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Platelets Express Activated P2Y12 Receptor in Patients with Diabetes

Running Title: *Hu et al.; Constitutively Active P2Y12 of Diabetic Platelets*

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Abstract

Background—Platelets from patients with diabetes are hyperactive. Hyperactivated platelets may contribute to cardiovascular complications and inadequate responses to antiplatelet agents in the setting of diabetes. However, the underlying mechanism of hyperactivated platelets is not completely understood.

Methods—We measured $P2Y_{12}$ expression on platelets from patients with type 2 diabetes mellitus (T2DM) and on platelets from rats with diabetes. We also assayed platelet $P2Y_{12}$ activation by measuring cAMP and VASP phosphorylation. The antiplatelet and antithrombotic effects of AR-C78511 and cangrelor were compared in rats. Finally we explored the role of the NFκB pathway in regulating $P2Y_{12}$ receptor expression in megakaryocytes.

Results—Platelet $P2Y_{12}$ levels are 4-fold higher in patients with T2DM compared to healthy subjects. $P2Y_{12}$ expression correlates with ADP-induced platelet aggregation ($r = 0.89$, $P < 0.01$). $P2Y_{12}$ in platelets from patients with diabetes is constitutively activated. Though both AR-C78511, a potent $P2Y_{12}$ inverse agonist, and cangrelor have similar antiplatelet efficacy on platelets from healthy subjects, AR-C78511 exhibits more powerful antiplatelet effects on diabetic platelets than cangrelor (aggregation ratio 36 ± 3% *vs* 49 ± 5%, respectively, $P < 0.05$). Using a $FeCl_3$-injury mesenteric arteriole thrombosis model in rats and an A-V shunt thrombosis model in rats, we found that the inverse agonist AR-C78511 has greater antithrombotic effects on diabetic GK rats than cangrelor (thrombus weight 4.9 ± 0.3 mg *vs* 8.3 ± 0.4 mg, respectively, $P < 0.01$). We also found that a pathway involving high glucose-ROS-NFκB increases platelet $P2Y_{12}$ receptor expression in diabetes.

Conclusions—Platelet $P2Y_{12}$ receptor expression is significantly increased and the receptor is constitutively activated in T2DM patients, which contributes to platelet hyperactivity and limits antiplatelet drug efficacy in T2DM.

Key Words: diabetes mellitus; cardiovascular disease; P2Y12 receptor; platelet; antiplatelet, antithrombotic, cangrelor, AR-C78511, inverse agonist

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Clinical Perspective

What is new?

- Platelets of type 2 diabetes mellitus (T2DM) patients express high levels of activated $P2Y_{12}$ receptor.
- The $P2Y_{12}$ inverse agonist AR-C78511 inhibits $P2Y_{12}$ activity of platelets from patients and rats with diabetes more than cangrelor, leading to a stronger antithrombotic effect of AR-C78511 in rats with diabetes.
- Increased platelet $P2_{Y12}$ receptor expression in diabetes is mediated by a high glucose-ROS-NFκB pathway.

What are the clinical implications?

- Our study may explain why thrombotic complications are more prevalent in patients with diabetes.
- Our data show why some antiplatelet drugs have limited efficacy in patients with diabetes, and provide a rationale for using $P2Y_{12}$ receptor inverse agonists to prevent and treat cardiovascular complications of diabetes.

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American Heart Association.

Circulation

Diabetes is a major cause of morbidity and mortality across the globe, leading to coronary artery disease, myocardial infarctions, and death.[1-4] Antiplatelet therapy treats and prevents cardiovascular complications in patients with diabetes. Platelets of patients with diabetes are characterized by increased reactivity, which contributes to not only the increased prevalence of cardiovascular complications in diabetics, but also the inadequate response to currently available antiplatelet agents such as clopidogrel and cangrelor compared with non-diabetics[5-7], resulting in significantly increased cardiovascular events and mortality associated with diabetes[5]. The mechanism underlying the increased platelet reactivity in patients with diabetes is not completely understood.

Increased platelet receptor expression and downstream signaling have been proposed to contribute to the hyperactivity of diabetic platelets[7, 8]. The $P2Y_{12}$ receptor is the most successful antiplatelet target and plays a central role in platelet activation and thrombosis[9], however, the role of $P2Y_{12}$ receptor in platelet hyperactivity of diabetes is still not clear. A gain of function $P2Y_{12}$ haplotype is associated with peripheral arterial disease, coronary artery disease[10-12] as well as ischemic cerebrovascular events[13]. Furthermore, increased $P2Y_{12}$ copy number is attributed to the gain of function of the platelet $P2Y_{12}$ receptor[12]. Platelets from patients with type 2 diabetes mellitus (T2DM) show $P2Y_{12}$ hyperactivity and decreased response to clopidogrel and cangrelor (AR-C69931MX)[6, 14]. Together, these studies suggest that $P2Y_{12}$ mutations or expression levels may contribute to platelet hyperactivity and impaired antiplatelet efficacy in patients with diabetes. However, $P2Y_{12}$ expression level on platelets of patients with T2DM has never been reported.

Previously we and others reported that constitutive activity of the $P2Y_{12}$ receptor [15-20] can result from $P2Y_{12}$ overexpression in recombinant systems[19, 20] or gain of function mutations[15, 17,

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[18]. Several $P2Y_{12}$ inverse agonists, which were believed to be therapeutically beneficial over neutral antagonists, including AR-C78511 and ticagrelor, were also reported[15-18]. Many of these studies were performed using an *in vitro* recombinant system[15, 17, 19, 20]. Using a transgenic mouse model, we found that constitutively activated $P2Y_{12}$ receptor increases platelet activation and arterial thrombosis[18]. Moreover, we have shown that the inverse agonist AR-C78511 inhibits platelet activation more robustly than cangrelor[15, 18], suggesting that a potent $P2Y_{12}$ receptor inverse agonist may have superior antithrombotic effects in patients with $P2Y_{12}$ gain of function mutation or expression increase.

We hypothesize that platelet $P2Y_{12}$ expression is increased in patients with T2DM, which activates platelet $P2Y_{12}$ signaling and leads to platelet hyperactivity in patients with diabetes. In this study, we found that platelet $P2Y_{12}$ receptor expression is enhanced in patients with T2DM and diabetic rats, correlating with platelet hyperactivity and increased $P2Y_{12}$ signaling. We elucidated the underlying mechanism of increased $P2Y_{12}$ expression, and then we demonstrated the therapeutic advantage of the inverse agonist AR-C78511 over the neutral antagonist cangrelor[6, 15, 18].

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Materials and Methods

Detailed Materials and Methods are described in the online-only Data Supplement.

Subjects

All experiments using human subjects were performed in accordance with the Declaration of Helsinki and approved by the Institutional Review Board Fudan University. Blood from 40 T2DM patients was obtained from Fudan University Huashan Hospital and Zhongshan Hospital. Peripheral artery disease was ruled out based on Ankle Branchial Indices (1.07 ± 0.07 for right

leg, 1.09 ± 0.06 for left leg, mean ± SD, n = 30) or carotid and femoral artery ultrasound examination (no plague, no arterial intimal thickening). Blood from 29 healthy volunteers recruited from Fudan University faculty and staff was used as control. All participants did not take any antiplatelet or other non-steroidal anti-inflammatory drugs for at least 14 days before blood collection and informed consent was obtained. The basic clinical data is presented in Supplemental Table 1. Blood (6 - 36 mL) was drawn from the antecubital vein and mixed with ACD buffer (6:1 vol/vol). Platelets were isolated and platelet suspension was prepared as described in Detailed Materials and Methods in the online-only Data Supplement.

Animal studies

The diabetic GK rats and Wistar rats were purchased from Shanghai Laboratory Animal Center (SLAC, Shanghai, China). All animal procedures were carried out in accordance with institutional guidelines at Fudan University. Rat platelets were prepared as described previously[21].

Platelet functional studies

Platelet aggregation, secretion, spreading, and clot retraction were measured as previously described[22, 23] and detailed in the online-only Data Supplement.

Cell culture

Human megakaryocytic cell line (Meg-01) was cultured in RPMI-1640 medium detailed in the online-only Data Supplement.

RT-PCR and real time PCR analysis

Total RNA was extracted from platelets and megakaryocytes with Trizol reagent (Invitrogen, Carlsbad, CA, USA). After RNA isolation, 1 μg of total RNA was reverse transcribed to cDNA using an RT-PCR kit (TaKaRa, Dalian, Japan). Real time PCR was performed by using specific

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primers (Supplemental Table 2).

Immunoblotting

Washed platelets were stimulated with or without agonists for the appropriate time and lysed in 5 x SDS-PAGE sample loading buffer and boiled for 10 minutes. Megakaryocytic cells were prepared as described above. Protein samples were subjected to immunoblotting.

cAMP assay

cAMP in platelets was assayed as we previously reported[23]. Briefly, washed platelets were preincubated with different agents for 3 minutes and reactions were terminated by addition of 0.1 M HCl. After incubating at room temperature for 20 minutes, the platelets were then centrifuged at 1,000 g for 10 min. The supernatant was decanted into a clean test tube and subjected to cAMP assay using cAMP EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instruction.

Rat bone marrow isolation and CD61+ megakaryocyte preparation

Rat bone marrow aspiration samples were collected in tubes containing EDTA. Mononuclear cells were isolated from bone marrow samples as reported by Boyum et al[24]. After removal of adherent cells, rat $CD61^+$ megakaryocytes population was enriched with CD61 MicroBeads (Miltenyi Biotech, Gladbach, Germany) according to the manufacturer's instruction as reported previously[25]. Flow cytometry was performed to verify the purity of the megakaryocyte sample. After the purification of megakaryocytes, cells were collected and processed for RNA and protein extraction.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed using the SimpleChIP enzymatic chromatin immunoprecipitation kit (Cell Signaling Technology) according to the manufacturer's

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protocol with minor modification. Briefly, Meg-01 cells or rat megakaryocytes were cross-linked with 1.5% (v/v) formaldehyde for 10 min, then stopped with 0.125 M glycine. Subsequently, Nuclei were collected and chromatin was sonicated to desired chromatin length (~500bp). Sonicated lysates were then diluted to 2 mL, with protease inhibitor cocktail, and 20 μL of this solution was removed for PCR analysis (input). After preclearing with Protein A/G Plus Agarose (Santa Cruz Biotechnology) for 1 hour at 4°C, antibody specific to NFκB p65 subunit was added and incubated at 4°C overnight on a rocking platform. Immune complexes were collected by addition of Protein A/G Plus Agarose for 1 hour at 4°C and the agarose beads were extensively washed with solutions of increasing ionic strength. Bound immune complexes were eluted and cross-links were reversed by incubating at 65°C for 4 hours. Samples were then treated with proteinase K, and DNA was purified using DNA purification spin columns. DNA was amplified for detection of the binding motif of transcription factors in $P2Y_{12}$ regulation region by PCR using the primers in supplemental Table 3.

Extracorporeal arterio-venous shunt (A-V shunt) thrombosis

The rat arterio-venous shunt thrombosis model was developed based on previously described procedures with minor modification[26]. Male rats were anesthetized by chloral hydrate (350 mg/kg, i.p.) on a heating pad at 37°C. Then the left jugular vein and right carotid artery were isolated and linked by a shunt catheter (SDR scientific, Sydney, Australia), which was composed of three parts including two 6 cm polyethylene (PE) 8040 catheters and a 6 cm PE 12080 catheter. A 6 cm long braided silk thread was placed in the central part of the shunt, to act as the thrombogenic substrate. Immediately after shunt preparation, 60 μg/kg cangrelor, AR-C78511 or an equal volume of saline was administered through caudal vein as a bolus followed by continuous infusion (6 μg/kg/min) throughout the experiment. Circulation was initiated 5

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minutes later and the extracorporeal shunt remained in place for 15 minutes. The shunt was then disconnected and the thread with thrombus was removed from the catheter. The wet thrombus attached in the silk thread was weighed immediately and calculated by subtracting the average weight of a 6 cm long braided silk thread.

Intravital microscopy of $FeCl_3$-injured thrombus formation in rat mesenteric arteriole

Intravital microscopy of $FeCl_3$ thrombus formation in rat mesenteric arteriole was performed as described previously with minor modification[22, 23]. Briefly, after a bolus injection of cangrelor (60 μg/kg), AR-C78511 (60 μg/kg) or saline via the tail vein, calcein-labeled rat platelets were injected into rats via lateral tail vein. Cangrelor and AR-C78511 were then continuously given at 6 μg/kg/min during the experiment. Thrombosis was induced by $FeCl_3$ 5 minutes later and recorded with intravital microscopy for 10 minutes.

Statistical analysis

All data are expressed as mean ± SEM. Unless otherwise stated, differences between two groups were analyzed by unpaired t-test when variances are equal and one-way ANOVA followed by Newman-Keuls test were used for multiple comparisons with Prism 5 (GraphPad Inc, San Diego) $P < 0.05$ was considered to be statistically significant.

Results

Increased platelet $P2Y_{12}$ expression and aggregation in patients with diabetes

We measured platelet mRNA in 40 patients and 29 healthy subjects (Figure 1A). $P2Y_{12}$ mRNA is significantly increased in platelets from T2DM patients. Platelet $P2Y_{12}$ protein level and platelet function of 20 patients and 12 healthy subjects were further analyzed. Platelet $P2Y_{12}$ protein expression is also significantly increased in patients with diabetes (Figure 1B). As

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expected, platelet aggregation in response to ADP is enhanced in patients with diabetes compared to healthy subjects (Figure 1C). Importantly, platelet $P2Y_{12}$ expression level is closely correlated with platelet aggregation in response to ADP ($r = 0.89$, $P < 0.01$, Figure 1D). Real time PCR analysis revealed that platelets from T2DM patients expressed similar ADP receptor $P2Y_1$, thrombin receptor PAR1 and PAR4, thromboxane receptor TP as healthy subjects (Figure 1 E).

