Biochemical and Biophysical Research Communications 449 (2014) 496–501

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

P66Shc mediates increased platelet activation and aggregation in hypercholesterolemia

Santosh Kumar *, Ajit Vikram, Young-Rae Kim, Julia S. Jacobs, Kaikobad Irani *

Cardiovascular Division, Department of Internal Medicine, University of Iowa Carver College of Medicine, IA City, IA 52242, USA

ARTICLE INFO

Article history:
Received 5 May 2014
Available online 15 May 2014

Keywords:
Hypercholesterolemia
p66Shc
Platelets
Reactive oxygen species

ABSTRACT

Background and hypothesis: Hypercholesterolemia leads to a prothrombotic phenotype. Platelet hyperactivity associated with hypercholesterolemia has been attributed, in part, to oxidative stress. P66Shc is a well-known determinant of cellular and organismal oxidative stress. However, its role in platelet biology is not known. We hypothesized that p66Shc mediates platelet hyperactivation and hyperaggregation in hypercholesterolemia.

Methods and results: P66Shc was expressed in both human and mouse platelets, as determined by qRT-PCR and immunoblotting. Mouse platelet p66Shc expression was upregulated by hypercholesterolemia induced by high-fat diet feeding. Compared to wild-type mice, high-fat diet-induced p66Shc expression in platelets was suppressed in transgenic mice expressing a short hairpin RNA targeting p66Shc (p66ShcRNAi). High-fat diet feeding of wild-type mice amplified surface P-selectin expression on platelets stimulated by the thrombin receptor agonist protease-activated receptor-4 (PAR4), and increased aggregation of platelets induced by thrombin. These exaggerated platelet responses induced by high-fat diet feeding were significantly blunted in p66ShcRNAi mice. Finally, thrombin-stimulated platelet reactive oxygen species were suppressed in p66ShcRNAi mice.

Conclusions: Hypercholesterolemia stimulates p66Shc expression in platelets, promoting platelet oxidative stress, hyperreactivity and hyperaggregation via p66Shc.

1. Introduction

A number of pathological states related to dyslipidemia, including atherosclerosis, diabetes, and metabolic syndrome are associated with increased platelet reactivity and thrombogenic potential [1,2]. Hypercholesterolemia in mice leads to increased platelet reactivity and thrombosis [3,4]. Reactive oxygen species (ROS) generated by platelets have been shown to promote platelet hyperreactivity in hypercholesterolemia [5]. ROS also regulate the activation of the platelet $\alpha_{IIb}\beta_{III}$ integrin receptor [6–8]. Moreover, platelets with higher levels of reactive oxygen species in patients with hyperlipidemia and heart failure further amplify the overall oxidative burden [9].

P66Shc is a member of the ShcA family of adaptor proteins that includes p46Shc and p52Shc. Unlike p46Shc and p52Shc, p66Shc regulates the redox state of cells and tissues by promoting oxidative stress [10]. Deletion of p66Shc increases life span in mice, and p66Shc-deficient mice are protected from systemic oxidative stress and atherogenesis induced by high-fat diet [10,11]. Observations that p66Shc expression is increased in white blood cells and monocytes of patients with coronary artery disease also suggest a possible role for it in human atherosclerotic disease [12]. Despite the well-recognized part for p66Shc in promoting atherosclerosis and vascular dysfunction, its role in platelet biology and thrombosis is not well understood. Therefore, we asked if p66Shc contributes to platelet hyperaggregability and hyperreactivity in hypercholesterolemia.

2. Materials and methods

2.1. Animals and diets

All experimental animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Iowa, Iowa city. The p66ShcRNAi transgenic mice were created in a B6SJL background as reported earlier [13]. Age-matched non-transgenic wild-type littermates were used as controls. All mice were maintained on a 12 h light–dark cycle (6PM–6AM) and had free access to food and water unless specified otherwise. P66ShcRNAi mice

* Corresponding authors.
E-mail addresses: santosh-kumar@uiowa.edu (S. Kumar), Kaikobad-irani@uiowa.edu (K. Irani).

http://dx.doi.org/10.1016/j.bbrc.2014.05.029
0006-291X/

and their littermate controls were fed normal diet (ND) and high fat diet (HFD, Harlan, TD.88137) starting at 8 weeks of age for 12 weeks.

2.2. Preparation of washed human platelets

Banked human platelet rich plasma (PRP) was obtained from the central blood bank. PGI_2 (10 nM, Sigma) was added to PRP and centrifuged at 1100*g* for 15 min. Platelet pellet so formed was re-suspended in small volume of erythrocyte lysis buffer (Qiagen). After 5 min incubation at room temperature, twice the volume of HEPES-Tyrode buffer pH-6.5 having EGTA (1 mM, Sigma) with PGI2 (10 nM, Sigma) and Apyrase (1 μg/ml, Sigma) was added and centrifuged at 1100*g* for 15 min to get the platelet pellet. Platelets were suspended in Tyrode buffer pH-7.4.

