Aldose Reductase–Mediated Phosphorylation of p53 Leads to Mitochondrial Dysfunction and Damage in Diabetic Platelets

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Background—Platelet abnormalities are well-recognized complications of diabetes mellitus. Mitochondria play a central role in platelet metabolism and activation. Mitochondrial dysfunction is evident in diabetes mellitus. The molecular pathway for hyperglycemia-induced mitochondrial dysfunction in platelets in diabetes mellitus is unknown.

Methods and Results—Using both human and humanized mouse models, we report that hyperglycemia-induced aldose reductase activation and subsequent reactive oxygen species production lead to increased p53 phosphorylation (Ser15), which promotes mitochondrial dysfunction, damage, and rupture by sequestration of the antiapoptotic protein Bcl-xL. In a glucose dose–dependent manner, severe mitochondrial damage leads to loss of mitochondrial membrane potential and platelet apoptosis (cytochrome c release, caspase 3 activation, and phosphatidylserine exposure). Although platelet hyperactivation, mitochondrial dysfunction, aldose reductase activation, reactive oxygen species production, and p53 phosphorylation are all induced by hyperglycemia, we demonstrate that platelet apoptosis and hyperactivation are 2 distinct states that depend on the severity of the hyperglycemia and mitochondrial damage. Combined, both lead to increased thrombus formation in a mouse blood stasis model.

Conclusions—Aldose reductase contributes to diabetes-mediated mitochondrial dysfunction and damage through the activation of p53. The degree of mitochondrial dysfunction and damage determines whether hyperactivity (mild damage) or apoptosis (severe damage) will ensue. These signaling components provide novel therapeutic targets for thrombotic complications in diabetes mellitus. (Circulation. 2014;129:1598-1609.)

Key Words: aldose reductase ▪ apoptosis ▪ diabetes mellitus ▪ mitochondria ▪ platelets

Diabetes mellitus (DM) is a complex disease characterized by absolute insulin deficiency or resistance to insulin, leading to hyperglycemia.1 The altered glucose metabolism can lead to many organ complications, including nephropathy, neuropathy, and retinopathy.2 Platelet abnormalities are one of the hallmarks of DM, contributing to the pathogenesis of many disease processes, including atherosclerosis and thrombosis.3 Several mechanisms have been proposed to explain the platelet abnormalities in DM, among which metabolic alterations and oxidative stress appear to play pivotal roles.4

Clinical Perspective on p 1609

Mitochondrial alterations contribute to the pathogenesis of DM.5 Given the central role of mitochondria in platelet metabolism and activation, mitochondrial alteration may contribute to platelet abnormalities in DM. Indeed, previous studies using isolated platelets from patients with type 2 DM have demonstrated low mitochondrial membrane potential compared with control subjects6 and upregulation of antioxidant enzymes (SOD2 and PRDX3), which indicates increased oxidative stress.7 Therefore, it is plausible that hyperglycemia-induced oxidative stress impairs mitochondrial function in DM platelets. Oxidative stress plays an important role in the development of microvascular and cardiovascular diabetic complications.8 In platelets, hyperglycemia-induced oxidative stress has been shown to contribute to platelet hyperreactivity.9,10 However, the role of hyperglycemia-induced oxidative stress in platelet mitochondrial function is unknown.

Platelets have also been demonstrated to possess critical signaling proteins (Bcl family proteins) known to regulate mitochondria-dependent apoptosis.11 Pharmacological inhibition of Bcl-xL with the BH3 mimetic compounds ABT-737 or ABT-263 triggers platelet apoptosis in vitro and acute thrombocytopenia in vivo,12,14 which suggests that Bcl-xL plays a crucial role in regulating the mitochondria-dependent apoptosis in platelets. ABT-737 treatment causes platelet mitochondrial damage with concomitant activation of caspase 3, which consequently leads to externalization of phosphatidylserine.15

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Despite these elegant studies demonstrating the presence of many stress and apoptosis signaling components, the pathway connecting these signaling components to hyperglycemia and mitochondrial damage, as well as their consequences in DM platelets, is not known.

We hypothesized that aldose reductase (AR) plays a key role in the pathway leading from hyperglycemia to platelet mitochondrial damage. AR, the first key enzyme in the polyol pathway, is well known to contribute to oxidative stress in DM.\(^6\) AR contributes to platelet activation and to hyperactivation in platelets under hyperglycemia.\(^9,17\) In the present study, we aimed to investigate the role of AR in diabetes-mediated mitochondrial dysfunction, as well as the signaling pathways involved in this process. We demonstrate that AR contributes to diabetes-mediated mitochondrial dysfunction and damage through the activation of p53. We report that the degree of mitochondrial dysfunction and damage determines whether hyperactivity (mild damage) or apoptosis (severe damage) will ensue.

### Methods

A more detailed description of the Methods can be found in the online-only Data Supplement.

### Patient Recruitment and Preparation of Human Platelet Samples

Venous blood was drawn from consenting volunteers (healthy and DM subjects) at Yale University School of Medicine (Human Investigation Committee No. 1005006865). Sixty-six subjects with type 2 DM (American Diabetes Association definition) and 8 healthy control subjects (HS) were recruited for the studies (online-only Data Supplement Table I). The platelet-rich plasma and washed platelets were collected by centrifugation.

### Development of Mouse Model for DM With Human AR Expression

Mice were injected with streptozotocin (50 mg/kg) intraperitoneally for 5 consecutive days to induce DM. Four weeks after streptozotocin administration, mice were maintained on a high-cholesterol diet for 12 weeks. Platelets were collected by centrifugation.