Enhanced pAkt and impaired pVASP downstream of $P2Y_{12}$ in platelets of patients with diabetes

We next explored $P2Y_{12}$ in platelets of patients with diabetes. We observed enhanced pAkt and impaired pVASP in response to ADP downstream of $P2Y_{12}$ in platelets of patients with T2DM compared with healthy subjects. Notably, in the absence of ADP stimulation, we detected significantly enhanced Akt phosphorylation and reduced VASP phosphorylation in platelets from T2DM patients compared with the healthy subjects, indicating the constitutive activation of resting platelet $P2Y_{12}$ receptor in T2DM patients due to elevated platelet $P2Y_{12}$ receptor expression (Figure 2).

Increased platelet $P2Y_{12}$ expression and platelet activation in diabetic rats

GK rats are non-obese Wistar rats which develop spontaneous T2DM in early life, making them ideal for T2DM research[27, 28]. We recapitulated our findings of T2DM patients using diabetic GK rats. Compared with Wistar rats, diabetic GK rats have increased platelet $P2Y_{12}$ expression both on mRNA and protein levels (Figure 3A and B). As expected, platelets from diabetic GK rats exhibited consistently increased aggregation in response to ADP, thrombin and AYPGKF (Figure 3C). When ATP release from platelet dense granules was simultaneously recorded, we found that platelets from diabetic GK rats also secreted more ATP in response to thrombin,

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AYPGKF and collagen in addition to enhanced aggregation compared to Wistar rats (supplemental Figure 1). We attribute the enhanced platelet activation in diabetic rats to increased $P2Y_{12}$ expression, as platelet $P2Y_1$, PAR4, and TP receptor expression levels are similar between Wistar and diabetic GK rats (Figure 3D). We also found that cyclooxygenase (COX-1 and COX-2) expression is unaltered in platelets and megakaryocytes of diabetic GK rats compared with healthy Wistar rats (supplemental Figure 2).

Clot retraction was also significantly accelerated in diabetic GK rats compared with Wistar rats of the same age (6 weeks old) (Figure 3E). Interestingly, diabetic GK rats 3 weeks of age have clot retraction that mirrors that of Wistar rats 6 weeks of age (Figure 3E). Correspondingly, we found that platelet $P2Y_{12}$ protein expression increased after 3 weeks of age in diabetic GK rats, while platelet $P2Y_{12}$ of Wistar rats did not change significantly with age until 7 weeks old (Figure 3F). In line with platelet $P2Y_{12}$ expression change, fasting blood glucose levels in diabetic rats also increased after 3 weeks of age and correlates with platelet $P2Y_{12}$ level (Figure 3F).

Enhanced pAkt and impaired pVASP downstream of $P2Y_{12}$ in platelets of diabetic rats

Similar to T2DM patients, when downstream signaling of $P2Y_{12}$ was investigated, platelets from diabetic GK rats demonstrated enhanced pAkt and impaired pVASP in response to ADP compared with Wistar rats. Importantly, as in patients with diabetes, platelets from diabetic GK rats also exhibited exacerbated Akt phosphorylation and reduced VASP phosphorylation compared to Wistar rat platelets in the absence of ADP stimulation (Supplemental Figure 3), indicating the constitutive activation of the platelet $P2Y_{12}$ receptor in diabetic GK rats resulting from enhanced platelet $P2Y_{12}$ receptor expression.

Increased NFκB activation upregulates $P2Y_{12}$ expression in diabetic platelets

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We next investigated the mechanism underlying the increased $P2Y_{12}$ expression in diabetic platelets. The $P2Y_{12}$ promoter contains a consensus NFκB binding site[29] (Figure 4A) and NFκB-mediated $P2Y_{12}$ upregulation has been reported in thrombin-stimulated human smooth muscle cells[30]. Compared with healthy subjects, platelets from T2DM patients exhibit augmented IκBα phosphorylation and degradation, while the NFκB subunit p65 remains unchanged (Figure 4B), suggesting NFκB activation in platelets from T2DM patients.

Similarly, we observed increased NFκB activation in diabetic GK rats (Figure 4C - E). In addition to the enhanced IκBα phosphorylation and degradation in platelets of diabetic rats and patients, IκBα phosphorylation and degradation were also increased in megakaryocytes from diabetic GK rats (Figure 4C). Furthermore, p65 from the nuclear extract of megakaryocytes from diabetic GK rats was also increased compared with Wistar rats (Figure 4D). Moreover, utilization of a ChIP assay revealed a significant increase in p65 binding to the $P2Y_{12}$ promoter area in diabetic rat megakaryocytes (Figure 4E). Collectively, these results strongly suggest that increased platelet $P2Y_{12}$ expression in diabetes is mediated by NFκB activation.

It is generally accepted that after phosphorylation by IKK, IκBα is ubiquitinated and degraded by proteasomes[31]. We measured IκBα ubiquitination by Co-Immunoprecipitation and Western blot in megakaryocytes from diabetic GK rats. As shown in Supplemental Figure 4 (panel A and B), there is enhanced IκBα ubiquitination in megakaryocytes from diabetic GK rats compared with healthy Wistar rats. We also quantified IκBα expression by real time PCR in platelets and megakaryocytes from diabetic GK rats and found that it was similar to healthy Wistar rats (Supplemental Figure 4, panel C). Together, these results support that the decreased IKBα in diabetic platelets observed in Figure 4 is mainly due to ubiquitination-mediated degradation.

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High glucose increases P2Y$_{12}$ expression in Meg-01 cells via NFκB activation

Increased platelet P2Y$_{12}$ expression in patients with diabetes and diabetic rats suggests that the upregulation of platelet P2Y$_{12}$ may be the result of hyperglycemia. To confirm this hypothesis, we treated Meg-01 cells, a human megakaryoblastic cell line, with different concentrations of glucose and quantified P2Y$_{12}$ expression. As shown in Figure 5, compared with normal glucose concentration (5 mM), 24 hours incubation with high glucose (15, 25 mM glucose) concentration-dependently increased P2Y$_{12}$ expression, which was reversed by NFκB inhibitor BAY 11-7082 (Figure 5A), supporting that hyperglycemia upregulates P2Y$_{12}$ in megakaryocytes and platelets in diabetes via NFκB activation.

In concert with the hyperglycemic-dependent increase of P2Y$_{12}$ expression in Meg-01 cells, IκBα phosphorylation and degradation in cell lysate and p65 from the nuclear extracts were also concentration-dependently elevated by high glucose and were inhibited by BAY 11-7082 (Figure 5A). Together, these results reveal that hyperglycemia-induced NFκB activation upregulates P2Y$_{12}$ expression in diabetic platelets.

Antioxidants down-regulate P2Y$_{12}$ expression in Meg-01 cells

We detected increased intracellular ROS in platelets from diabetic GK rats and high glucose-treated megakaryocytic Meg-01 cells (supplemental Figure 5). We also found that high glucose-induced P2Y$_{12}$ upregulation and NFκB activation in Meg-01 cells were blocked by N-acetylcysteine (NAC), an antioxidant and glutathione precursor (Figure 5B), and dithiothreitol (DTT), a structurally different antioxidant (Figure 5B).

Phorbol myristate acetate (PMA) was reported to stimulate Meg-01 cell differentiation and platelet production[32]. We thus explored whether PMA-stimulated Meg-01 cells also express more P2Y$_{12}$ under high glucose condition. As expected, PMA increased P2Y$_{12}$ expression in

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Meg-01 cells both under normal (5 mM) and high glucose (15 and 25 mM) conditions, with higher $P2Y_{12}$ expression in the presence of higher glucose concentration (Figure 5C). Finally, as in diabetic rat megakaryocytes (Figure 4D), our ChIP assay exhibited increased p65 binding to the $P2Y_{12}$ promoter in Meg-01cells treated with high glucose (25 mM) in comparison with normal glucose (Figure 5D). Correspondingly, mRNA level analysis of $P2Y_{12}$ in Meg-01 cells by real time PCR also revealed $P2Y_{12}$ upregulation by high concentrations of glucose, which was completely inhibited by NFκB inhibitor BAY 11-7082 (Figure 5E), verifying the enhanced $P2Y_{12}$ transcription by high glucose mediated by NFκB activation. Taken together, these *in vitro* findings on megakaryoblastic Meg-01 cells corroborate the findings in diabetic rats that hyperglycemia upregulates $P2Y_{12}$ transcription via NFκB activation leading to increased platelet $P2Y_{12}$ expression in diabetes.

Increased proinflammatory cytokines in platelets of diabetes

Real time PCR analysis revealed that the proinflammatory cytokines IL-1β, TNFα, and IL-8 in platelets from T2DM patients were more than 4.7 times higher than the healthy subjects. Additionally, cell adhesion molecules ICAM-1 and VCAM-1 were also more than 2 times higher in T2DM patient platelets (supplemental Figure 6A). We did not observe an alteration in IL-6. Similar results were observed in platelets from diabetic GK rats (supplemental Figure 6B). Next, it is important to clarify whether the upregulation of proinflammatory cytokines and adhesion molecules in diabetes is the consequence or cause. To address this issue, we treated Meg-01 cells with high glucose and determined the proinflammatory cytokines in Meg-01 cells by real time PCR. Our results show that high glucose concentration-dependently enhanced mRNA levels of proinflammatory cytokines IL-1β, TNFα, IL-8 and adhesion molecules ICAM-1 and VCAM-1 in Meg-01 cells, which was abrogated by NFκB inhibitor Bay 17-7082

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(Supplemental Figure 6C). These data support the hypothesis that upregulation of proinflammatory cytokines and adhesion molecules in diabetes is a result of NFκB activation due to hyperglycemia.

Superior antiplatelet effect of inverse agonist AR-C78511 over cangrelor on platelets of patients with diabetes

Previously we demonstrated the superior antiplatelet effect of inverse agonist AR-C78511 over AR-C69931MX (cangrelor) using murine platelets expressing constitutively active chimeric $P2Y_{12}$ receptor[18]. Because we have shown that the increased $P2Y_{12}$ receptors in platelets from T2DM patients are also constitutively activated, we tested whether or not AR-C78511 bears superior antiplatelet activity over cangrelor. As shown in Figure 6A, platelets from T2DM patients demonstrated enhanced response to ADP compared to platelets from healthy donors, which was inhibited more significantly by AR-C78511 than cangrelor. As a control, both cangrelor and AR-C78511 exhibited similar antiplatelet activity when platelets from healthy donors were used (Figure 6A, left panel).

We further analyzed whether platelet $P2Y_{12}$ level influences the antiplatelet activity of cangrelor and AR-C78511. Figure 6B shows that the anti-aggregatory activity of both cangrelor and AR-C78511 decreased as platelet $P2Y_{12}$ expression level increased. However, the antiplatelet activity of AR-C78511 is influenced significantly less than cangrelor. On platelets expressing higher $P2Y_{12}$, AR-C78511 exhibited stronger antiplatelet activity compared with cangrelor (Figure 6B, $P < 0.01$).

The superior antiplatelet activity of AR-C78511 over cangrelor is attributed to its potent $P2Y_{12}$ inverse agonist activity. Consistent with the decreased pVASP in platelets of T2DM patients (Figure 2A), intracellular cAMP, another measure of $P2Y_{12}$-Gi activation, in

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unstimulated platelets from T2DM patients is also significantly lower than that of healthy subjects (Figure 6C), further confirming the constitutive activation of platelet $P2Y_{12}$ receptor in T2DM patients. As expected, inverse agonist AR-C78511, rather than cangrelor, reversed the cAMP decrease in platelets of T2DM patients (Figure 6C), in line with our previous findings on platelets from transgenic mice expressing constitutively activated chimeric $P2Y_{12}$ receptor[18]. As a control, at the same concentration, both cangrelor and AR-C78511, effectively antagonized ADP-induced cAMP reduction in platelets of healthy subjects (Supplemental Figure 7).

Superior antiplatelet effect of inverse agonist AR-C78511 over cangrelor on platelets of diabetic rats

The superior antiplatelet activity of AR-C78511 on human diabetic platelets was successfully recapitulated using platelets from diabetic GK rats. As shown in Figure 6D, in the range of 1.5 - 10 nM, both cangrelor and AR-C78511 similarly inhibited ADP-induced platelet aggregation of Wistar rats (Figure 6D, left panel). In contrast, AR-C78511 exhibited significantly stronger inhibition on ADP-induced platelet aggregation of diabetic GK rats; compared with AR-C78511, the concentration-effect curve of cangrelor right-shifted (P <0.01), indicating that the antiplatelet effect of cangrelor is impaired in diabetic GK rats (Figure 6D, right panel).

Further comparison of the responses of Wistar and diabetic GK rat platelets to cangrelor demonstrated that concentration-effect curve of cangrelor on diabetic GK rat platelets right-shifted ($P < 0.01$) (Figure 6E, left panel), while AR-C78511 did not ($P > 0.05$) (Figure 6E, right panel), indicating that antiplatelet activity of cangrelor decreased in diabetic rats while AR-C78511 remained the similar antiplatelet activity on both normal and diabetic rat platelets. These data further verified the superior antiplatelet effect of AR-C78511 over cangrelor on diabetic rats.

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In accordance with the reduced intracellular cAMP of platelets from patients with diabetes, cAMP in platelets of diabetic rats was also drastically lower than Wistar rat platelets (Figure 6F, left panel). Consistent with the findings on human platelets, AR-C78511 reversed the intracellular cAMP reduction in resting platelets from diabetic GK rats while the same concentration of cangrelor did not (Figure 6F, left panel). When platelets were stimulated with ADP, both cangrelor and AR-C78511 effectively reversed ADP-induced cAMP decrease in platelets of Wistar rats. When diabetic GK platelets was tested, however, cangrelor did not reverse ADP-elicited intracellular cAMP reduction, while AR-C78511 raised intracellular cAMP level close to that in unstimulated platelets of Wistar rats (Figure 6F, right panel). These results suggest that AR-C78511 also inhibits the constitutive activity of platelet $P2Y_{12}$ due to increased expression in diabetic rats in addition to antagonism on ADP-induced $P2Y_{12}$ activation.