2.3. Preparation of washed mouse platelets

Blood was collected in Acid Citrate Dextrose buffer and centrifuged at 100*g* for 15 min. Supernatant (platelet rich plasma, PRP) was isolated carefully. To the PRP, PGI_2 (10 nM, Sigma) was added and centrifuged at 1100*g* for 15 min. Platelet pellet so formed was resuspended in small volume of Erythrocyte Lysis buffer (Qiagen). After 5 min incubation at room temperature, twice the volume of HEPES-Tyrode buffer pH-6.5 with EGTA (1 mM, Sigma), PGI2 (10 nM, Sigma), and Apyrase (1 μg/ml, Sigma) was added and centrifuged at 1100*g* for 15 min to get the platelet pellet. Platelets were suspended in Tyrode buffer pH-7.4.

2.4. Immunoblotting for p66Shc

Platelet pellets were lysed by repeated freeze-thaw in protein lysis buffer (Tris 50 mM, EDTA 5 mM, Triton X-100 1%, NaCl-150 mM, glycerol 5%). Lysates were centrifuged at 10,000*g* for 10 min. Supernatant was collected and protein content was estimated using Bradford reagent (Biorad). Equal amount of protein was loaded onto gel (10% acrylamide) and was run at 100 V for stacking and 140 V for resolving using Tris-Glycine buffer. Proteins were transferred to nitrocellulose membrane (Bio-Rad) by wet transfer at 100 V. Membrane was blocked with 5% non-fat milk prepared in Tris buffered Saline having 0.1% Tween 20 (TBST) for 30 min at room temperature followed by overnight incubation at 4 °C with primary antibody against ShcA (1:1000 dilution, BD Bioscience). After washing with 0.1% TBST for 30 min (5, 5, 10 &10 min cycle), membrane was incubated with HRP-linked secondary antibody (1:2500 dilution, Santa Cruz Biotechnology) for 30 min at room temperature. Protein bands were detected by chemiluminescence kit (Super Signal West Femto visualization substrate, Thermo scientific).

2.5. RNA isolation and cDNA preparation

Platelet pellets were lysed in 1 ml TRIzol (Invitrogen) and 200 μl of Chloroform was added. The tube was vortexed vigorously to get a homogenous mixture and was centrifuged to get the aqueous supernatant containing RNA. Ice-cold isopropanol was added to the supernatant and centrifuged at 4 °C to get the RNA pellet. The RNA pellet was washed with 75% ethanol twice and allowed to dry. Milli-Q water was used to dissolve the RNA pellet and quantitated by spectrophotometry (Biorad) and also run on agarose gel to check the quality of RNA. For performing reverse transcription, SuperScript II reverse transcriptase (Invitrogen) was used. Reaction was carried out as per the manufacturer's protocol. Briefly, 500 ng RNA was incubated with 200 ng of random primers & dNTP mix at 65 °C for 5 min and quickly chilled on ice. Dithiothreitol (DTT), RNaseOUT and 5× First-Strand Buffer was added to the tube and incubated at room temperature for 2 min. SuperScript IIRT (1 μl) was added and mixed. After 10 min incubation at room temperature mixture was maintained at 42 °C for 50 min. Reaction was terminated by heating at 70 °C for 15 min.

2.6. Real time PCR for p66Shc

Real time PCR was done for p66Shc using GAPDH as internal control. A reaction mixture was prepared having 40 ng of cDNA, forward and reverse primers, and SYBER Green Mix (Thermo Scientific). Reaction was carried out for 40 cycles. Primer sequences for human p66Shc are as follows: forward, 5′-AAG TAC AAT CCA CTC CGG AAT GA-3′ and reverse, 5′-GG GCC CCA GGG ATG AAG-3′ and for human GAPDH are forward, 5′-ATG GCA TCA AGA AGG TGG TG-3′ and reverse, 5′-CAT ACC AGG AAA ATG AGC TTG-3′. The primer sequences for mouse p66Shc are forward, 5'-GAC GAT AGT CCG ACT ACC CTG TGT-3' and reverse, 5'-CAG CAG GAT TGG CCA GCT T-3' and for mouse GAPDH are forward, 5'-GGC AAA TTC AAC GGC ACA GT-3' and reverse, 5'-CGC TCC TGG AAG ATG GTG AT-3'. The data was analyzed with ABI Prism 7000 SDS software.