### Ligation of Mouse Carotid Artery

The carotid artery ligation model was performed as described previously.\(^18\) Briefly, the left common carotid artery was ligated near the carotid bifurcation. After 7 days, mice were euthanized. The left and right arteries were fixed in 4% paraformaldehyde, embedded in OCT compound, and sectioned for analysis.

### Confocal Microscopy

The washed platelets were settled on glass-bottomed dishes for 30 minutes, fixed with 4% paraformaldehyde, and stained with specific antibodies. The stained platelets were observed with a Nikon Eclipse-Ti (Nikon Instruments, Melville, NY) confocal microscope with a 100× oil immersion lens.

### Determination of Mitochondrial Membrane Potential, Phosphatidylserine Externalization, and Mitochondrial Superoxide Production

To assess the changes in mitochondrial membrane potential (\(\Delta \Psi_m\)) and phosphatidylserine externalization by use of flow cytometry, platelets were stained with 40 nmol/L tetramethylrhodamine methyl ester (TMRM), followed by staining with 4 \(\mu\)g/mL annexin V. To measure mitochondrial superoxide production, platelets were stained with 5 \(\mu\)mol/L MitoSOX Red (Life Technologies, Carlsbad, CA).

### Western Blotting, Immunoprecipitation, and Subcellular Fractionation

Protein samples were subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose membrane and probed with the indicated antibodies. For immunoprecipitation, platelet lysates were incubated with agaro-conjugated p53 antibody, and the immunoprecipitated proteins were eluted in SDS loading buffer for Western blot analysis. Subcellular fractionation was performed with a mitochondria isolation kit (Pierce Biotechnology, Rockford, IL).

### Statistical Analysis

All data were expressed as means±SD. The parametric t test was performed for comparisons of 2 groups. The parametric 1-way ANOVA, followed by Tukey multiple comparison test, was used to assess difference between >2 groups within the same study. Analysis was performed with Prism software (GraphPad Software, Inc, La Jolla, CA). A difference of \(P<0.05\) was considered significant.

### Results

#### Severe Mitochondrial Damage Occurs in DM Platelets

To initially assess mitochondrial function in DM platelets compared with platelets from control subjects, we measured the \(\Delta \Psi_m\) by TMRM staining. TMRM is readily sequestered by the mitochondria, but its fluorescence is rapidly lost when \(\Delta \Psi_m\) is dissipated. Compared with healthy platelets, DM platelets exhibited reduced \(\Delta \Psi_m\) (Figure 1A). According to fluorescence-activated cell sorting analysis, only 10% of platelets were TMRM negative in HS. In contrast, ≈80% of DM platelets and platelets treated with ABT-737 (Bcl-2 inhibitor) were TMRM negative (Figure 1B). As positive controls, healthy platelets treated with anti-annexin A and rotenone (inhibitors for mitochondrial complex I and III, respectively) also demonstrated a greater TMRM-negative population (Figure 1B). In addition to \(\Delta \Psi_m\) dissipation, mitochondrial superoxide production was increased significantly with rotenone and anti-annexin A (positive controls; Figure 1C). Further supporting the severe mitochondrial damage, the mitochondrial permeability transition pore (MPTP) was also increased, as demonstrated by the loss of retained calcein fluorescence in the mitochondria with a cobalt chloride–quenching assay (Figure I in the online-only Data Supplement). Ionomycin was used as a control to trigger complete MPTP permeability. Using electron microscopy, we also observed direct evidence of mitochondrial damage. The mitochondria from DM platelets exhibited varying degrees of damage and rupture of the outer mitochondrial membrane compared with HS platelets, which predominantly showed the classic double-membrane mitochondria without outer mitochondrial membrane damage (Figure 1D). The complete electron microscopy field is provided in Figure IIA in the online-only Data Supplement, and a lower-power field showing distinct morphological differences in HS versus DM platelets is shown in Figure IIB in the online-only Data Supplement. Collectively, these results demonstrate that platelet mitochondrial dysfunction and damage are characteristics of DM platelets.
DM Platelets Exhibit Increased p53 Phosphorylation

To identify the key components that lead to mitochondrial dysfunction in DM platelets, we used a proteome profiler array (R&D Systems, Minneapolis, MN) to compare platelets from HS (n=8 pooled) and patients with type 2 DM (n=8 pooled). The most consistent observation was reduced expression of Bcl-xL by 2.5-fold in DM and, interestingly, increased phosphorylation of p53 at serine 15 by 2-fold (Figure IIIA and IIIB in the online-only Data Supplement). No change was found in the expression of the other Bcl-2 family proteins (Figure IIIC in the online-only Data Supplement).

In both the cytosolic and mitochondrial fractions of platelets, the expression of p53 and its phosphorylation at serine 15 were markedly upregulated in DM compared with HS, but no detectable changes in Bax and Bak were observed (Figure 2A). Using high-power confocal microscopy, we demonstrate that in DM platelets, the level of p53 phosphorylated at serine 15 was increased, and much of it localized to the mitochondria, whereas Bcl-xL was diminished (Figure 2B). The complete field where these platelets were selected is shown in Figure IVA in the online-only Data Supplement. In addition to phosphorylation, we found that glutathionylation and acetylation of p53 were increased in DM platelets (Figure IVB in the online-only Data Supplement).