Enhanced thrombus formation in diabetic rats and the superior antithrombotic effect of AR-C78511 over cangrelor in diabetic rats

The superior antiplatelet activity of inverse agonist AR-C78511 over cangrelor on platelets from patients with diabetes and diabetic rats strongly suggests that it may translate into a superior *in vivo* antithrombotic effect in diabetes. Not surprisingly, diabetic GK rats exhibited increased thrombus formation using both $FeCl_3$-injured mesenteric arteriole thrombosis model (Figure 7A) and A-V shunt thrombosis model (Figure 7B). Of note, compared with 6 weeks old diabetic GK rats, thrombus size and weight of 3 weeks old diabetic GK rats did not increase, similar to that of 6 weeks old Wistar rats, which is consistent with our observations on clot retraction, blood sugar, and platelet $P2Y_{12}$ protein expression (Figure 3E & F). Both AR-C78511 and cangrelor treatment effectively inhibited thrombus formation similarly in Wistar rats. In diabetic rats, both AR-

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C78511 and cangrelor effectively inhibited thrombus formation, but AR-C78511 was much more effective than cangrelor on both thrombosis models, especially on A-V shunt model (Figure 7). AR-C78511was first developed by AstraZeneca, PD and PK of AR-C78511 were not reported previously. We assayed the plasma concentration of AR-C78511 in healthy Wistar and diabetic GK rats under the antithrombotic conditions. As in Supplemental Figure 8, plasma concentration of AR-C78511 is similar in healthy and diabetic rats. The concentration of AR-C78511 during infusion is around 900 ng/ml (1.55 μM), high enough to inhibit platelet activation and thrombosis.

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Discussion

In this study we demonstrate that (1) patients with T2DM have enhanced platelet $P2Y_{12}$ receptor expression; (2) hyperglycemia triggers ROS production, activates NFκB and increases expression of the prothrombotic platelet $P2Y_{12}$ receptor; (3) increased $P2Y_{12}$ receptor is constitutively activated in diabetic platelets; (4) the $P2Y_{12}$ receptor inverse agonist AR-C78511 is more effective in inhibiting platelet activation and thrombosis compared to cangrelor in platelets of patients with diabetes. Our results suggest that increased expression and constitutive activation of platelet $P2Y_{12}$ contributes to platelet hyperactivity, prevalence of cardiovascular complications, and hampered antiplatelet efficacy in diabetes. It also indicates that $P2Y_{12}$ receptor antagonists with potent inverse agonist activity may have superior antiplatelet efficacy in diabetes, especially in patients with increased platelet $P2Y_{12}$ expression.

According to the two-state model of GPCR activation (Supplemental Figure 9), GPCRs can be activated in the absence of agonists, exhibiting the same effect as agonist-induced activity. This effect, produced in the absence of agonists, is called constitutive activation.

Receptor mutation or increased expression increases GPCR constitutive activity. Constitutive activity has been reported in numerous GPCRs and has been associated with specific diseases[16, 18, 33-36]. Neutral antagonists, which block agonist binding to the receptors, do not inhibit the constitutive activity of GPCRs[33, 37]. Inverse agonists, which shift the balance to the inactive state, inhibit the constitutive activity of GPCRs, and therefore bear therapeutic advantage over neutral antagonists[16, 17, 36]. Most of the clinically important medicines targeting GPCRs have inverse agonist activity to some extent[36, 38].

$P2Y_{12}$ plays a central role in platelet activation[9] and is a major antiplatelet target. We show that T2DM patients have enhanced platelet $P2Y_{12}$ receptor expression, corroborating prior reports of $P2Y_{12}$ signaling pathway upregulation and platelet hyperactivity in diabetes[6]. Furthermore, platelet $P2Y_{12}$ is constitutively activated in patients with diabetes, which may underlie platelet hyperactivity and dampened response to antiplatelet agents in diabetes[6, 14]. Prasugrel and cangrelor have minimal inverse agonist activity on constitutively activated $P2Y_{12}$ receptor[15, 16, 18]. Not surprisingly, the potent $P2Y_{12}$ receptor inverse agonist AR-C78511 inhibits the constitutively active $P2Y_{12}$ and exhibits superior antiplatelet efficacy over cangrelor on platelets from both patients with diabetes and diabetic rats. We defined the prothrombotic role of the upregulated and constitutively activated $P2Y_{12}$ in diabetic rats. Finally and more importantly, we provided evidence that the superior antiplatelet activity of inverse agonist AR-C78511 can be translated into superior antithrombotic effect over cangrelor in diabetic rats using two thrombus models. In agreement with our findings, ticagrelor, which inhibits platelet activation greater than prasugrel and clopidogrel in patients with diabetes[39-41], was recently reported to be a potent $P2Y_{12}$ receptor inverse agonist[16].

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We propose that the NFκB signal pathway increases $P2Y_{12}$ expression in platelets from subjects with diabetes. First, we detected increased NFκB signaling in platelets of both patients with diabetes and diabetic rats, as well as in megakaryocytes of diabetic rats. Second, the $P2Y_{12}$ promoter contains an NFκB binding site and we demonstrated significantly higher binding of NFκB subunit p65 to the $P2Y_{12}$ promoter of diabetic rat megakaryocytes. Finally, using a megakaryocytic cell line treated with high concentrations of glucose, we demonstrated that hyperglycemia enhances $P2Y_{12}$ expression in an NFκB dependent manner.

It has been proposed that hyperglycemia increases ROS[42-44]and NFκB is the target of ROS[45, 46]. We found that the hyperglycemia-ROS-NFκB pathway mediates $P2Y_{12}$ upregulation in diabetic platelets (Figure 8) based on 1) increased $P2Y_{12}$ expression and NFκB activation in platelets from patients with diabetes (Figure 1A, B) and rats (Figure 3A, B); 2) increased NFκB activation in megakaryocytes of diabetic rats (Figure 4); 3) hyperglycemia induces $P2Y_{12}$ high expression and NFκB activation in megakaryocytic Meg-01 cells (Figure 5A); 4) increased intracellular ROS in platelets from diabetic GK rats and in megakaryocytic Meg-01 cells; 5) antioxidants abolish high glucose-induced $P2Y_{12}$ high expression and NFκB activation (Figure 5B); 6) NFκB inhibition abolishes high glucose-induced $P2Y_{12}$ high expression (Figure 5A, E).

In conclusion, the $P2Y_{12}$ receptor, a major receptor in platelet activation and thrombosis, has increased expression and activation in subjects with diabetes. To our knowledge, this is the first report to show the constitutive activation of $P2Y_{12}$ receptor in disease. We also demonstrated that hyperglycemia-ROS-NFκB mediates platelet $P2Y_{12}$ upregulation, which may explain the prothrombotic state in diabetes and the impaired antiplatelet efficacy. Our study sheds new insight into the pathogenesis of cardiovascular complications of diabetes. It also provides guidance on antiplatelet therapy to prevent and treat cardiovascular complications in

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patients with diabetes. $P2Y_{12}$ receptor antagonists with potent inverse agonist activity may have superior antiplatelet and antithrombotic efficacy in patients with diabetes, especially for the patients with increased platelet $P2Y_{12}$ expression.

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Disclosures

None

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Figure Legends

Figure 1. Increased platelet $P2Y_{12}$ expression and aggregation in patients with T2DM. A: Increased $P2Y_{12}$ expression in platelets from patients with T2DM compared with healthy subjects analyzed by real time PCR. Unpaired t-test with Welch's correction was used. **B:** Increased $P2Y_{12}$ expression in platelets from patients with T2DM analyzed by Western blot. Representative immunoblots of platelet $P2Y_{12}$ and GAPDH from 4 healthy subjects and 5 patients with T2DM (upper) and summary data (lower) from 12 healthy subjects and 20 patients with T2DM. **C**: Increased platelet aggregation in response to ADP in T2DM patients. Representative tracings (upper) and summary data are presented, unpaired t-test with Welch's correction was used. **D**: Platelet aggregation induced by 10 μM ADP is well-correlated with protein level of the platelet $P2Y_{12}$ receptor in 12 healthy subjects and 20 patients with T2DM. Each solid circle represents a different individual (Pearson correlation, GraphPad Prism 5). **E**: Similar expression of platelet $P2Y_1$, thromboxane A2 receptor (TP), protease-activated receptor-1 (PAR1) and protease-activated receptor-4 (PAR4) in patients with T2DM compared with healthy subjects detected by real time PCR.

Figure 2. Enhanced pAkt and impaired pVASP downstream of $P2Y_{12}$ in platelets of patients with T2DM compared with healthy subjects. A: Decreased VASP phosphorylation in resting and ADP-stimulated platelets of patients with T2DM compared with healthy subjects. **B:** Increased Akt phosphorylation in resting and ADP-stimulated platelets of patients with T2DM compared with healthy subjects. Representative immunoblots (upper) and summary data (lower) are given, unpaired t-test with Welch's correction was used.

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Figure 3. Increased platelet $P2Y_{12}$ expression and platelet activation in diabetic rats compared with Wistar rats. A: Increased $P2Y_{12}$ expression in platelets from diabetic GK rats compare to Wistar rats, analyzed by real time PCR. Unpaired t-test with Welch's correction was used. **B**: Increased platelet $P2Y_{12}$ expression in diabetic GK rats compare to Wistar rats, analyzed by Western blot. Representative immunoblots of platelet $P2Y_{12}$ and GAPDH from 4 Wistar rats and 5 diabetic GK rats are presented. **C**: Increased platelet aggregation in response to multiple agonists in diabetic GK rats compared with healthy Wistar rats. Representative tracings (upper) and summary data are given; two-way ANOVA followed by Bonferroni post-tests were used. **D**: Similar expression of platelet $P2Y_1$, thromboxane A2 receptor (TP), protease-activated receptor-1 (PAR1) and protease-activated receptor-4 (PAR4) in diabetic GK rats platelets compared with healthy Wistar rats platelets, detected by real time PCR. **E**: Age-dependent increase of clot retraction in GK diabetic rats compared with Wistar rats. Representative results of 5 experiments (left) and summary data are presented; one-way ANOVA for repeated measures followed by Newman-Keuls test were used. **F**: Age-dependent increase of platelet $P2Y_{12}$ expression and fasting blood glucose in GK diabetic rats. Representative results of 5 experiments and summary data are presented. Dotted lines: combined baseline values of fasting blood glucose (4.3 ± 0.7 mM) and platelet $P2Y_{12}$ (0.09 ± 0.04) of Wistar rats (n = 3).

Figure 4. NFκB activation mediates increased platelet $P2Y_{12}$ expression in T2DM patients and diabetic GK rats. A: Schematic diagram showing the NFκB binding position in $P2Y_{12}$ promoter. **B:** Increased NFκB activation in platelets from T2DM patients. IκBα phosphorylation and degradation were increased in platelets from T2DM patients compared with healthy subjects. Unpaired t-test with Welch's correction was used for p-IκBα and p65 in platelets. **C**: Increased

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NFκB activation in platelets and megakaryocytes from diabetic GK rats. GAPDH was used as loading control. **D:** Increased p65 in nuclear extract of megakaryocytes from diabetic GK rats. H3 was used as loading control. **E:** Increased p65 binding in $P2Y_{12}$ promoter region of megakaryocytes from diabetic GK rats. p65 binding to $P2Y_{12}$ promoter was determined by ChIP. The right panel shows the amplification of $P2Y_{12}$ promoter region containing NFκB binding motif after ChIP from rat megakaryocytes; left panel demonstrates total DNA input. GAPDH was used as control to show precipitation specificity. Results shown are representative of at least 3 experiments using platelets and megakaryocytes from different rats.

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Figure 5. High glucose induces increased $P2Y_{12}$ expression in Meg-01 cells via activation of NFκB signal pathway. A: high glucose concentration-dependently increased $P2Y_{12}$ expression, IκBα phosphorylation and degradation in Meg-01 cells (upper panel) and p65 translocation into the nuclei (bottom panel). BAY 11-7082, a NFκB inhibitor, reversed the effects of high glucose. **B:** Antioxidant NAC and DTT reversed high glucose induced increase of $P2Y_{12}$ expression, NFκB activation in Meg-01 cells. **C:** High glucose concentration-dependently enhanced $P2Y_{12}$ expression in Meg-01 cells, which was further increased by PMA stimulation. **D:** High glucose enhanced NFκB binding to $P2Y_{12}$ promoter of Meg-01 cells as determined by ChIP. The right panel demonstrates amplification of the $P2Y_{12}$ promoter region containing the NFκB binding motif in Meg-01 cells; left panel demonstrates total DNA input. GAPDH was used as a control to show precipitation specificity. Meg-01 cells were cultured with different concentrations of glucose in the presence or absence of NFκB inhibitor, antioxidants or PMA as indicated for 24 hours before assay, mannitol was used to adjust the osmotic pressure. Results shown are representative of at least 3 separate experiments run on different days. **E**: NFκB inhibitor BAY

11-7082 abolished hyperglycemia-induced $P2Y_{12}$ upregulation in Meg-01 cells analyzed by real time PCR. Data are expressed as mean ± SEM, n =4.

Figure 6. Superior antiplatelet effect of AR-C78511 over cangrelor on platelets from T2DM patients and diabetic rats. **A:** Cangrelor and AR-C78511 similarly inhibited platelet aggregation of healthy subjects (left panel) while AR-C78511 exhibited superior antiplatelet activity over cangrelor in platelets from T2DM patients (right panel). Washed platelets were used. One-way ANOVA for repeated measures followed by Newman-Keuls test were used. **B:** Increased $P2Y_{12}$ expression on platelets dampened the antiplatelet activity of both AR-C78511 and cangrelor with AR-C78511 less influenced, especially at high $P2Y_{12}$ expression level. Platelet aggregation ratio in the presence of cangrelor or AR-C78511 (data is from Figure 6A) was plotted against $P2Y_{12}$ receptor expression level (data is from Figure 1B). Different slopes of regression lines indicate that platelet $P2Y_{12}$ expression level influences the antiplatelet activities of cangrelor and AR-C78511. **C:** AR-C78511, not cangrelor, reversed the reduced intracellular cAMP in platelets of T2DM patients. cAMP in platelets from T2DM patients was significantly lower than healthy platelets, which was reversed by AR-C78511 rather than cangrelor. Data from 12 healthy subjects and 20 patients with T2DM are presented. **D:** Superior antiplatelet activity of AR-C78511 over cangrelor on diabetic rat platelets. Typical tracings and corresponding summary analysis are provided. **E:** Wistar and diabetic GK rat platelets responded similarly to AR-C78511 (right panel) while the response of diabetic GK rat platelets to cangrelor was impaired. **F:** Reduced cAMP in resting platelets of diabetic GK rats was reversed by AR-C78511. Compared to Wistar rats, cAMP in resting platelets of diabetic GK rats was dramatically lower, which was reversed by AR-C78511, but not by cangrelor (left panel). Both

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cangrelor and AR-C78511 completely antagonized ADP-induced cAMP decrease in platelets of both Wistar rats and diabetic GK rats, while AR-C78511 further raised cAMP level in platelets of diabetic GK rats to that of resting platelets of Wistar rats (right panel). Data are expressed as mean ± SEM, n = 5 – 6. For panel B, D and E, linear regressions were performed and the difference between two lines was determined by the method of "Test whether the slopes and intercepts are significantly different" using Prism.