2.7. Expression of P-selectin

Expression of platelet surface P-selectin was determined by Flow Cytometry in whole blood. Blood from mouse saphenous vein was collected in EDTA-coated tubes (Microvette, Sarstedt). Three microliters of whole blood was added to 50 μl of HEPES-Tyrode buffer, pH-7.4, and incubated with either pan-platelet marker (CD41-PE, BD Bioscience) or/and P-selectin (CD62p-FITC, BD Bioscience) antibody as well as mouse isotype control IgG (tagged with PE/FITC, BD Bioscience) for 30 min in the dark. Samples were stimulated with PAR-4 (0.5 mM, Sigma) for 15 min and were further diluted by adding 50 μl diluted sample to 450 μl 0.2% formaldehyde saline. FACSCalibur (BD Bioscience) was calibrated with calibrating beads for optimal acquisition and voltage was set for the differential distribution of platelets, RBCs and WBCs on the forward and sidewise scatter distribution windows. Platelets were identified by scatter gating and were further gated for CD41 labeling and a minimum of 10,000 events (platelets) were acquired. Samples were evaluated for P-selectin expression at baseline as well as after PAR-4 treatment. Data was analyzed using FACS Scan software.

2.8. Whole blood aggregation

Whole blood aggregation was measured by an impedance aggregometer (Chrono-log, Model 590). Blood was collected in citrated buffer from mice by cardiac puncture and was diluted in the ratio of 1:4 with saline in a plastic sample cuvette and pre-warmed for 5 min at 37 °C with a stirring speed of 1200 rpm. An electrode was inserted, and electrical impedance was measured. Change in impedance in response to thrombin (1 unit/ml), was measured over time (1 h). Individual aggregation values for each experiment were calculated as a percentage of maximal calibrated impedance.

2.9. ROS level in platelets

Levels of platelet-derived intracellular ROS were measured as described previously with minor modifications [14]. Washed platelets were prepared as described above and pre-incubated with 10 μmol/l of the redox-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes) for 30 min in dark. Platelets were activated with 0.1 U/ml of human thrombin. After 2 min platelets were fixed in 2%-paraformaldehyde and incubated on ice for 15 min. Fluorescence was measured by flow cytometry

(LSR II with UV, BD) acquiring a minimum of 10,000 events per sample and mean fluorescence was calculated using FlowJo software.

2.10. Statistical analysis

All experiments were performed at least three times. Data are expressed as mean ± SEM. Statistical analysis was performed with SigmaStat. Data in which two conditions were compared were tested using the Student's *t*-test. Data in which more than two conditions were compared in a single experiment were tested using ANOVA or repeated measures of ANOVA as appropriate. Correlation between variables was evaluated using the Pearson product method. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. P66Shc is expressed in human and mouse platelets

First, we determined p66Shc expression in human and mouse platelets at both the mRNA and protein level by qRT-PCR and western blotting. P66Shc mRNA and protein were expressed in human platelets (Fig. 1A and C), although the expression level was lower compared to that in human umbilical vein endothelial cells (HUVEC). Lack of significant contamination of platelet preparation with leukocytes was confirmed by lack of amplification of the leukocyte marker CD45 (Fig. 1B). P66Shc was also detectable in mouse platelets by qRT-PCR (Fig. 1D). The amplification curves indicated that p66shc was less expressed in mouse than human platelets (Fig. 1A and D). Nonetheless, these findings show that p66Shc is expressed in circulating platelets, albeit to a lesser degree than in nucleated endothelial cells.

3.2. High fat diet feeding of mice increases platelet p66Shc expression

We next questioned if hypercholesterolemia leads to increase in platelet p66Shc. C57Bl/6 mice were fed a high-fat diet (HFD) consisting of 21% fat (42% kcal from fat) for 12 weeks, resulting in an increase in serum cholesterol from 96.2 ± 2.7 mg/dl to 222.7 ± 21.1 mg/dl. High-fat diet feeding resulted in a 2.5-fold increase in platelet p66Shc (Fig. 1E). We also determined the effect of HFD feeding on platelet p66Shc in mice expressing a p66Shc shRNA transgene (p66ShcRNAi) [13]. Compared to wild-type mice, high-fat diet-induced platelet p66Shc was markedly blunted in p66shcRNAi mice (Fig. 1E). Thus hypercholesterolemia induces platelet p66Shc expression that is inhibited in p66ShcRNAi mice.

3.3. P66Shc mediates hypercholesterolemia-induced hyperactivation of platelets

Platelet activation involves the secretion of granular contents which results in P-selectin (CD62P) expression on the platelet surface. Expression of surface P-selectin mediates adhesion of platelets to one another and to other cells including leucocytes and endothelial cells [15]. We asked if p66Shc mediates surface expres-