Previous studies have suggested that p53 can induce mitochondrial opening by interacting with Bcl-xL. Bcl-xL coimmunoprecipitated with p53 increased markedly in DM platelets by 3-fold compared with HS (Figure 2B), which can be attributed to phosphorylated p53. Detection was limited in healthy platelets because of low levels of phosphorylated p53 (Figure 2C). Together with the colocalization studies from confocal microscopy, our results showed that Bcl-xL was sequestered by the increased phosphorylated p53.
p53. In platelets, Bcl-x<sub>L</sub> exerts its prosurvival reaction by binding to the proapoptotic mediators Bax and Bak at the mitochondria.<sup>20</sup> Therefore, because Bcl-x<sub>L</sub> is sequestered by phosphorylated p53, Bax and Bak would mediate the outer membrane permeabilization, leading to the release of cytochrome c from the intermembrane space.<sup>21</sup> The loss
of cytochrome c in the intermembrane space can no longer inhibit reactive oxygen species formation in the electron transport, which leads to increased oxidative stress and further mitochondrial permeability. In addition to the interaction between p53 and Bcl-xL, recent data have demonstrated that Bcl-xL alone can stabilize the ΔΨm by interacting with ATP synthase. ATP synthase activity was not changed in the diabetic platelets (Figure IA in the online-only Data Supplement). p53 has been associated with regulation of mitochondrial respiration. We found that mitochondrial ATP levels were reduced in diabetic platelets, which suggests that ATP generation from oxidative phosphorylation was impaired in DM platelets. Interestingly, cytosolic ATP was increased in diabetic platelets, which suggests that increased glycolytic flux may ensue in DM platelets (Figure IB in the online-only Data Supplement). These findings are supported by a previous study that showed that platelets from patients with type 2 DM are characterized by high levels of total ATP but low ΔΨm.

AR Contributes to Hyperglycemia-Induced p53 Phosphorylation in Human Platelets

To understand whether acute hyperglycemia contributes to p53 phosphorylation, human platelets isolated from HS were incubated with 5 mmol/L glucose or 25 mmol/L glucose (HG) for 2 hours. Platelets incubated with HG showed increased p53 phosphorylation localized to mitochondria (Figure 3A); however, in contrast to DM platelets, no reduction in Bcl-xL expression was observed in normal platelets treated with HG (Figure 3A). The increased phosphorylation of p53 at serine 15 was further confirmed by Western blot (Figure 3B). In contrast to DM platelets, total p53 expression was not changed after incubation of healthy platelets with HG.

It is well recognized that phosphorylation of p53 at serine 15 is mediated by oxidative stress. Therefore, we hypothesized that hyperglycemia-induced oxidative stress (mediated by AR) phosphorylated p53 in platelets, which translocated to the mitochondria with Bcl-xL. Treatment with epalrestat (10 μmol/L), an AR inhibitor (ARI), and the antioxidant

Figure 4. Alteration of mitochondrial function in platelet isolated from transgenic diabetic mouse expressing human aldose reductase. A, Mitochondrial membrane potential (ΔΨm) was measured by staining with tetramethylrhodamine methyl ester (TMRM) 40 nmol/L, and phosphatidylserine externalization was assessed with FITC–annexin V. Murine platelets were treated with ABT-737 10 μmol/L to serve as a positive control for apoptosis. Data are presented as percentage of TMRM or annexin V–positive cells ±SD (n=6; ***P<0.001 compared with corresponding non-DM). B, Mitochondrial superoxide production was labeled with MitoSOX Red 5 μmol/L. MFI indicates mean fluorescence intensity. Data are presented as mean fluorescence ±SD (n=4; ***P<0.001 compared with non-DM). C, Activation of the mitochondrial permeability transition pore was assessed by loss of retained calcein fluorescence. Data are presented as a percentage of retained calcein fluorescence ±SD (n=4; ***P<0.001 compared with non-DM). D, Total expression of p53, Bcl-xL, phosphorylated p53 (serine 15), and active caspase 3 was determined by Western blot. Representative blots are shown (n=4). E, Expression of Bax, Bak, and cytochrome c (Cyto c) was determined by Western blot in cytosolic and mitochondrial fractions. Representative blots are shown (n=4). COX IV indicates cytochrome c oxidase subunit 4.
N-acetyl-cysteine attenuated the HG-induced phosphorylation of p53 (Figure 3A and 3B). Treatment with pifithrin-μ, an inhibitor of p53 that binds to mitochondrial Bcl-xL protein,30 attenuated the interaction with Bcl-xL but had no effect on the phosphorylation of p53 (Figure 3B and 3C). Treatment with Z-DEVD-FMK (a downstream caspase 3 inhibitor) also showed no effect on the phosphorylation of p53. The binding of Bcl-xL with immunoprecipitated p53 also increased in platelets incubated with HG, which is comparable to DM (Figure 3C), which suggests that hyperglycemia enhanced phosphorylation of p53 and protein-protein interaction with Bcl-xL. Treatment with an ARI also attenuated the interaction between Bcl-xL and p53, although the effect was less than with pifithrin-μ (Figure 3C).

We have previously shown in human platelets that high glucose can induce the phosphorylation of p38α mitogen-activated protein kinase (MAPK) through AR activity,9 and it is known that p53 can be phosphorylated by the activation of p38α MAPK.28,31 In both DM and HG-treated platelets, the phosphorylation of p38α MAPK was increased, which was attenuated by ARI and SB239063 (a p38α MAPK inhibitor).