Figure 7. Increased thrombosis in diabetic GK rats and the superior antithrombotic effects of inverse agonist AR-C78511 over cangrelor. A: Intravital microscopy of $FeCl_3$-induced thrombosis in mesenteric arterioles of Wistar or diabetic GK rats. Rats were intravenously given 60 μg/kg cangrelor, AR-C78511, followed by continuous infusion 6 μg/kg/min or same volume of normal saline 5 minutes before $FeCl_3$ injury. Typical thrombus formation at 5 and 10 min after $FeCl_3$ injury (A1) and statistical analysis of thrombus size at 10 minutes (A2) were given. Dotted lines in A1 indicate vessel walls. **B**: Thrombus weight in rat A-V shunt model. Rats were intravenously given 60 μg/kg cangrelor and AR-C78511 or same volume of normal saline, followed by 6 μg/kg/min continuous infusion during the experiment. Thrombus formation was induced and measured as described under Materials and Methods. Of note, thrombus formation of 3 weeks old diabetic GK rats did not increase compared with 6 weeks old diabetic GK rats.

Figure 8. Working model for $P2Y_{12}$ upregulation in diabetes and the superior antiplatelet activity of $P2Y_{12}$ inverse agonist. Hyperglycemia activates the NFκB signaling pathway, which upregulates $P2Y_{12}$ expression on platelets, and increases platelet activity and thrombosis in diabetes. High expression of $P2Y_{12}$ confers the constitutive activation, which is inhibited by the

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$P2Y_{12}$ inverse agonist AR-C78511, but not by the neutral antagonist cangrelor. The inverse agonist activity of AR-C78511 confers its superior antiplatelet and antithrombotic activity in type 2 diabetes mellitus.

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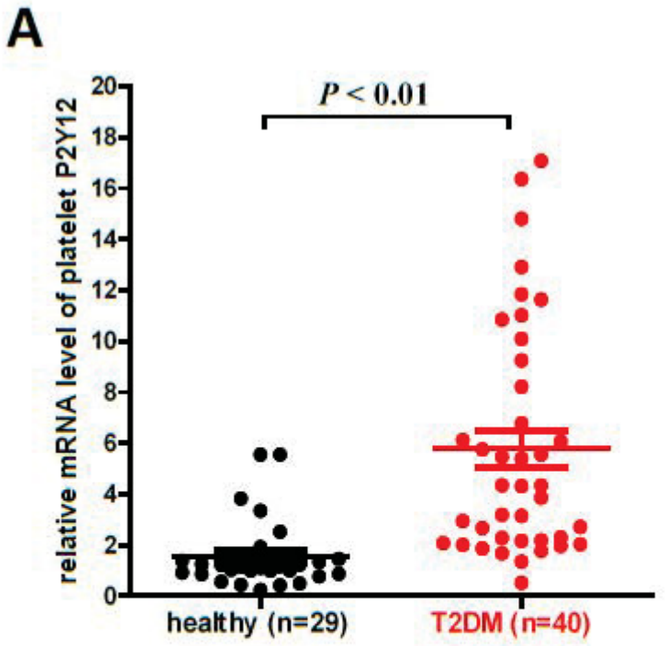

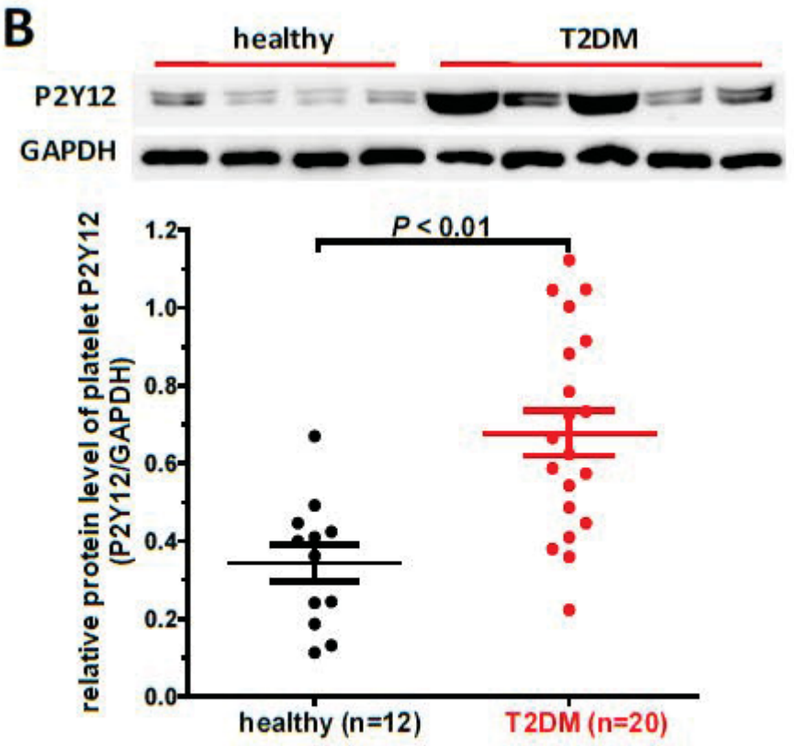

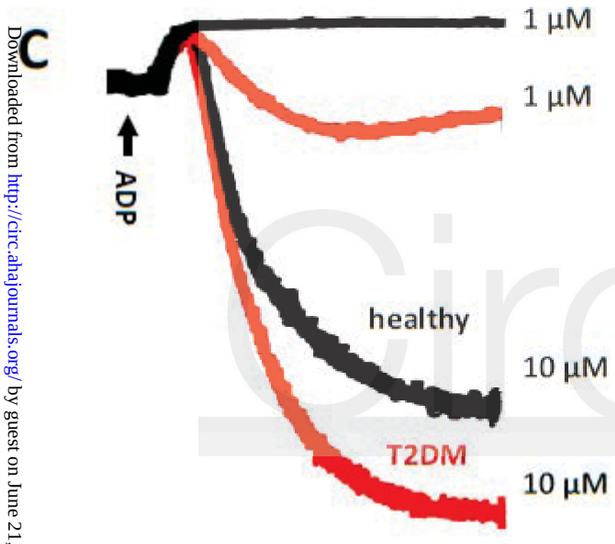

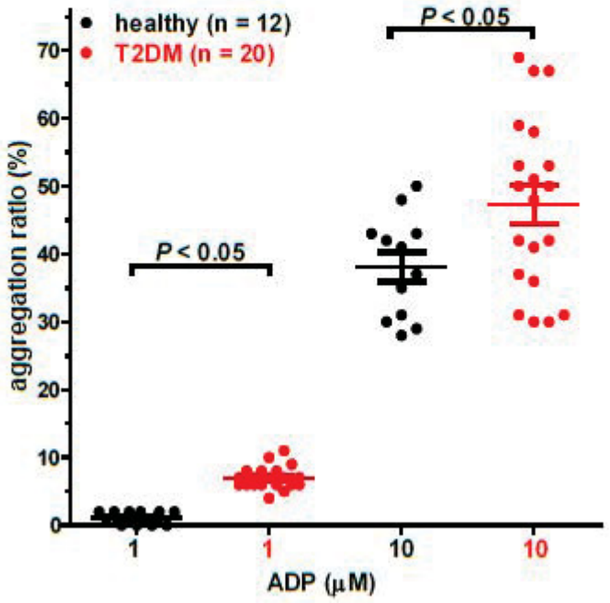

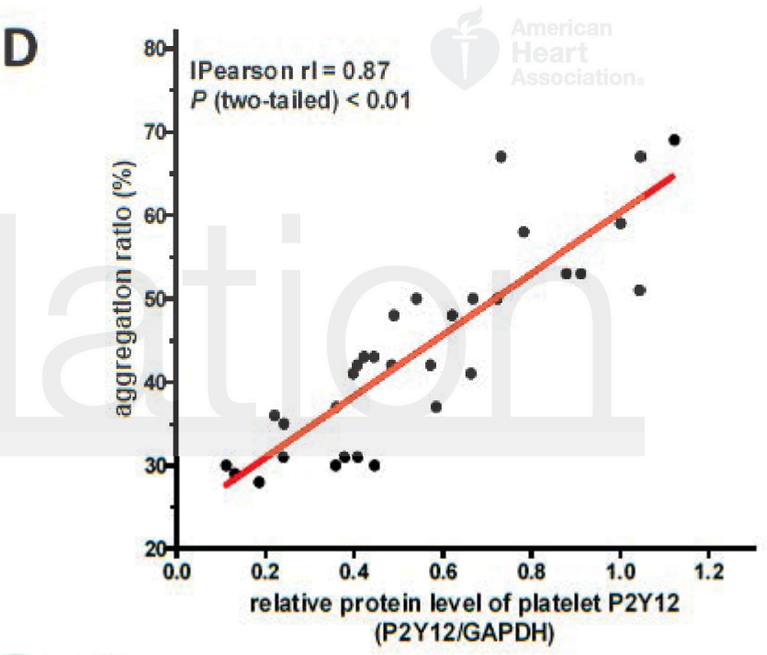

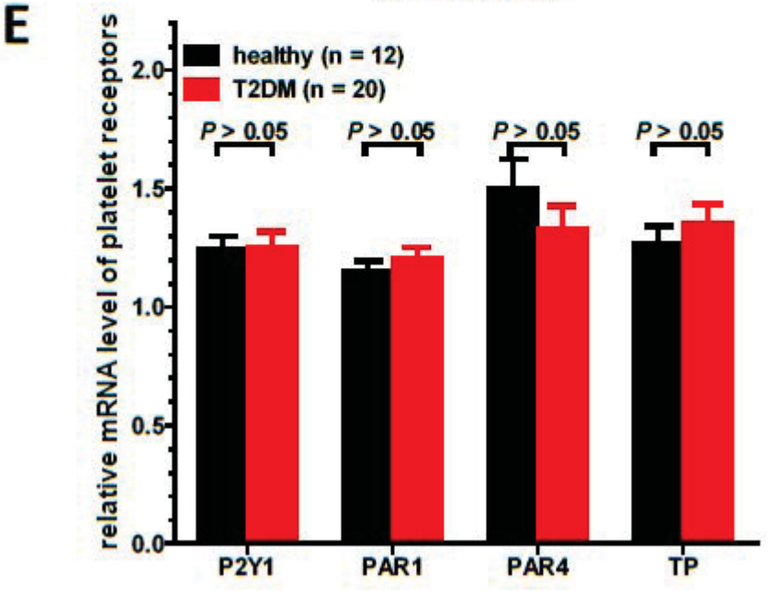

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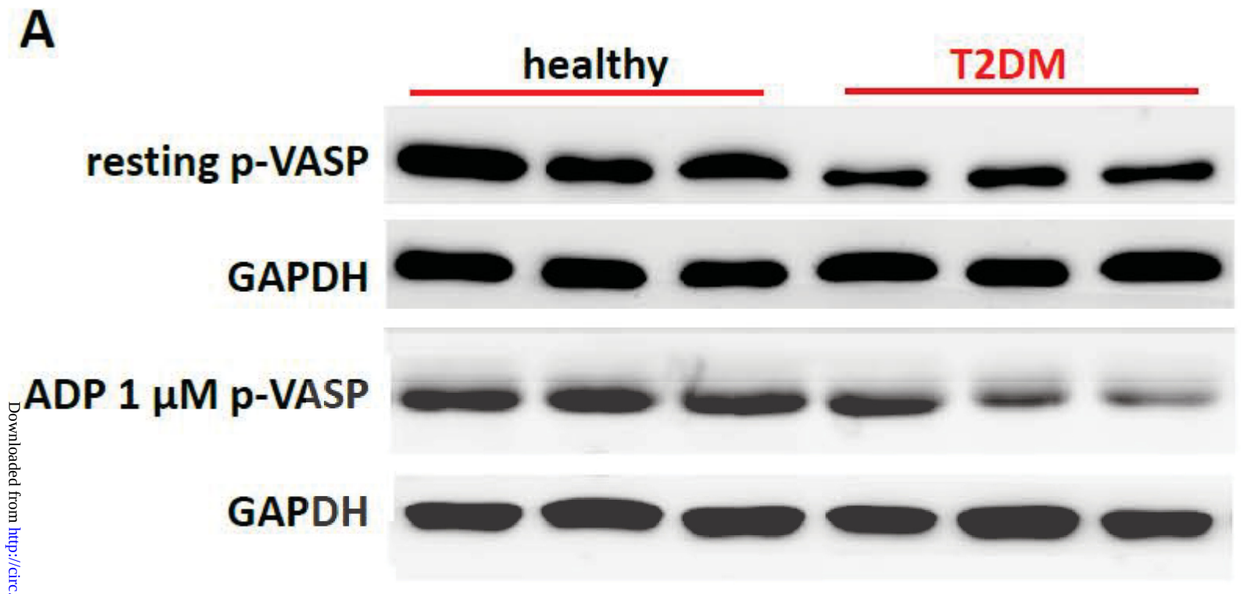