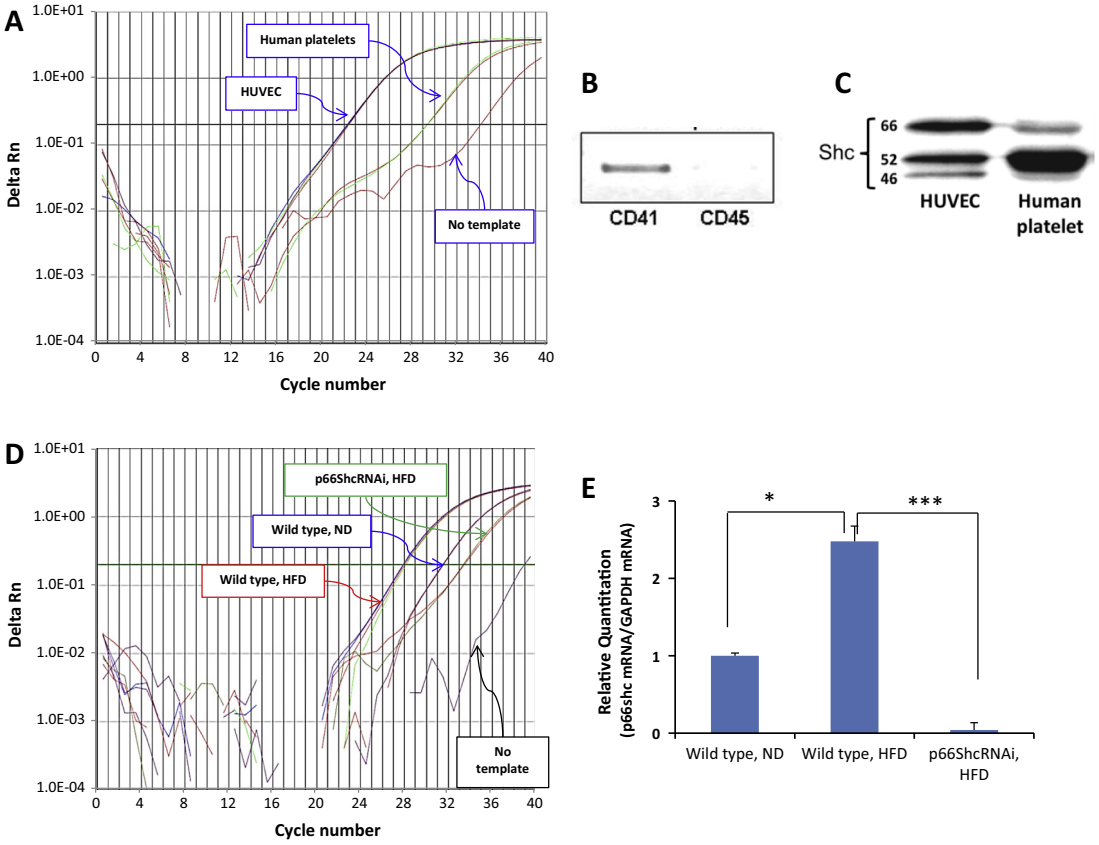

Fig. 1. P66Shc is expressed in platelets and is upregulated with high-fat diet. (A) RT-PCR curves showing amplification of p66shc mRNA in human platelets and HUVEC. (B) PCR amplification from human platelet cDNA of the platelet-specific marker (CD41) and leucocyte-specific marker (CD45). (C) Immunoblot showing protein expression of p66shc in HUVEC and human platelet lysates. (D) Representative RT-PCR curves showing amplification of p66shc mRNA, and change in expression of p66shc mRNA, in wild-type mice and transgenic mice expressing p66shc shRNA (p66shcRNAi) on normal chow diet (ND) or high-fat diet (HFD) for 12 weeks. (E) Quantitative RT-PCR showing expression of p66Shc mRNA normalized to GAPDH mRNA, in platelets of wild-type mice and p66shcRNAi mice on ND or HFD. All the values are shown as mean ± SEM. $^{*}P$ = <0.05, $^{***}P$ = <0.001. n = 3.

sion of P-selectin on platelets. A thrombin receptor activating peptide PAR-4 (protease-activated receptor-4; AYPGKF) agonist was used to activate platelets in whole blood, and platelet surface P-selectin expression was assessed by flow cytometry. PAR-4 stimulated platelet surface P-selectin expression in wild-type mice (Fig. 2A). Importantly, HFD feeding augmented PAR-4-stimulated P-selectin expression (Fig. 2A and B). In comparison to wild-type mice on HFD, PAR-4-stimulated P-selectin expression was significantly reduced in p66ShcRNAi mice on HFD (Fig. 2A and B). Thus, p66Shc mediates hypercholesterolemia-induced hyperactivity of platelets.

3.4. P66Shc mediates hypercholesterolemia-induced platelet hyperaggregation

Hypercholesterolemia enhances platelet aggregation [15,16]. Therefore, we asked if p66Shc mediates thrombin-stimulated hyperaggregation of platelets in hypercholesterolemia. In wild-type mice, HFD feeding resulted in significant increase in maximal and total aggregation of platelets in whole blood (Fig. 3A–C). These measures of platelet aggregation exaggerated by HFD feeding were negated in p66ShcRNAi mice (Fig. 3A–C). Thus, platelet hyperaggregation induced by hypercholesterolemia is dependent, in part, on p66Shc.