Inhibition of p38α MAPK also reduced the HG-induced p53 phosphorylation, which suggests that AR activity increased the phosphorylation of p38α MAPK, leading to p53 activation in human platelets under the HG condition (Figure 3D). With regard to mitochondrial function, HG platelets exhibited mild mitochondrial alterations, including reduced ∆Ψm and MPTP opening. Moreover, annexin V-positive platelets were also increased modestly under the HG condition. All of these changes were attenuated by treatment with ARI or pifithrin-μ (Figure VA and VB in the online-only Data Supplement).

Taken together, the present results demonstrated that a 2-hour hyperglycemia treatment was sufficient to activate p53 and led to mild mitochondrial dysfunction by increasing its phosphorylation and binding to Bcl-xL, and this was caused, at least in part, by AR activation and reactive oxygen species production in platelets.

Platelet Mitochondrial Dysfunction in Transgenic DM Mice Expressing Human AR

Wild-type mice have very little native AR (analogous to a functional knockout). The lack of AR may be a key reason

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**Figure 5.** Requirement of aldose reductase for hyperglycemia-induced mitochondrial dysfunction in mouse platelet. A, Mitochondrial membrane potential (∆Ψm) was measured by staining with tetramethylrhodamine methyl ester (TMRM) 40 nmol/L, and phosphatidylserine externalization was assessed by labeling with FITC–annexin V. Platelets of mice expressing human aldose reductase (huAR) were incubated with different concentrations of glucose (ie, 5, 15, and 25 mmol/L), and the role of aldose reductase was determined by pretreatment with an aldose reductase inhibitor (ARI), epalrestat 10 μmol/L. For TMRM staining (top right), data are presented as mean percentage of TMRM-positive platelets ±SD (n=4). ***P<0.001, *P<0.05 compared with 5 mmol/L glucose group in the absence of ARI; ##P<0.01 compared with 5 mmol/L glucose group in the presence of ARI. For annexin V staining (bottom right), data are presented as mean percentage of annexin V–positive platelets ±SD (n=4). ***P<0.001 compared with 5 mmol/L glucose group in the absence of ARI; ###P<0.001 compared with 5 mmol/L glucose group in the presence of ARI. B, Activation of the mitochondrial permeability transition pore was assessed by loss of retained calcine fluorescence. Data are presented as mean percentage of retained calcine fluorescence ±SD (n=4). **P<0.01 compared with huAR-ATP; #P<0.05 compared with huAR incubated with 25 mmol/L glucose. C, Phosphorylation of p33 was assessed by Western blot in huAR platelets incubated with normal or 25 mmol/L glucose. The level of phosphorylated p33 was normalized to total p33 expression. Representative blots are shown (n=4). Gluc indicates glucose.
why mice are resistant to the deleterious effect of hyperglycemia. We therefore induced DM in a mouse model in which human AR (huAR) was expressed and had activity comparable to that found in human platelets (Figure VIA in the online-only Data Supplement). Blood glucose levels were significantly elevated in DM mice, and no significant difference was found in body weight (Figure VIB in the online-only Data Supplement).

Consistent with the findings in DM patients, huAR transgenic murine platelets exhibited dissipation of $\Delta\Psi_m$ and phosphatidylserine externalization (apoptosis) under DM conditions. A clear reduction in the TMRM-positive population and significant increases in the annexin V–positive population were also observed in mouse platelets treated with ABT-737 10 $\mu$mol/L (Figure 4A). Likewise, mitochondrial superoxide and MPTP opening were increased significantly in DM murine platelets (Figure 4B and 4C). All of these results suggested that mitochondrial function is severely altered in DM murine platelets where cellular apoptosis occurs.

We further studied whether phosphorylation of p53 at serine 15 was also found in DM murine platelets. Consistent with our findings in human platelets, the expression of p53 and its phosphorylation at serine 15 was increased in DM murine platelets (Figure 4D). Expression of Bcl-x$_L$ was reduced and caspase 3 activation and cytochrome c release were increased in DM murine platelets. Confirming our human data, the subcellular level of Bax and Bak was not changed in DM murine platelets compared with non-DM murine platelets (Figure 4E).

Together, these mouse studies further validate our observations in human platelets, in which mitochondrial function is altered in DM platelets, likely from AR-dependent activation of p53. Moreover, platelet apoptosis may ensue.

AR Contributes to Acute Hyperglycemia-Induced Mitochondrial Dysfunction by Increasing P53 Phosphorylation in Non-DM Murine Platelets

To further understand how AR contributes to hyperglycemia-induced mitochondrial alterations, huAR transgenic murine platelets (non-DM) were subjected to different concentrations of glucose for 2 hours. For incubation with HG, $\Delta\Psi_m$ was significantly dissipated with concomitant phosphatidylserine externalization in huAR transgenic murine platelets.
in a dose-dependent manner, and the effect was markedly attenuated by the treatment with an ARI (Figure 5A). The opening of the MPTP also increased with treatment with glucose 25 mmol/L and was restored by treatment with an ARI (Figure 5B). This further supports a role of AR as a key player in hyperglycemia-induced mitochondrial dysfunction. Consistent with mitochondrial dysfunction, phosphorylation of p53 at serine 15 was increased significantly in murine platelets incubated with glucose 25 mmol/L, which was attenuated by AR inhibition (Figure 5C). Taken together, our results demonstrate that huAR expression in murine platelets exacerbates hyperglycemia-induced mitochondrial dysfunction through p53 activation.