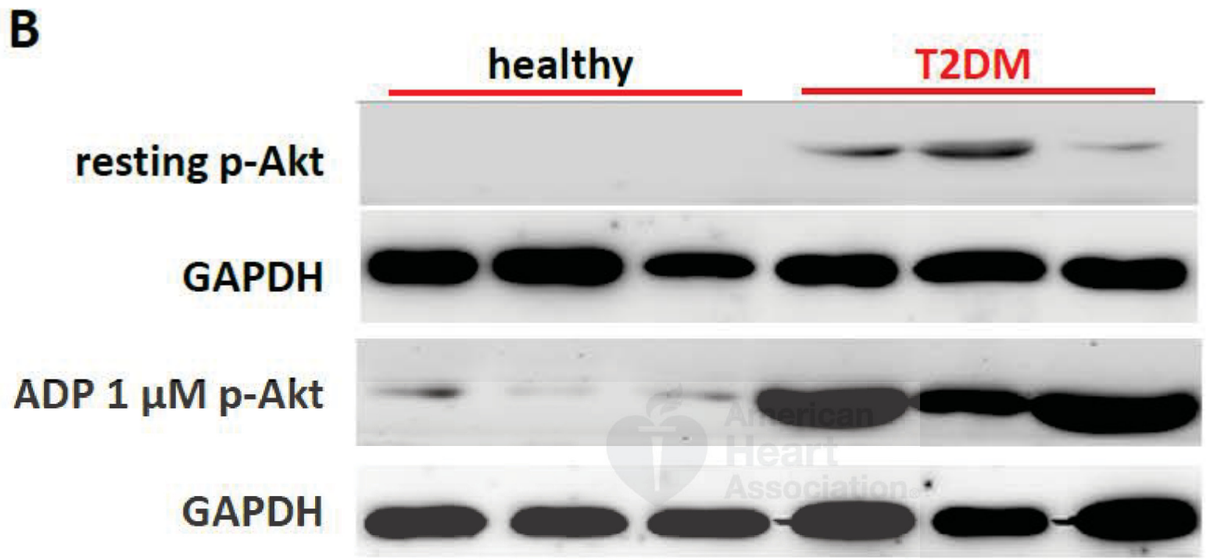

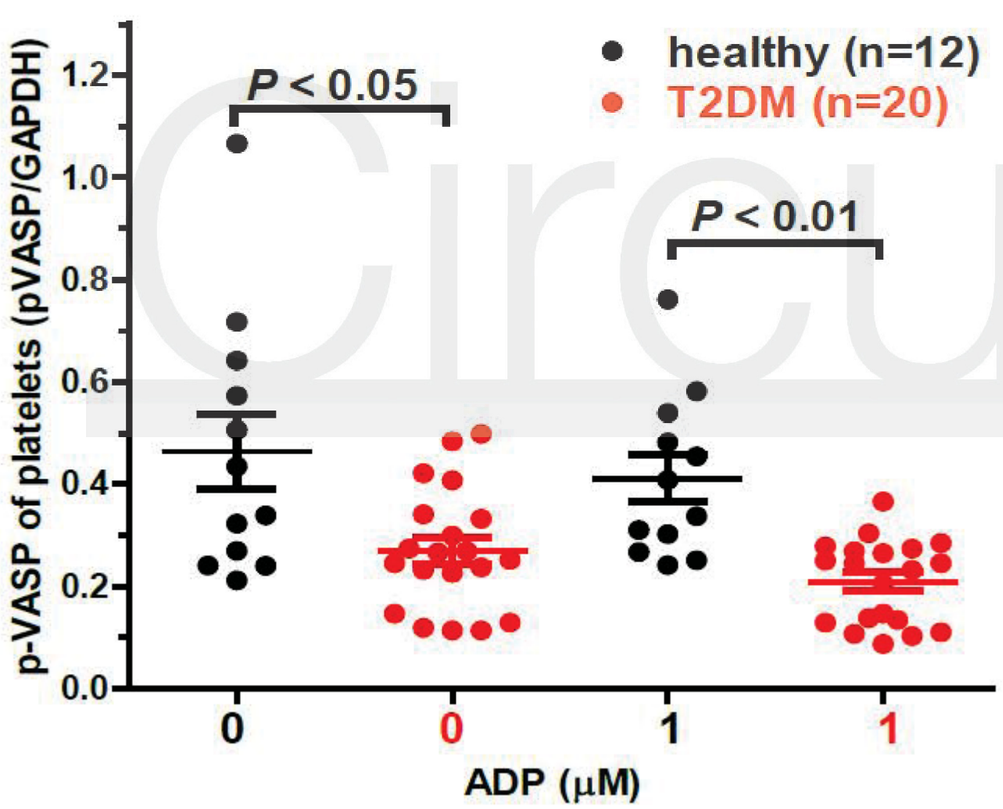

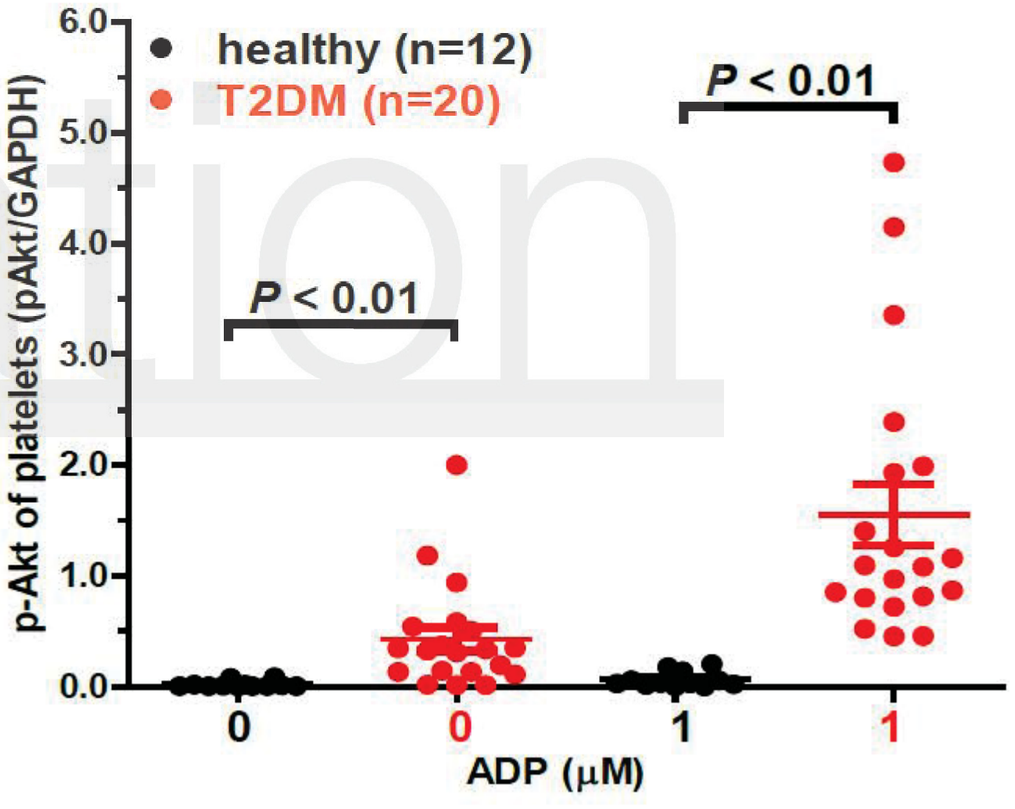

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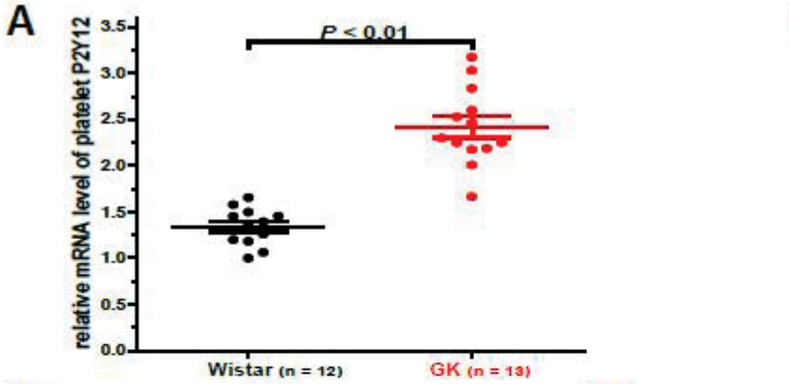