3.5. P66Shc mediates hypercholesterolemia-induced platelet oxidative stress

P66Shc regulates reactive oxygen species (ROS) in many cell types and tissues [17]. ROS in platelets promote their activation and aggregation [7]. Therefore, we determined the role of p66Shc in regulating platelet ROS. Using H_2DCF-DA as the ROS sensor, ROS in washed platelets were quantified by FACS. Thrombin increased platelet ROS in both wild-type and p66shcRNAi mice (Fig. 4A and B). In addition, thrombin-stimulated platelet ROS were significantly enhanced with HFD feeding of wild-type mice (Fig. 4A and B). In comparison to wild-type mice, HFD did not augment thrombin-stimulated platelet ROS in p66ShcRNAi mice (Fig. 4A and B). Therefore, p66Shc mediates oxidative stress in activated platelets induced by hypercholesterolemia.

4. Discussion

P66Shc promotes hypercholesterolemia-associated vascular dysfunction and vascular disease. P66Shc-deficient mice are protected from systemic oxidative stress, endothelial dysfunction and atherogenesis induced by high-fat diet [11,18]. However, to date, the role of p66Shc in regulating platelet function has not been investigated. Our work identifies p66Shc as an important player in platelet hyperactivity and hyperaggregation associated with hypercholesterolemic states. It dovetails with recent findings that p66Shc expression is epigenetically upregulated in endothelial cells by low-density lipoprotein cholesterol [19,20]. Although platelets are anucleate, a similar hypercholesterolemia-stimulated up-regulation of p66Shc in precursor megakaryocytes, from which platelets inherit their protein complement, would explain the increase in platelet p66Shc we observed with HFD feeding. However, we cannot exclude the possibility that post-transcriptional mechanisms present in platelets, such as miRNA-mediated mRNA and protein regulation [21], may also contribute to increase in platelet p66Shc.

ShcA proteins are expressed in platelets, and play an important role in platelet signal transduction [22]. Although the contribution of p66Shc to platelet signaling is debatable, there is evidence that it at least participates in some of the molecular mechanisms important for platelet activation. In response to thrombin, p66Shc is tyrosine phosphorylated and binds to the Src homology-2 (SH2) domain of platelet Grb-2, an adaptor protein which further interacts with the kinases PyK2 and FAK, thus participating in thrombin-induced platelet activation [22]. Contrary correlative evidence shows lack of co-precipitation of p66Shc with integrin receptor subunit $\alpha_{IIb}\beta_{III}$ in activated platelets, suggesting that p66Shc does not participate in platelet activation [23]. Although we did not look at its binding partners in platelets, p66Shc may promote platelet hyperactivation via a parallel pathway which may be independent of binding to its conventional partners-via promoting oxidative stress which has been linked to platelet activation [8]. It is also noteworthy that under baseline conditions (mice on a normal chow diet) platelet aggregation was largely preserved in p66ShcRNAi mice, suggesting that under normal physiologic conditions knockdown of p66Shc is not sufficient to inhibit

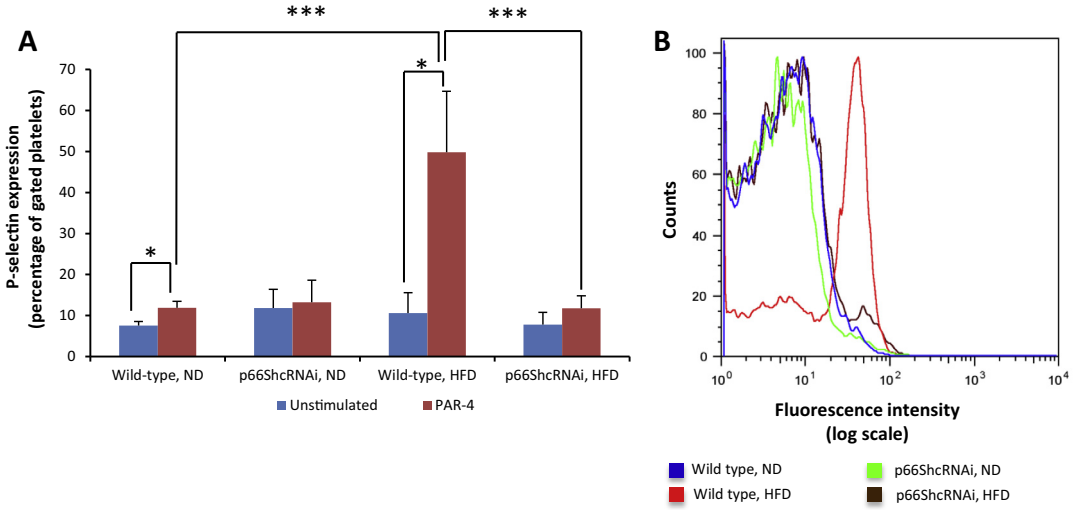

Fig. 2. P66shc mediates hypercholesterolemia-induced platelet P-selectin expression. (A) Percent of gated platelets expressing surface P-selectin in wild-type and p66shcRNAi mice on normal diet (ND) or high-fat diet (HFD). Blood was either unstimulated or stimulated with the thrombin receptor agonist PAR-4. All values are shown as mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$; NS = nonsignificant; $n = 3$–9/group. (B) Representative histograms showing fluorescence intensity of P-selectin expression on PAR-4-stimulated platelets from wild-type mice and p66shcRNAi mice on ND or HFD.

