min</td>
<td>6 min</td>
<td>Before</td>
</tr>
</tbody>
</table>

B

Time to thrombosis (sec)

- p=0.0002
- p<0.0001
Figure 5

A

AGEs (µg/ml)

- wt
- cd36⁻/⁻

Chow  DBD  STZ  WD

B

Ig G

Chow  DBD  STZ

C

AGE staining scores

- wt
- cd36⁻/⁻

Chow  DBD  STZ

p = 0.006  p = 0.003  p = 0.4
Figure 6

A

B

C

Chow    DBD    STZ

Ig G

0

2

1

2

5

4

wt

cd36⁻⁻

p-JNK2, JNK2

Chow    DBD    STZ

p-JNK2, JNK2 ratio

p=0.1    p=0.005    p=0.001

p-JNK2 staining score

p=0.001    p=0.03    p=0.6
Figure 7

A

<table>
<thead>
<tr>
<th>AGE-BSA</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>cd36/−</td>
</tr>
<tr>
<td>AGES (μg/ml)</td>
<td>AGES (μg/ml)</td>
</tr>
<tr>
<td>30 ± 5</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>10 ± 2</td>
<td>5 ± 1</td>
</tr>
</tbody>
</table>

B

**AGE-BSA**

<table>
<thead>
<tr>
<th>Time</th>
<th>Before</th>
<th>1 min</th>
<th>2 min</th>
<th>4 min</th>
<th>6 min</th>
<th>occlusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>wt</em></td>
<td><img src="image1" alt="image" /></td>
<td><img src="image2" alt="image" /></td>
<td><img src="image3" alt="image" /></td>
<td><img src="image4" alt="image" /></td>
<td><img src="image5" alt="image" /></td>
<td><img src="image6" alt="image" /></td>
</tr>
<tr>
<td><em>cd36/−</em></td>
<td><img src="image7" alt="image" /></td>
<td><img src="image8" alt="image" /></td>
<td><img src="image9" alt="image" /></td>
<td><img src="image10" alt="image" /></td>
<td><img src="image11" alt="image" /></td>
<td><img src="image12" alt="image" /></td>
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</tbody>
</table>

**BSA**

<table>
<thead>
<tr>
<th>Time</th>
<th>Before</th>
<th>1 min</th>
<th>2 min</th>
<th>4 min</th>
<th>6 min</th>
<th>occlusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>wt</em></td>
<td><img src="image13" alt="image" /></td>
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<td><em>cd36/−</em></td>
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<td><img src="image20" alt="image" /></td>
<td><img src="image21" alt="image" /></td>
<td><img src="image22" alt="image" /></td>
<td><img src="image23" alt="image" /></td>
<td><img src="image24" alt="image" /></td>
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</table>

**Time to thrombosis (sec)**

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>cd36/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE-BSA</td>
<td><img src="image25" alt="image" /></td>
<td><img src="image26" alt="image" /></td>
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<tr>
<td>BSA</td>
<td><img src="image27" alt="image" /></td>
<td><img src="image28" alt="image" /></td>
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</tbody>
</table>

*p<0.01* *p<0.05* *p>0.05*
Advanced glycation end products induce a prothrombotic phenotype in mice via interaction with platelet CD36

Weifei Zhu, Wei Li and Roy L. Silverstein