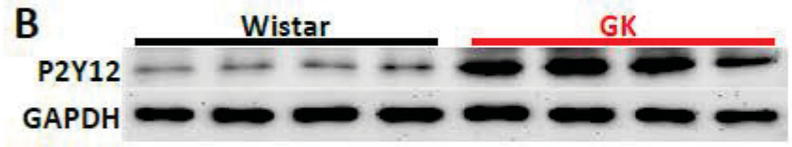

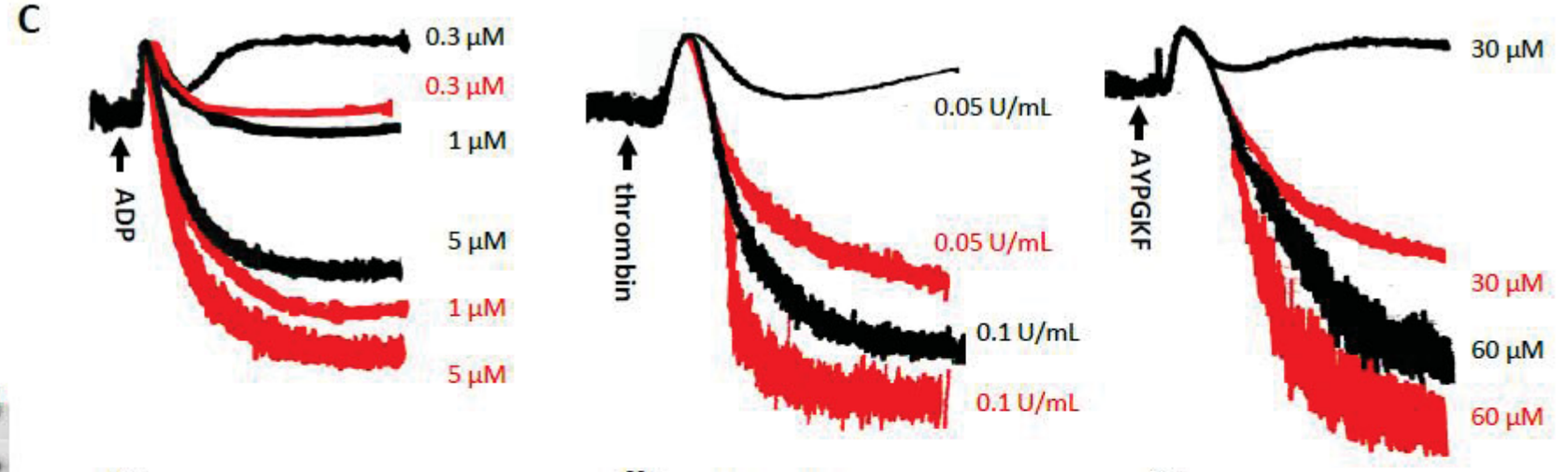

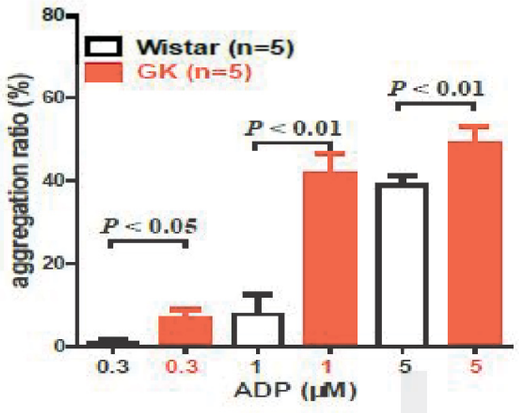

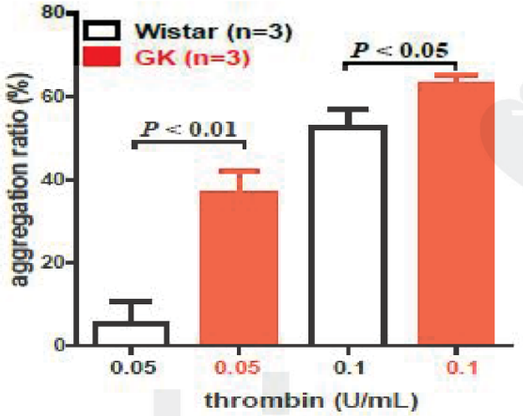

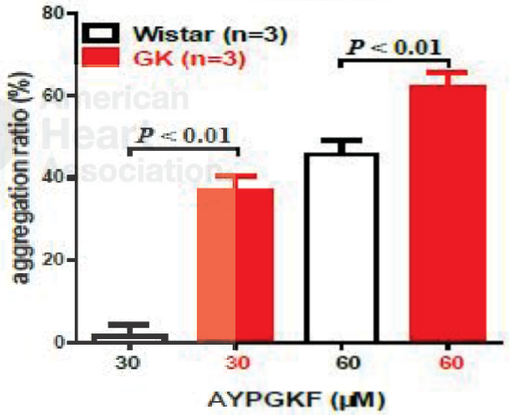

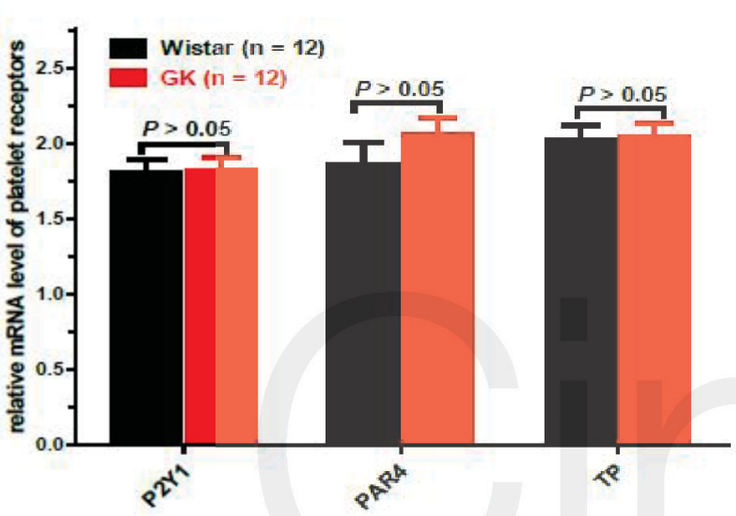

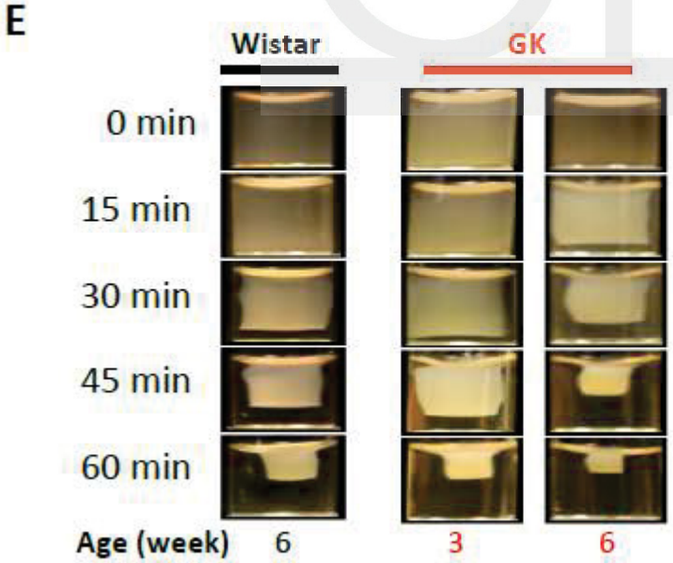

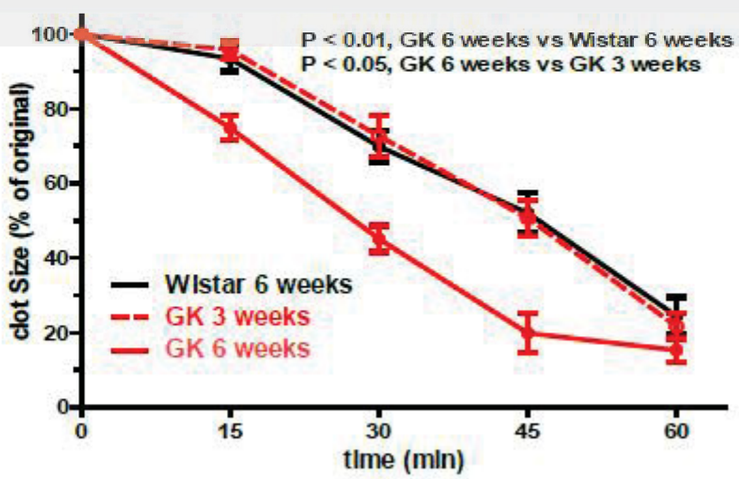

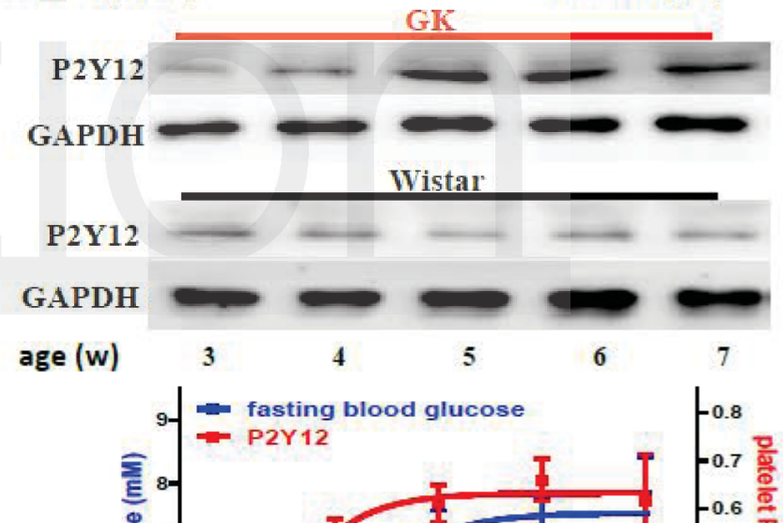

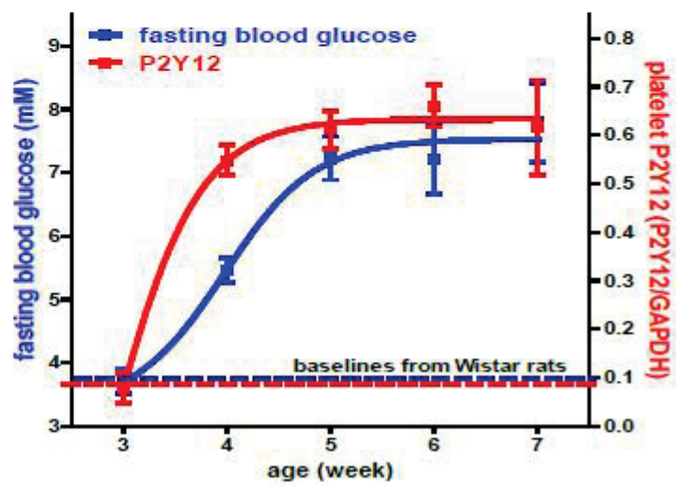

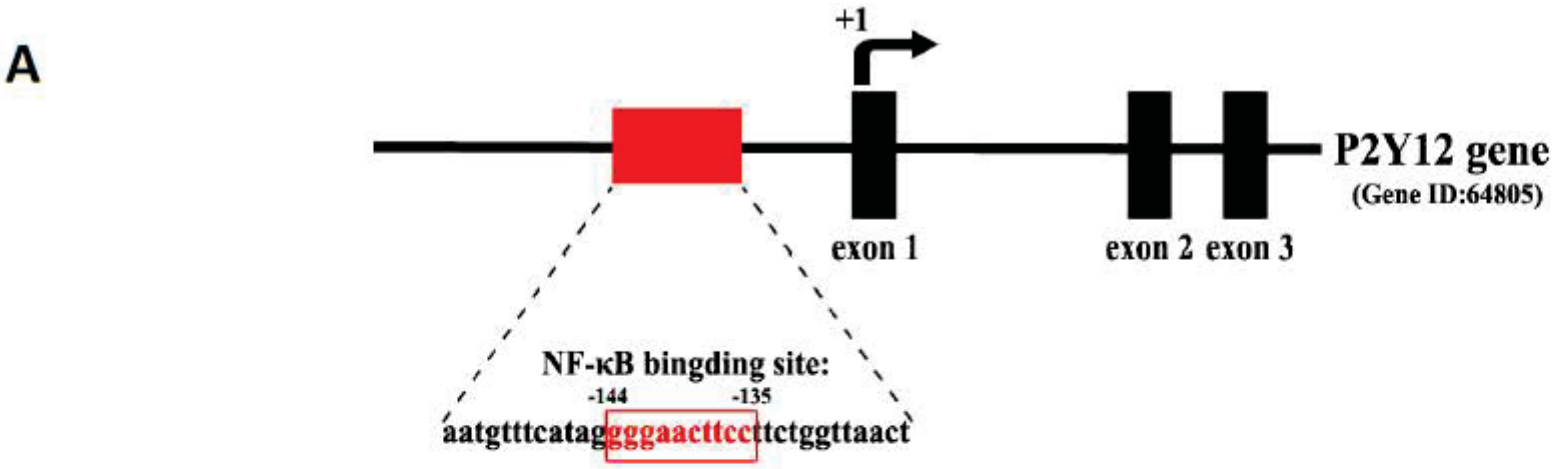