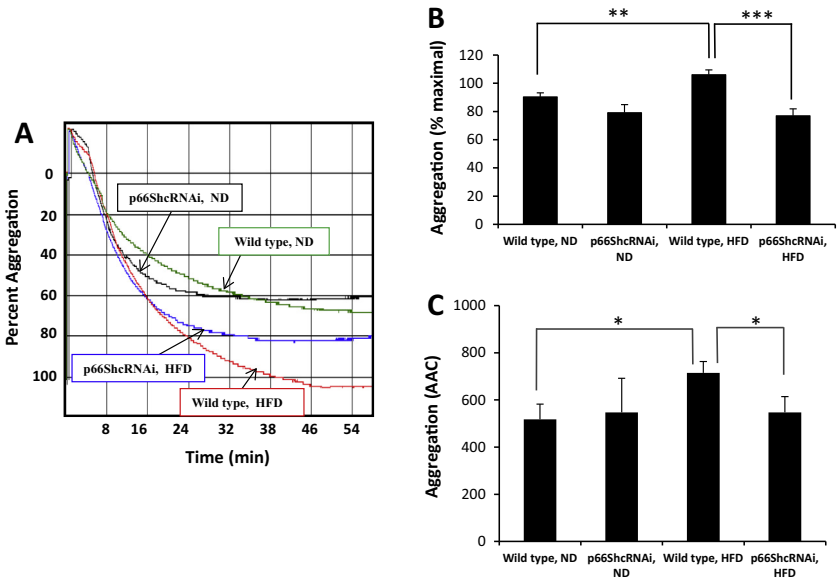

Fig. 3. P66shc mediates hypercholesterolemia-induced platelet hyperaggregation. (A) Representative thrombin-induced impedance aggregation curves of whole blood from wild-type and p66ShcRNAi mice on ND or HFD. (B) Percent of maximal possible thrombin-induced aggregation of whole blood from wild-type and p66shcRNAi mice on ND or HFD. (C) Area above curve (AAC) for thrombin-induced aggregation of whole blood from wild-type and p66shcRNAi mice on ND or HFD. All values are shown as mean ± SEM. (n = 6–24) $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

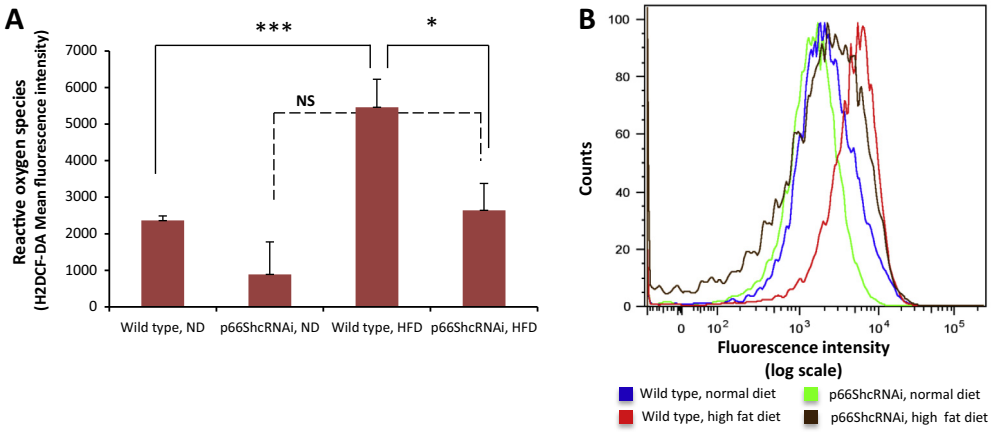

Fig. 4. P66shc regulates thrombin-stimulated platelet ROS. (A) ROS, measured as mean fluorescence intensity of the redox-sensitive probe H_2DCF-DA, in thrombin-stimulated washed platelets from wild-type and p66ShcRNAi mice on ND or HFD. All the values are shown as mean ± SEM. $^{*}P < 0.05$; $^{***}P < 0.001$; NS = nonsignificant; n = 4–9/group. (B) Representative histograms of H_2DCFDA fluorescence intensity in thrombin-stimulated washed platelets.

homeostatic platelet function. However, with hypercholesterolemia (mice on HFD), p66Shc's role in platelet aggregation became evident, suggesting that only in hypercoagulable states does p66Shc take center stage in governing an exaggerated platelet response.