Platelet Activation and Apoptosis Are Distinct States in DM Human Platelets

In DM human platelets, the dissipation of \( \Delta \Psi_m \) is so severe that there is a concomitant increase in phosphatidylserine externalization (Figure 6A). Such severe mitochondrial damage and apoptosis were visible as a paucity of red staining (dissipation of \( \Delta \Psi_m \)) inside the green rings (phosphatidylserine externalization) when the platelets were stained with TMRM. Treatment with ABT-737 10 \( \mu \)mol/L was also found to reduce \( \Delta \Psi_m \) and increase phosphatidylserine externalization (Figure 6A). Also, compared with HS, a larger population of annexin V–positive platelets (apoptotic platelets) was found in both DM platelets and platelets treated with ABT-737 (3% versus 30% and 70%, respectively; Figure 6B). Using fluorescence-activated cell sorting analysis, we also showed that DM platelets exhibited an increase in the apoptotic population compared with HS platelets in a glucose (in vivo) dose-dependent manner (Figure 6C). Consistent with severe mitochondrial dysfunction and phosphatidylserine externalization, active caspase 3 and cytochrome c release were also increased in the DM platelets (Figure 6D and 6E).

Because both activation and apoptosis can ensue in DM platelets, we sought to understand the role of mitochondrial dysfunction in platelet activation. Platelets were divided into high \( \Delta \Psi_m \) (red population) and low \( \Delta \Psi_m \) (blue population) according to TMRM fluorescence with flow cytometry. Platelet activation in response to ADP 1 \( \mu \)mol/L can be demonstrated by an increase in size and a shape change of the cell population to a narrower, elongated shape. Platelet activation was observed in the red population of platelets incubated with either 5 or 15 mmol/L glucose and in DM platelets, which suggests that platelets with high \( \Delta \Psi_m \) contribute to platelet activation. However, ABT-737–treated platelets exhibited primarily a blue population, so platelet activation in the red population was not found (Figure VIIA in the online-only Data Supplement). In addition, increased P-selectin expression as a marker of platelet activation was found in platelets incubated with either 5 or 15 mmol/L glucose but not in ABT-737–treated platelets (Figure VIIIB in the online-only Data Supplement).

Figure 7. Aldose reductase exacerbates thrombus formation after carotid artery ligation in diabetic mice. A, Hematoxylin-and-eosin staining of serial cross sections from a ligated carotid artery from nontransgenic (NTg) mice and transgenic mice expressing human aldose reductase (huAR), with or without diabetes mellitus (DM), after 7 days (bar, 10 \( \mu \)m; n=4). B, Percentage of thrombosis in ligated carotid artery was calculated by dividing the area of thrombus by the area of the vessel lumen (area of thrombus/area of lumen from multiple contiguous sections). Data are presented as mean±SD (n=4 mice). ***P<0.001 and **P<0.01 compared with NTg; ###P<0.001 and #P<0.05 compared with huAR; $P<0.05 compared with NTgDM. C, Platelet deposition in thrombus after carotid artery ligation was assessed by immunohistochemical detection of platelets with FITC-CD41 antibody in NTg and huAR mice. Nucleated cells were visualized by DAPI staining (bar, 10 \( \mu \)m; n=4). D, Aggregation of mouse platelet was measured by light transmission aggregometer in response to collagen 10 \( \mu \)g/mL. Data are presented as mean±SD (n=4). ***P<0.001 and *P<0.05 compared with NTg; ###P<0.001 and #P<0.05 compared with huAR; $P<0.05 compared with NTgDM.
Data Supplement). These findings indicate distinct pools of platelets arising from hyperglycemia. Depending on the severity of the mitochondrial damage caused by hyperglycemia, platelets with a large decrease in $\Delta\Psi_m$ do not respond to agonist stimulation (apoptotic platelets), but those with a mild decrease in $\Delta\Psi_m$ (active platelets) or no decrease in $\Delta\Psi_m$ (normal platelets) were able to respond to ADP stimulation. Thus, mitochondrial dysfunction appears to play a central role in determining platelet activation or apoptosis under conditions of hyperglycemia.

**Diabetes-Induced Platelet Hyperreactivity Leads to Arterial Thrombosis**

It is well established that DM platelets can be hyperactive, as demonstrated by our aggregation studies in human platelets (Figure VIII in the online-only Data Supplement). Previous studies have shown that increased platelet apoptosis, as indicated by increased phosphatidylserine externalization, leads to increased procoagulant activity, which enhances thrombosis in rats. Moreover, apoptosis induces increased platelet turnover, and the ensuing larger, younger platelets are more active, which further contributes to increased thrombosis.

To further study the role of AR in platelet function in vivo, carotid artery ligation was performed in our mouse model, and thrombus formation was assessed by hematoxylin-and-eosin and immunofluorescent staining. After 7-day carotid artery ligation (blood stasis model), thrombus formation was increased under DM conditions, particularly in the presence of huAR (Figure 7A and 7B). Platelet deposition, as determined by platelet marker CD41 staining, was also increased in huAR mice compared with nontransgenic mice (Figure 7C). Under non-DM conditions, thrombus formation was not increased significantly in huAR mice compared with nontransgenic mice. Platelets from DM mice showed increased aggregation in response to collagen 10 $\mu$g/mL, and platelet aggregation was increased in huAR mice compared with nontransgenic mice (Figure 7D). These results support that huAR expression contributes to DM-induced platelet hyperreactivity and apoptosis, which leads to increased arterial thrombosis.

**Discussion**

We demonstrate for the first time that AR-mediated p53 phosphorylation contributes to $\Delta\Psi_m$ dissipation, MPTP activation, and mitochondrial dysfunction, damage, and rupture in DM platelets (Figure 8). This can result in caspase 3 activation and phosphatidylserine externalization in DM human platelets, features typical of apoptosis. These mechanistic studies provide new insights into platelet abnormalities and potential therapy (eg, ARIs) in DM to prevent cardiovascular complications.