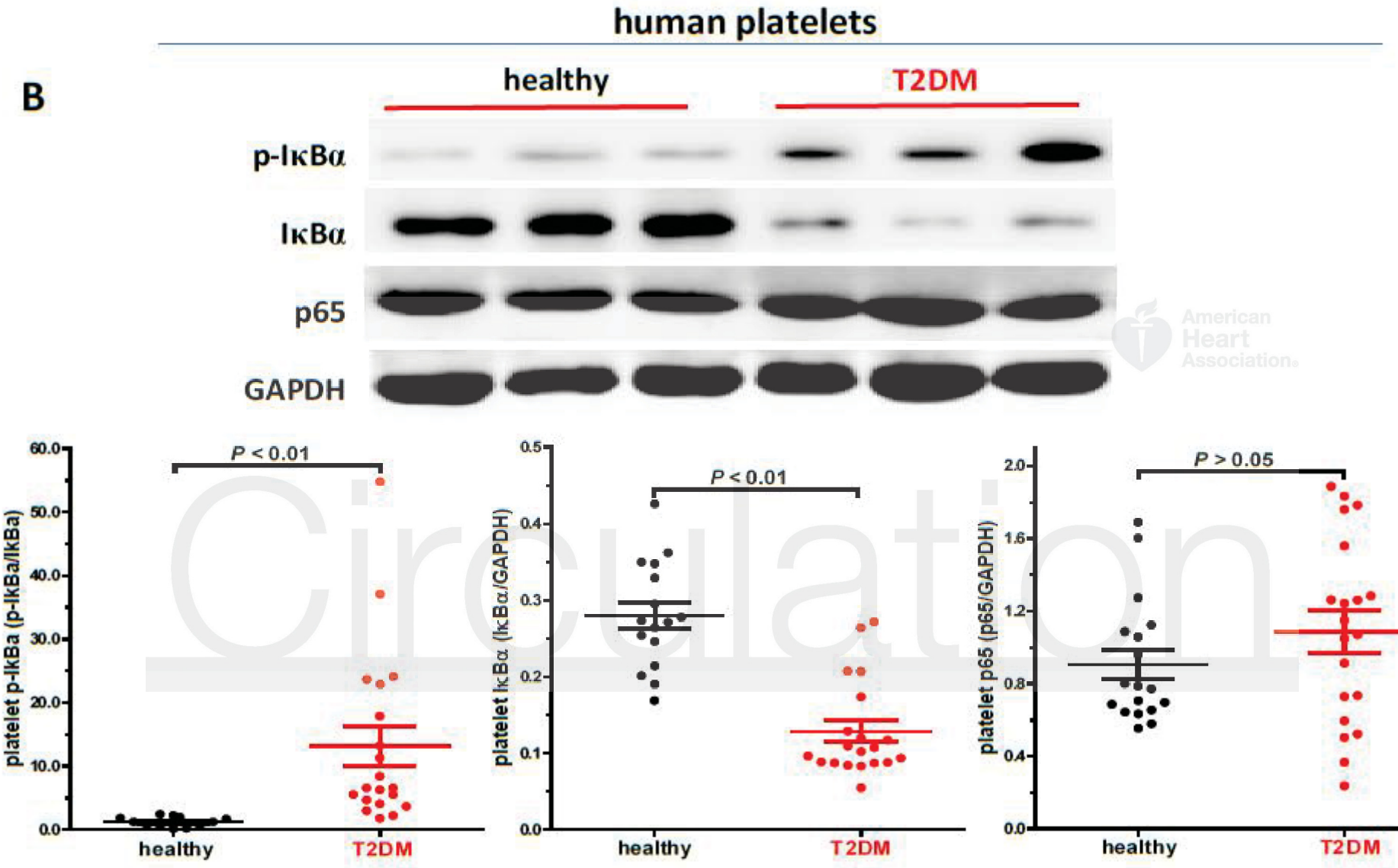

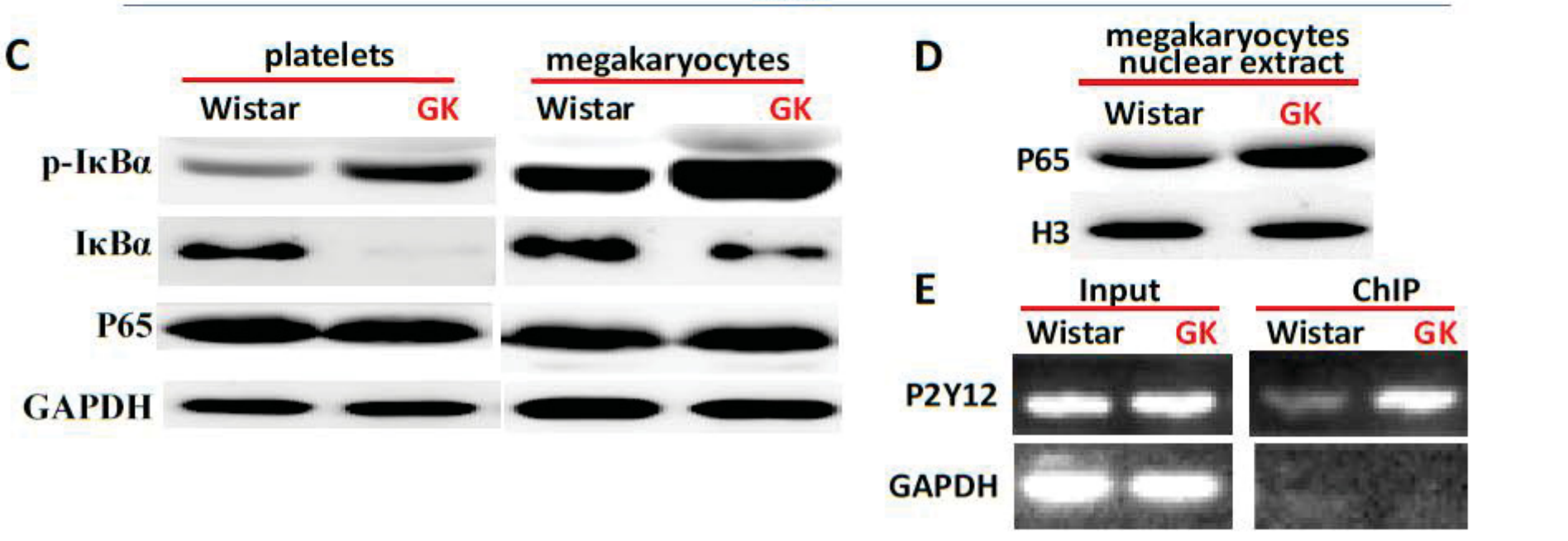

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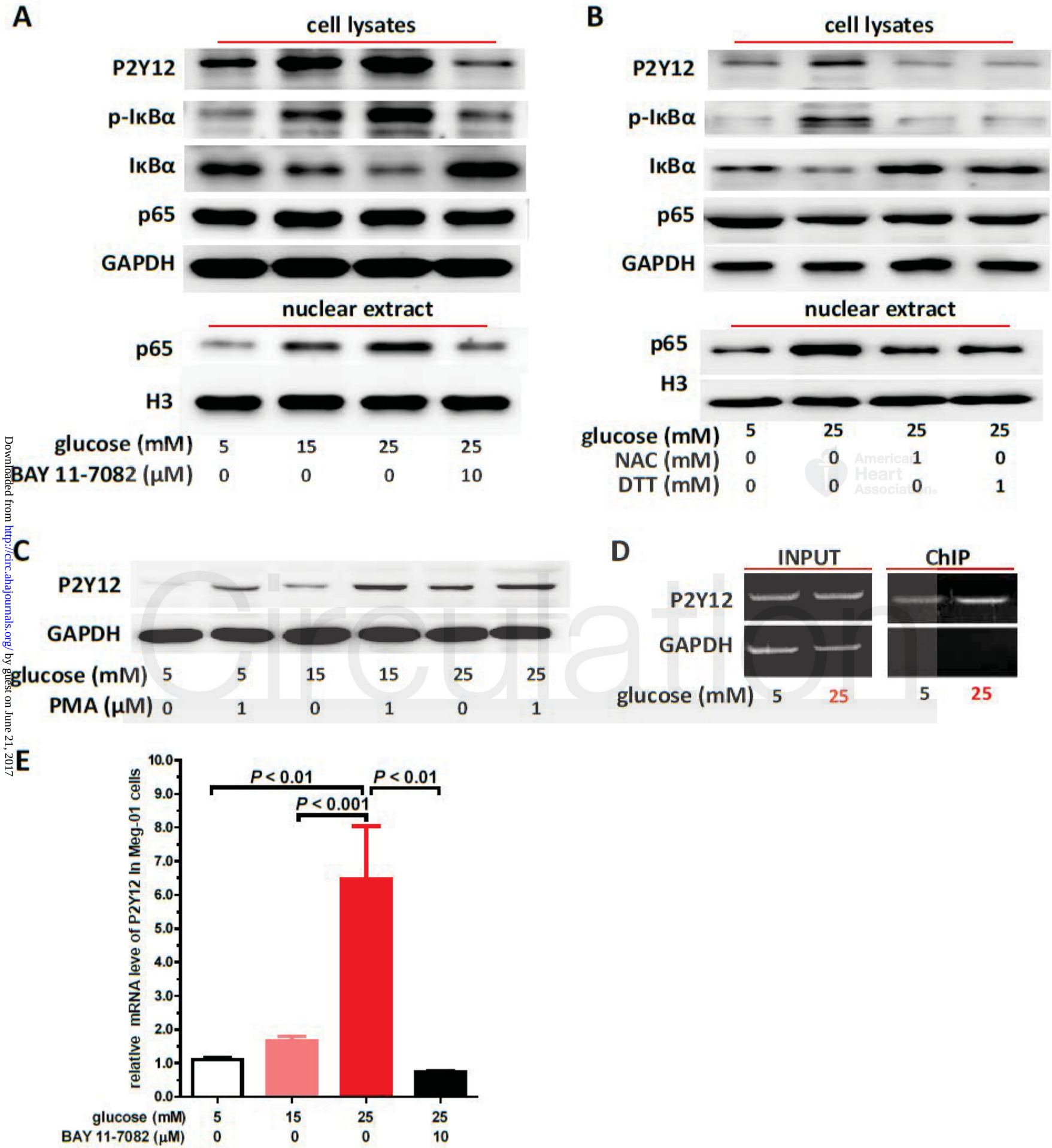

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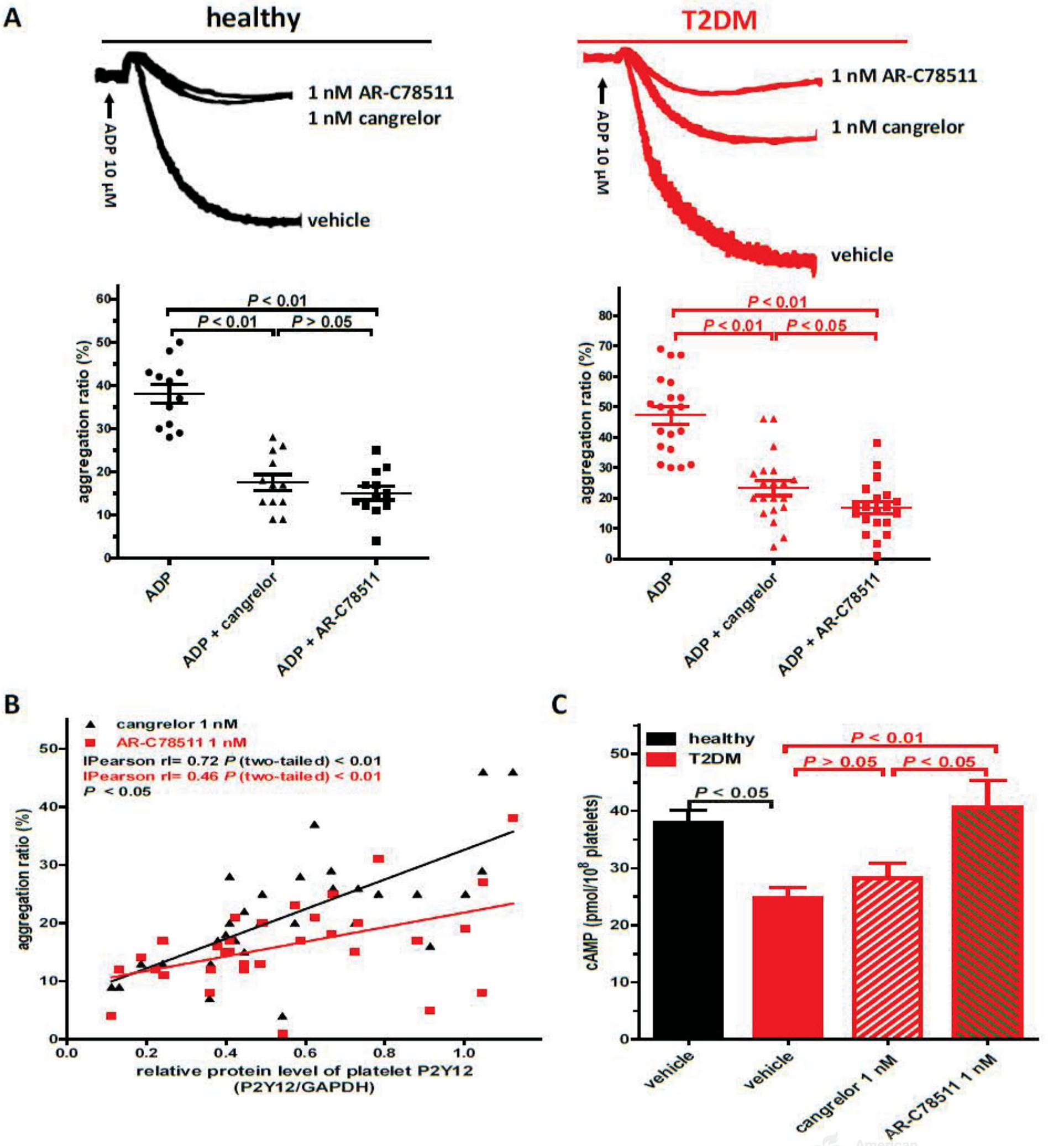
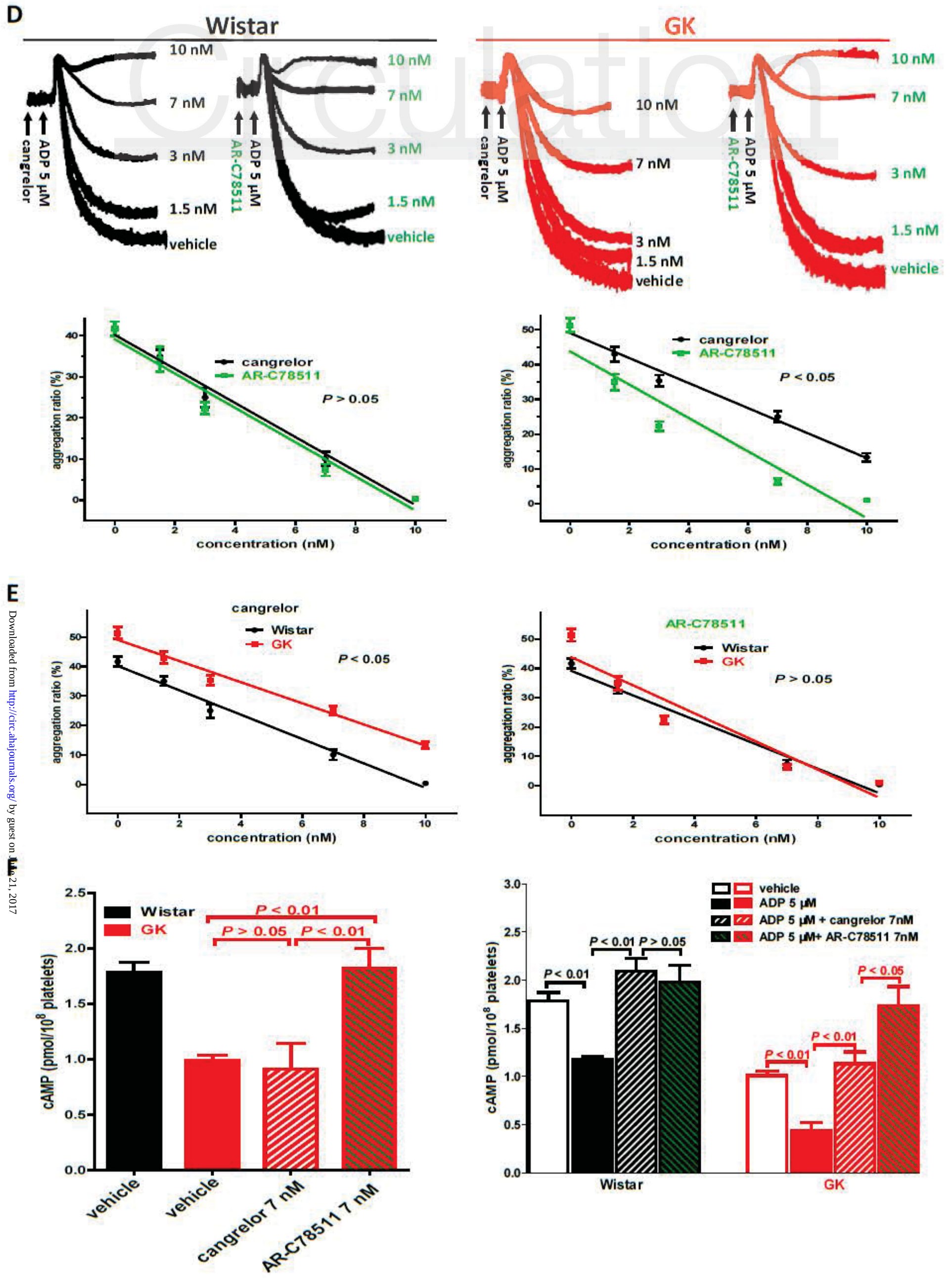
human platelets



rat platelets



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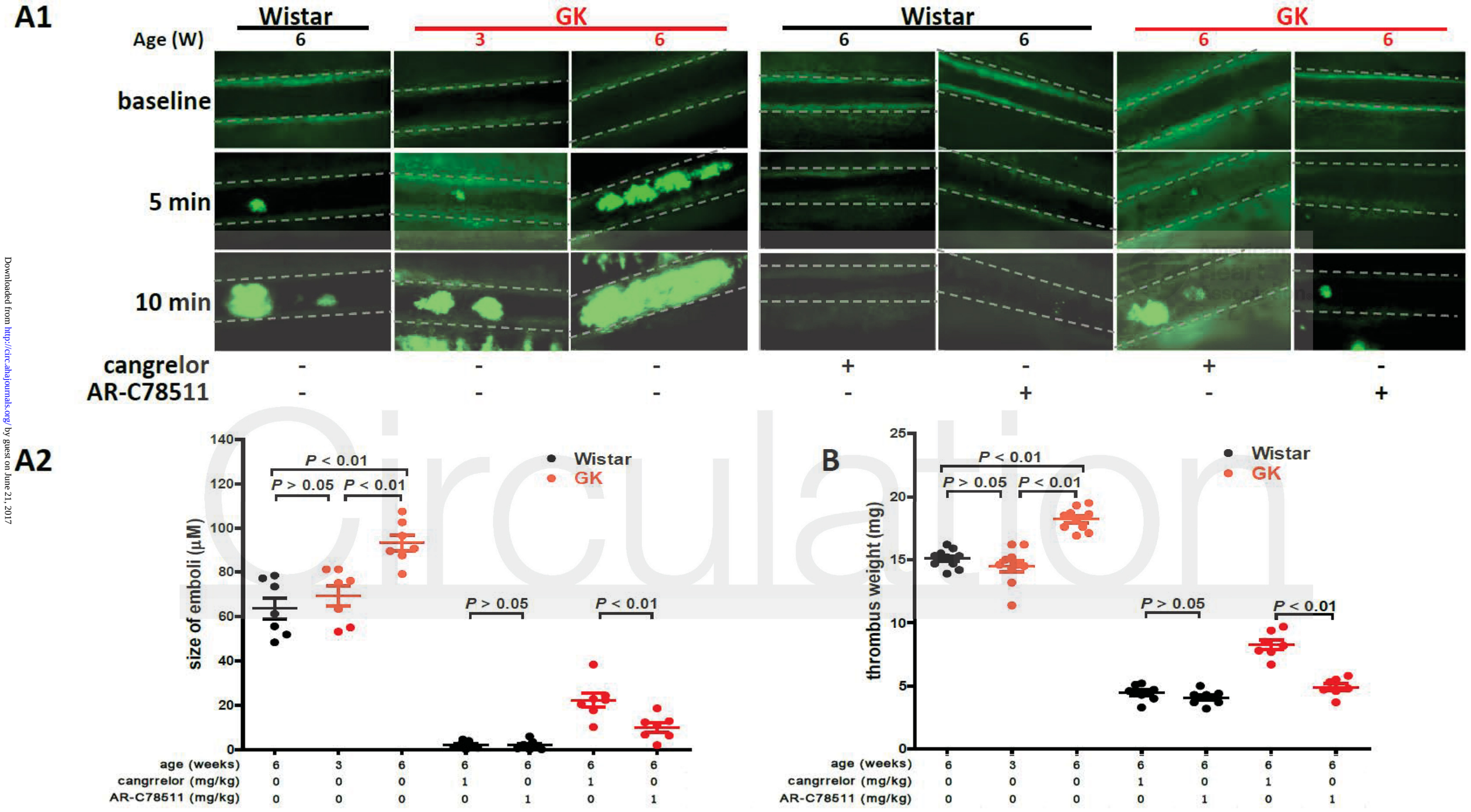