Hyperlipidemia enhances platelet activation and promotes thrombosis [24]. Studies in animals and humans have shown a direct relationship between increased blood cholesterol level and platelet reactivity [1]. Hyperlipidemia is also known to increase platelet ROS [25] which has also been noted in other pro-thrombotic conditions such as ageing, and diabetes [14,26]. Interestingly, p66Shc is upregulated in many of these same prothrombotic conditions raising the possibility that it may play an important part in platelet hyperreactivity in these pathophysiological conditions as well.

In summary, our findings demonstrate that p66shc, much like its role in other cell types, promotes platelet oxidative stress in response to activation of the thrombin receptor. They also show that p66shc mediates hypercholesterolemia-stimulated platelet hyperactivation and hyperaggregation. Thus, p66shc may be a new molecular therapeutic target in hypercoagulable states.

Acknowledgments

This work was supported by a postdoctoral fellowship from the American Heart Association to S.K. (11POST6880001) and by the University of Iowa Endowed Professorship in Cardiovascular Medicine (K.I.). We thank Dr. Zubair Karim and Dr. Sanjana Dayal for help with washed platelet study. The data presented herein were obtained at the Flow Cytometry Facility, which is a Carver College of Medicine/Holden Comprehensive Cancer Center core research facility at the University of Iowa. The Facility is funded through user fees and the generous financial support of the Carver College of Medicine, Holden Comprehensive Cancer Center, and Iowa City Veteran's Administration Medical Center.

References

[1] F. Santilli, N. Vazzana, R. Liani, M.T. Guagnano, G. Davi, Platelet activation in obesity and metabolic syndrome, Obes. Rev. 13 (2012) 27–42.

[2] G. Davi, C. Patrono, Platelet activation and atherothrombosis, N. Engl. J. Med. 357 (2007) 2482–2494.

[3] A.S. Plump, J.D. Smith, T. Hayek, K. Aalto-Setala, A. Walsh, J.G. Verstuyft, E.M. Rubin, J.L. Breslow, Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells, Cell 71 (1992) 343–353.

[4] D.T. Eitzman, R.J. Westrick, Z. Xu, J. Tyson, D. Ginsburg, Hyperlipidemia promotes thrombosis after injury to atherosclerotic vessels in apolipoprotein E-deficient mice, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 1831–1834.

[5] K.Y. Stokes, J.M. Russell, M.H. Jennings, J.S. Alexander, D.N. Granger, Platelet-associated NAD(P)H oxidase contributes to the thrombogenic phenotype induced by hypercholesterolemia, Free Radic. Biol. Med. 43 (2007) 22–30.

[6] K. Irani, Y. Pham, L.D. Coleman, C. Roos, G.E. Cooke, A. Miodovnik, N. Karim, C.C. Wilhide, P.F. Bray, P.J. Goldschmidt-Clermont, Priming of platelet alphaIIbbeta3 by oxidants is associated with tyrosine phosphorylation of beta3, Arterioscler. Thromb. Vasc. Biol. 18 (1998) 1698–1706.

[7] A.J. Begonja, S. Gambaryan, J. Geiger, B. Aktas, M. Pozgajova, B. Nieswandt, U. Walter, Platelet NAD(P)H-oxidase-generated ROS production regulates alphaIIbbeta3-integrin activation independent of the NO/cGMP pathway, Blood 106 (2005) 2757–2760.

[8] L. Iuliano, J.Z. Pedersen, D. Pratico, G. Rotilio, F. Violi, Role of hydroxyl radicals in the activation of human platelets, Eur. J. Biochem. 221 (1994) 695–704.

[9] A.J. Ijsselmuiden, R.J. Musters, G. de Ruiter, L. van Heerebeek, F. Alderse-Baas, M. van Schilfgaarde, A. Leyte, G.J. Tangelder, G.J. Laarman, W.J. Paulus, Circulating white blood cells and platelets amplify oxidative stress in heart failure, Nat. Clin. Pract. Cardiovasc. Med. 5 (2008) 811–820.

[10] E. Migliaccio, M. Giorgio, S. Mele, G. Pelicci, P. Reboldi, P.P. Pandolfi, L. Lanfrancone, P.G. Pelicci, The p66shc adaptor protein controls oxidative stress response and life span in mammals, Nature 402 (1999) 309–313.

[11] C. Napoli, I. Martin-Padura, F. de Nigris, M. Giorgio, G. Mansueto, P. Somma, M. Condorelli, G. Sica, G. De Rosa, P. Pelicci, Deletion of the p66Shc longevity gene reduces systemic and tissue oxidative stress, vascular cell apoptosis, and early atherogenesis in mice fed a high-fat diet, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 2112–2116.