We were surprised to discover that p53 played an important role in hyperglycemia-mediated platelet mitochondria dysfunction. In addition to its role in transcription, p53 has been demonstrated to have a direct apoptogenic role at the mitochondria. A table is presented that summarizes the signaling component changes under each condition; normal glucose in healthy subjects, high glucose (healthy subjects), and recurrent high glucose (subjects with diabetes mellitus). DM indicates diabetes mellitus; HS, healthy subjects; Mito, mitochondrial; Mod, moderate; PS, phosphatidylserine; and ROS, reactive oxygen species.

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phosphorylation of p53 at serine 15 was also found to contribute to mitochondrial dysfunction, which was significantly attenuated by treatment with ARI and the antioxidant N-acetylcysteine. Reactive oxygen species production is an early event in p53-induced damage. AR activity can induce phosphorylation of p38 MAPK by increasing oxidative stress under hyperglycemic conditions, which leads to phosphorylation of p53. Consistent with these studies, the present results showed that pharmacological inhibition of p38α MAPK attenuated the phosphorylation of p53 under hyperglycemia, which indicates that AR activity contributes to phosphorylation of p53 through the oxidative stress and activation of p38α MAPK.

Bcl-xL plays a central role in the regulation of platelet survival. Moreover, loss of Bcl-xL results in thrombocytopenia. Consistent with previous studies that showed Bcl-xL can be downregulated in various DM tissues, we also found that expression of Bcl-xL was reduced in DM platelets but not in acute hyperglycemia. The present study and another now demonstrate that p53 phosphorylated at serine 15 can translocate into mitochondria and sequester Bcl-xL, leading to dissipation of ∆Ψm. These results in human platelets showed that AR contributed to p53 phosphorylation and its binding to Bcl-xL through hyperglycemia-induced oxidative stress, which led to mitochondrial damage (Figure 8).

It has been suggested previously that platelet activation and apoptosis are closely related, because ∆Ψm dissipation, caspase 3 activation, and phosphatidylserine externalization have been found with thrombin-stimulated platelets. Previous studies showed that activation of Bax (a proapoptotic protein that physically targets mitochondria) generates a subpopulation of highly activated platelets, which suggests that mitochondria and Bcl-2 family proteins participate in the process of platelet activation and mitochondrial damage and apoptosis. Therefore, it is plausible that mitochondrial alteration and the resultant apoptotic signaling (caspase 3 activation and phosphatidylserine externalization) are triggered in DM platelets, contributing to thrombus formation. Indeed, patients with type 2 DM exhibit platelet hyperreactivity both in vitro and in vivo, coupled with biochemical evidence of persistently increased thromboxane-dependent platelet activation, accelerated turnover of platelets, increased platelet apoptosis, and excessive thrombus formation. Consistent with such studies, we provide evidence that both platelet activation and apoptosis ensue in DM platelets, and these 2 distinct processes are dependent on the activity of AR and the resultant mitochondrial damage. Phosphatidylserine externalization promotes thrombin formation, a marker for procoagulant activity. Moreover, with increased apoptosis, increasing numbers of younger platelets are released, and these larger, younger platelets are more active. Taken together, the present findings suggest that both platelet activation and apoptosis synergistically contribute to increased thrombosis in DM.

Currently, more than 19.7 million adults (8.3%) in the United States have diagnosed DM, and 38.2% of the US adult population has prediabetes. Sixty-five percent of patients with DM will die of thrombotic cardiovascular events in which platelets play key roles. Of great concern is that 10% to 40% of patients with DM are biochemically resistant to aspirin, the drug most commonly used to prevent and treat heart attacks and strokes. New therapies targeting the underlying mechanism for platelet dysfunction are urgently warranted. On the basis of our studies, we now propose that drugs targeting platelet AR/reactive oxygen species/p53 may be effective against hyperglycemia-induced platelet dysfunction.

Study Limitations
The inherent fragile nature of platelets, particularly those from DM patients, required that studies be performed within a few hours of blood acquisition, and thus, many DM patient recruits were required; however, the reproducibility and consistency from the use of 66 heterogeneous patients with DM adds strength to our conclusions. Inherent differences are known to exist between mouse and human platelets, particularly because mouse platelets lack AR. However, conferring p53 induction and mitochondrial sensitivity by overexpressing human AR supports the importance of this hyperglycemia-induced signaling mechanism.

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Disclosures
None.

References


Currently, more than 19.7 million adults (8.3%) in the United States have diagnosed diabetes mellitus, and 38.2% of the US adult population has prediabetes. Most diabetic patients will die of thrombotic cardiovascular events. Of great concern is that many diabetic patients are biochemically resistant to aspirin, the drug most commonly used to prevent and treat such thrombotic events. There is a need to provide new, effective antiplatelet drug targets. We report a novel pathway that gives rise to mitochondrial damage and increased thrombosis in diabetes mellitus. Using both human and mouse platelets from diabetic and normal subjects, we report a unique hyperglycemia-mediated platelet aldose reductase, reactive oxygen species, p53-mediated pathway that leads to mitochondrial dysfunction and damage. This is particularly intriguing because platelets do not have a nucleus, which normally mediates many of the well-recognized functions of p53. Moreover, we demonstrate that this mitochondrial damage can lead to 2 distinct pathways, apoptosis and platelet hyperactivity, both of which can promote thrombus formation. A mouse carotid ligation model was used to produce moderate blood stasis, which demonstrated that hyperglycemia-induced aldose reductase activation and mitochondrial dysfunction enhanced arterial thrombosis in vivo. This explains in part the enhanced risk for thrombosis observed in human patients with diabetes. These pathway components may provide novel targets for pathway-specific therapy to combat increased thrombosis in patients with diabetes.