[12] A. Bosutti, G. Grassi, M. Zanetti, A. Aleksova, M. Zecchin, G. Sinagra, G. Biolo, G. Guarnieri, Relation between the plasma levels of LDL-cholesterol and the expression of the early marker of inflammation long pentraxin PTX3 and the stress response gene p66ShcA in pacemaker-implanted patients, Clin. Exp. Med. 7 (2007) 16–23.

[13] C.S. Kim, S.B. Jung, A. Naqvi, T.A. Hoffman, J. DeRicco, T. Yamamori, M.P. Cole, B.H. Jeon, K. Irani, P53 impairs endothelium-dependent vasomotor function through transcriptional upregulation of p66shc, Circ. Res. 103 (2008) 1441–1450.

[14] S. Dayal, K.M. Wilson, D.G. Motto, F.J. Miller Jr., A.K. Chauhan, S.R. Lentz, Hydrogen peroxide promotes aging-related platelet hyperactivation and thrombosis, Circulation 127 (2013) 1308–1316.

[15] A.D. Blann, S.K. Nadar, G.Y. Lip, The adhesion molecule P-selectin and cardiovascular disease, Eur. Heart J. 24 (2003) 2166–2179.

[16] K. Prathap, The natural history of platelet-rich mural thrombi in systemic arteries of hypercholesterolaemic monkeys: light- and electron-microscope observations, J. Pathol. 110 (1973) 203–212.

[17] P. Pinton, R. Rizzuto, P66Shc, oxidative stress and aging: importing a lifespan determinant into mitochondria, Cell Cycle 7 (2008) 304–308.

[18] I. Martin-Padura, F. de Nigris, E. Migliaccio, G. Mansueto, S. Minardi, M. Rienzo, L.O. Lerman, M. Stendardo, M. Giorgio, G. De Rosa, P.G. Pelicci, C. Napoli, P66Shc deletion confers vascular protection in advanced atherosclerosis in hypercholesterolemic apolipoprotein E knockout mice, Endothelium 15 (2008) 276–287.

[19] T. Yamamori, A.R. White, I. Mattagajasingh, F.A. Khanday, A. Haile, B. Qi, B.H. Jeon, A. Bugayenko, K. Kasuno, D.E. Berkowitz, K. Irani, P66shc regulates endothelial NO production and endothelium-dependent vasorelaxation: implications for age-associated vascular dysfunction, J. Mol. Cell Cardiol. 39 (2005) 992–995.

[20] Y.R. Kim, C.S. Kim, A. Naqvi, A. Kumar, S. Kumar, T.A. Hoffman, K. Irani, Epigenetic upregulation of p66shc mediates low-density lipoprotein cholesterol-induced endothelial cell dysfunction, Am. J. Physiol. Heart Circ. Physiol. 303 (2012) H189–H196.

[21] S. Nagalla, C. Shaw, X. Kong, A.A. Kondkar, L.C. Edelstein, L. Ma, J. Chen, G.S. McKnight, J.A. Lopez, L. Yang, Y. Jin, M.S. Bray, S.M. Leal, J.F. Dong, P.F. Bray, Platelet microRNA-mRNA coexpression profiles correlate with platelet reactivity, Blood 117 (2011) 5189–5197.

[22] T. Ohmori, Y. Yatomi, N. Asazuma, K. Satoh, Y. Ozaki, Involvement of proline-rich tyrosine kinase 2 in platelet activation: tyrosine phosphorylation mostly dependent on alphaIIbbeta3 integrin and protein kinase C, translocation to the cytoskeleton and association with Shc through Grb2, Biochem. J. 347 (2000) 561–569.

[23] K.J. Cowan, D.A. Law, D.R. Phillips, Identification of shc as the primary protein binding to the tyrosine-phosphorylated beta 3 subunit of alpha IIbbeta 3 during outside-in integrin platelet signaling, J. Biol. Chem. 275 (2000) 36423–36429.

[24] S.J. Shattil, R. Anaya-Galindo, J. Bennett, R.W. Colman, R.A. Cooper, Platelet hypersensitivity induced by cholesterol incorporation, J. Clin. Invest. 55 (1975) 636–643.

[25] P.F. Monteiro, R.P. Morganti, M.A. Delbin, M.C. Calixto, M.E. Lopes-Pires, S. Marcondes, A. Zanesco, E. Antunes, Platelet hyperaggregability in high-fat fed rats: a role for intraplatelet reactive-oxygen species production, Cardiovasc. Diabetol. 11 (2012) 5.

[26] P.C. Redondo, I. Jardin, J.M. Hernandez-Cruz, J.A. Pariente, G.M. Salido, J.A. Rosado, Hydrogen peroxide and peroxynitrite enhance Ca2+ mobilization and aggregation in platelets from type 2 diabetic patients, Biochem. Biophys. Res. Commun. 333 (2005) 794–802.