Supplemental Material
Supplemental Figure 1: (A) Activation of mitochondrial permeability transition pores (MPTP) was assessed by loss of retained calcein fluorescence. (mean fluorescence ± SD, n=3; bar=10 µm). ***P<0.001 compared with HS. (B) The activity of ATP synthase in platelets from healthy subjects (HS) and diabetic patients (DM). Data are presented as mean ± SD (for HS, n=3; For DM, n=4). (C) The mitochondrial and total ATP levels in HS and DM platelets. Data are presented as mean ± SD (For HS, n=5; For DM, n=4). **P<0.01 compared with HS.
**Supplemental Figure 2:** (A) Representative EM field demonstrating a number of platelets from a healthy subject (HS) or a diabetes mellitus subject (DM). (B) Representative higher power EM field demonstrating a small number of platelets from a healthy subject (HS) or a diabetes mellitus subject (DM).
Supplemental Figure 3: Expression profiles of apoptosis-related proteins in platelets from DM patients. Washed platelets were isolated from healthy subjects (HS) & type 2 (T2DM) DM patients. Platelets from eight different patients in each group were pooled. (A) The relative expressions of 35 apoptosis-related proteins were measured using a human apoptosis array (R&D Systems). The intensity of the dot blots were analyzed by Image Lab software, and normalized to HS. Data are presented as fold change. (B) The changes of Bcl-2 family proteins and phosphorylated p53 in platelet from HS and DM patients were further assessed by Western blotting. Representative blots are shown (n=4).
Supplemental Figure 4: (A) Lower powered confocal field from where the single platelets in Figure 3B was taken (bar=10 µm). (B) The level of acetylation, glutathionylation and phosphorylation of p53 in DM platelets (n=4).
Supplemental Figure 5: The role of AR in hyperglycemia-induced mitochondrial dysfunction in human platelets. Platelets were isolated from healthy subjects (HS), washed, and incubated with 25 mM glucose (HG) for 2 hours. (A) Platelet $\Delta\Psi_m$ was measured by staining with 1 µM Tetramethylrhodamine methyl ester (TMRM) under NG and HG conditions, and PS externalization was assessed by labeling with Annexin-V. Platelets incubated with HG were either pre-treated with 10 µM epalrestat for 30 min (HG+ARI) or 20
µM Pifithrin-µ (HG+PFT-µ). Representative images are shown (n=8; bar=10 µm). (B) $\Delta \Psi_m$ and PS externalization were also measured by FACS. Quantification of $\Delta \Psi_m$ was presented as mean fluorescence ± SD, and PS externalization was presented as percentage of Annexin-V positive platelets ± SD (n=8). **$P<0.01$ & *$P<0.05$ compared with NG; #$P<0.05$ compared with HG. (C) MPTP opening was assessed by loss of retained calcein fluorescence in mitochondria using FACS. Quantification of data was presented as mean fluorescence ± SD (n=3). **$P<0.01$ compared with NG.
Supplemental Figure 6: Human aldose reductase expressed in diabetic mouse platelets.

(A) The expression and activity of aldose reductase in platelets isolated from non-transgenic (NTg) and huAR-transgenic (huAR) mouse with or without STZ-induced DM. Representative blots are shown (n=4). AR activity was defined as the μmole of NADPH consumption/minute/mg protein. Data were presented as mean ± SD (n=4). ***P<0.001 & **P<0.01 compared with NTg. (B) Fasting blood glucose level (upper panel) and body weight (lower panel) were assessed. The mice were starved for 6 hours before measuring the blood glucose level (mean ± SD, n=8 **P<0.01 compared with NTg; ##P<0.01 compared with huAR).
Supplemental Figure 7: Effect of hyperglycemia-induced mitochondrial dysfunction on mouse platelet reactivity. (A) Platelets of non-DM and DM huAR mice were isolated.

Tang: Mitochondrial damage in DM platelets.
Non-DM platelets were further incubated with either 5 mM or 15 mM glucose for 2 hours. Platelets treated with 10 μM ABT-737 served as a control. Murine platelets were gated by TMRM fluorescence into two populations, TMRM positive (red) and TMRM negative (blue). Flow cytometry of isolated murine platelets showing FSC vs SSC in the absence (top) and presence (bottom) of 1μM ADP. (B) Representative data showing P-selectin expression in the resting and ADP-stimulated murine platelets. Data was presented as mean fluorescence intensity (MFI) of FITC-P-selectin ± SD (n=4). ***P<0.001 compared with the resting platelet in the corresponding groups.
Supplemental Figure 8: Inhibition of AR attenuates platelet hyperaggregability in DM patients. Percentage of light transmission, an index of platelet aggregation, was measured in both HS and DM platelet suspension in response to 1 µg/ml collagen for 10 min. Platelets isolated from DM patients were treated with 10 µM epalrestat (ARI) for 30 min before the experiments. Quantification of data is presented as percentage of light transmission. Data are expressed as mean ± SD (n=9 for both HS and DM). *P<0.05 compared with HS; #P<0.05 compared with DM.
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Medications (%)

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**Supplemental Table 1: Clinical characteristics of healthy control and patients with type 2 diabetes mellitus (T2DM).**
Supplemental Methods:

Human platelet preparation

Platelet rich plasma was prepared by differential centrifugation of 27 ml of blood drawn by venipuncture into 3 ml of 3.8% trisodium citrate (w/v). Platelet-poor plasma (PPP) was obtained by centrifugation at 1400 g (25°C for 10 min). PRP was adjusted with PPP to $2-3 \times 10^8$ platelets/ml. For washed platelets, the platelets were washed twice and resuspended at $2-3 \times 10^8$ platelets/ml.

Development of mouse model for DM with human AR expression

To develop mice that mimic human subjects with high cardiovascular risk, huAR-LDLR$^{-/-}$ mice with human AR (huAR) transgene expression on a C57BL/6J background (B6.Cg-Ldlr$^{tm1Her}$Tg(H2-K-AKR1B1)1Tj/J; stock no. 006877 ) were purchased from Jackson Labs. To study the effects of recurrent episodes of hyperglycemia on platelets rather than single high glucose treatment, we induced DM in mice using streptozotocin (STZ). Eight week old mice were divided into 2 groups; one half were injected with STZ (50 mg/kg) intraperitoneally for 5 days to induce recurrent episodes of acute hyperglycemia (DM), and the other half were used as non-DM controls. Four weeks after STZ administration, DM and non-DM mice were maintained on high cholesterol diet (HCD) for 12 weeks, fasted 6 hours and sacrificed for blood sampling. Glucose was measured from the tail-tip with a glucometer.
The Institutional Animal Care and Use Committee at Yale University approved all animal protocols (IACUC #11413 & #11539).

**Murine platelet preparation**

Blood (0.7-1 mL) was directly aspirated from the right cardiac ventricle into 1.8% sodium citrate (pH 7.4). Citrated blood from several mice of identical genotype was pooled, and diluted with equal volume of HEPES/Tyrode’s buffer. PRP was prepared by centrifugation at 100 g for 10 min and then used for measuring platelet aggregation in response to 10 µg/ml of collagen. Washed platelets were prepared from PRP by centrifugation at 5000 g for 2 mins. The platelet pellets were resuspended in HEPES/Tyrode’s buffer in the presence of 3 µg/mL apyrase.

**Ligation of mouse carotid artery**

The left common carotid artery was partially ligated near the carotid bifurcation. All mice recovered from surgery and showed no symptoms of stroke. Mice were euthanized seven days after carotid artery ligation. Left and right arteries were fixed in 4% paraformaldehyde. Arteries were embedded in OCT compound, and serial sections (5 µm thick) were cut for analysis by hematoxylin-eosin staining and immunofluorescent staining. Five sections spanning most of the vessel segments from each mouse were analyzed for Tang: Mitochondrial damage in DM platelets.
Confocal microscopy

Washed platelets were allowed to settle on glass-bottom dishes for 30 min before fixing for 15 mins with 4% paraformaldehyde in PBS. After fixation, platelets were permeabilized for 10 minutes using 0.5% triton-X 100 in PBS with 3% BSA, and incubated with different antibodies, including mouse anti-OxPhos Complex V (Invitrogen), rabbit anti-Bcl-xL (Cell Signaling), Alexa Fluor 488 conjugated anti-p53 phosphorylated at serine 15 (Cell Signaling), or rabbit anti-cleaved Caspase-3 antibodies at 1:250 dilution in PBS with 10% BSA at 4°C overnight. The platelets were then washed and incubated with Alexa Fluor 546 conjugated anti-mouse or Alexa Fluor 647 conjugated anti-rabbit antibodies (Invitrogen).

Determination of mitochondrial membrane potential ($\Delta \Psi_m$), phosphatidylserine (PS) externalization and mitochondrial superoxide production

To assess the $\Delta \Psi_m$ and PS externalization simultaneously, platelet suspensions ($5 \times 10^6$ platelets/ml) were incubated with 40 nM TMRM at 37°C for 30 mins, followed by staining with 4 µg/ml annexin V at room temperature for 15 mins. To measure mitochondrial superoxide production, platelets were stained with 5 µM MitoSOX™ Red for 10 mins at 37 °C. The fluorescent intensity was detected by flow cytometry (LSRII). Platelets were identified and gated by their characteristic forward and side scatter properties. 10,000
platelets were analyzed from each sample.

**Western blotting, subcellular fractionation and immunoprecipitation**

The protein samples were subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose membrane and probed with the indicated antibodies. Primary antibodies against the following proteins were used according to the manufacturer’s instructions: Bcl-xL, Bcl-2, Bax, Bak, Bid, Bad, phosphorylated Bad, p53, phosphorylated p53 (serine 15), p38α, phosphorylated p38α, cleaved caspase-3 and cytochrome C (Cell Signaling). The blots were reprobed with a β-tubulin antibody (Santa Cruz Biotechnology) for normalization. For subcellular fractionation, the blots were re-probed with antibodies against COX IV (Cell Signaling), a mitochondrial marker, and β-tubulin (Santa Cruz Biotechnology), a cytoplasmic marker for normalization. Quantification was performed using Image Lab software. For immunoprecipitation, platelet lysates were incubated with agarose conjugated p53 antibody (Calbiochem) at 4 °C overnight, and agarose conjugated mouse IgG was used as control. After washing, the immunoprecipitated proteins were eluted in SDS loading buffer for Western analysis. Quantification of Bcl-xL binding to p53 was performed using Image Lab software.