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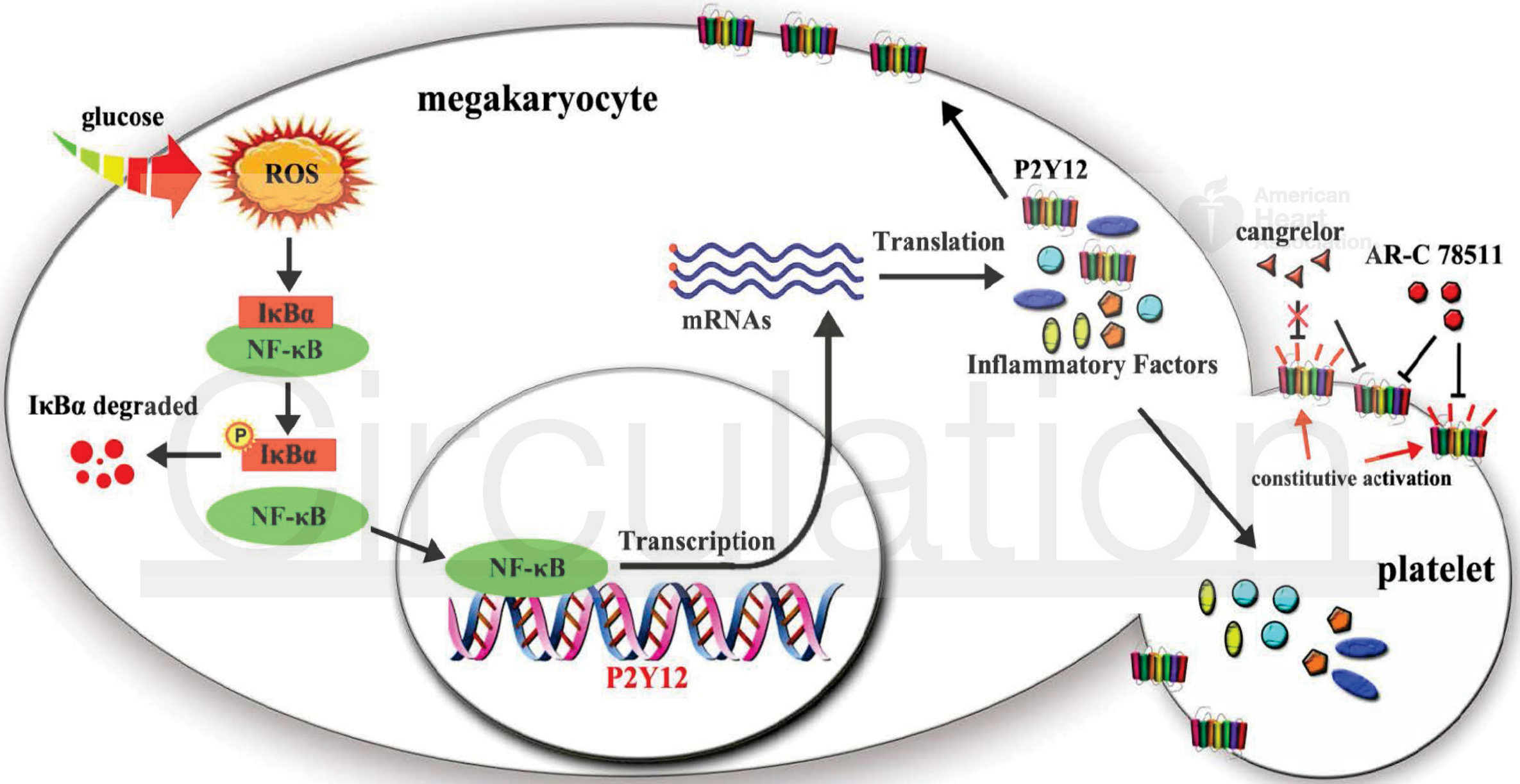

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Platelets Express Activated P2Y12 Receptor in Patients with Diabetes

Liang Hu, Lin Chang, Yan Zhang, Lili Zhai, Shenghui Zhang, Zhiyong Qi, Hongmei Yan, Yan Yan, Xinping Luo, Si Zhang, Yiping Wang, Satya P. Kunapuli, Hongying Ye and Zhongren Ding

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SUPPLEMENTAL MATERIAL

Expanded Materials and Methods

Materials

ADP, fibrinogen, thrombin, and collagen were purchased from Chrono-Log (Havertown, PA). FITC-labeled phalloidin, apyrase, PGE1, Phorbol 12-myristate 13-acetate, and calcein acetoxymethyl ester were from Sigma-Aldrich (St Louis, MO). Cangrelor (AR-C69931MX) was from Biochempartner (Shanghai, China). AR-C78511 was synthesized by Institute of Materia Medica, Beijing University of Chemical Technology (Beijing, China). The anti-$P2Y_{12}$ antibody was from Alomone Labs (Jerusalem, Israel). The anti-p65, anti-phospho-IκB and anti-GAPDH antibodies were from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA). The anti-phospho-Akt and anti-phospho-VASP were from Cell Signaling Technology (Beverly, MA). The anti-IκB and anti-histone H3 antibodies were from Proteintech (Proteintech Group, Chicago, IL).

Platelet isolation and platelet suspension preparation

Human blood was drawn from the antecubital vein and mixed with ACD (85 mM sodium citrate, 71.38 mM citric acid, and 27.78 mM glucose) solution (6:1 vol/vol). Platelet-rich plasma prepared as previously described[1, 2] was filtered through a Sepharose 2B column (Sigma-Aldrich, Saint Louis, MO) equilibrated in Tyrode's solution (pH 7.35) to isolate platelets[3, 4]. Platelets can also be separated by centrifuging platelet-rich plasma at 900 g for 10 min and resuspending platelet pellets in Tyrode buffer[2].

Rat blood was drawn from the abdominal aorta using a syringe containing ACD as anticoagulant. Platelet-rich plasma and platelet suspension in Tyrode buffer was prepared similarly as described above.

Platelet functional studies

Platelet aggregation in response to agonists with/without antagonists was measured using a lumi-aggregometer (Model 400VS; Chrono-Log) under stirring conditions (900 rpm) at 37℃ as described previously[2, 4]. In some experiments, platelet dense granule secretion was simultaneously monitored by measuring ATP release using CHRONO-LUME reagent (Chrono-Log)[4]. The aggregation tracings and ATP release tracings were recorded using a recorder (Model 707; Chrono-Log) connected to the aggregometer. Aggregation amplitude

can be measured from the aggregation tracings, calculated as aggregation ratio and used to reflect platelet aggregation extent.

For clot retraction, rat platelets were processed as previously described [4]. Briefly, 2 mg/mL fibrinogen was added to rat platelets suspended in Tyrode buffer and dispensed in 0.3 mL aliquots into cuvettes. Clot retraction was induced by stimulation with thrombin (1.0 U/mL) at 37°C and monitored by taking photographs at indicated time points using a digital camera. Clot surface area was quantified using NIH Image J software. Results were expressed as percentage of retraction (% = $area_t$ / $area_{t0}$ × 100%).

Cell culture

Human megakaryocytic cell line (Meg-01) was cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 20 mM L-glutamine for at least 5 passages allowing for adaptation prior to use. Cells were treated in medium with different concentration of glucose (5, 15 and 25 mM) for 24 hours, and mannitol was added to maintain equivalent osmotic pressure. For experiments with pharmacological inhibition of intracellular reactive oxygen species (ROS) or p-IκB, 1 mM *N*-acetyl-*L*-cysteine (NAC), 1 mM DL-dithiothreitol (DTT) or 10 μM BAY 11-7082 was added. At the end of the treatment, cells were collected and processed for RNA and protein extraction.

In vitro ubiquitin-conjugation assay

Immunoprecipitation assays were performed as previously reported with minor modification[5, 6]. Briefly, cytoplasmic extracts prepared from megakaryocytes of diabetic GK rats were incubated with 1.0 μg anti-IκBα antibody at 4°C on a rotating device overnight, followed by incubation with 20 μl Protein A/G PLUS-Agarose at 4°C for 3 hours; pellets were collected by centrifugation and washed with RIPA buffer for 5 times. Boiled samples were analyzed by immunoblotting with anti-ubiquitin antibody.

Measurement of intracellular ROS generation

Intracellular ROS generation in platelets from diabetic GK rats and megakaryocytic Meg-01 cells induced by high glucose were measured using fluorescent probe 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) as described with modification[7]. Briefly, the platelets and Meg-01 cells were resuspended in PBS and incubated with 10 μM DCFH-D probe (Sigma) for 15 min at 37 °C. Cells were washed once with PBS and aliquoted into a 96-well plate (10^5 cells in 100 μL in each well) for ROS detection. Then intracellular ROS were detected by

immunofluorescence microscope. Images were acquired with the Olympus IX73 microscope equipped with a Olympus DP80 camera and Cell Sense software. Intracellular ROS was also measured by fluorescence microplate reader (SpectraMax M5, Molecular Devices) with excitation and emission wavelengths set at 488 and 525 nm, respectively. The ROS accumulation was expressed as percentage of control (as 100 %).

Determination of plasma concentration of AR-C78511

Sample preparation

Blood samples (0.25 mL) were collected through the suborbital vein from rats into heparinized tubes at 0, 2, 5, 10, 20, 35 and 50 min after a bolus intravenous injection of AR-C78511 (60 μg/kg) followed by 20 min of continuous infusion (6 μg/kg/min), and then immediately centrifuged at 4000 rpm for 10 min. Harvested plasma samples were stored at −80°C until analysis. Plasma 20 μL was precipitated by 100 μL I.S. working solution (verapamil 25 ng/mL in methanol: acetonitrile (50:50, v/v). These samples were mixed on a vortex mixer for 1 min, centrifuged for 5 min at 15000 rpm, and then the supernatant was mixed with isometrical water for 30 seconds before injection.

LC-MS/MS

The analyses were performed on an Acquity ultra performance liquid chromatography (UPLC) system (Waters Corporation, Milford, MA, USA) coupled to a Xevo TQ-S mass spectrometer (Waters Corporation, Milford, MA, USA). Chromatographic separation was performed using an Acquity UPLC BEH C18 (1.7 μm 2.1*50 mm) column supplied by Waters at a flow of 0.5 mL/min. Gradient elution were used with a mobile phase composed of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid).

The Xevo TQ-S mass spectrometer was equipped with an electrospray ionization probe and was operated in the positive ion mode. The ionspray voltage was kept at 3000 V at a temperature of 500 ◦C. The Desolvation gas flow was 1000 L/h. The cone voltages were 20 V. The mass transitions for quantitation were 582 → 295 for AR-C78511 with collision energy 22 V and 455 → 150 for verapamil (I.S.) with collision energy 34 V.

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Supplemental Table 1. Characteristics of study population

	healthy	T2DM
n	29	40
male	16	22
female	13	18
Age	53 ± 4.1	57 ± 6.3
HbA1c (%)	5.7 ± 0.6	8.8 ±1.2*
fasting plasma glucose (mmol/L)	4.9 ± 0.2	11.4 ± 0.9**

*$P < 0.05$ compared with the healthy, **$P < 0.01$ compared with healthy.

T2DM: type 2 diabetes mellitus

Supplemental Table 2. The primers used for real-time PCR

Target gene	Homo sapiens			Wistar rats		
	Primer 5’ → 3’	Accession No.	Product (bp)	Primer 5’ → 3’	Accession No.	Product (bp)
P2Y12	TCACCCAGGTCCTCTTCCC TGTTCCCAGTTTGGCATCAC	**NM_176876.2**	**200**	ATTGATAACCATTGACCGATAC GCAGTGACAGCAGGAACA	**NM_022800.1**	**125**
P2Y1	CTCTATGGCAGCATCTTGT CTGAGGTGGTGTCGTAAC	**NM_002563.4**	**220**	AGGTCAAGAAGAAGCAACAT CAGCAGATAGCAACAAGGA	**NM_012800.1**	**198**
PAR1	GCATAAGCTGCTGCATCGAC TTAACCTCCCAGCAGTCCCT	**NM_001992.4**	**221**			
PAR4	GGTGGTGTCCTCATAAGATAA TCCTTCTGCCTCAGTCTC	**NM_003950.3**	**221**	GCCTCACCACCATACTCT AGCACCAGAAGCACATTG	**NM_053808.1**	**343**
TP	CTGTCCTTCCTGCTGAAC CGCTCTGTCCACTTCCTA	**NM_001060.5**	**420**	TTCGGGCTCATGTTTGCTCT ACCATGATGCCTACGAGCTG	**NM_017054.1**	**167**
TNF- α	TCAACCTCCTCTCTGCCATC CCAAAGTAGACCTGCCCAGA	**NM_000594.3**	**187**	GCCACCACGCTCTTCTGTC GCTACGGGCTTGTCACTCG	**NM_012675.3**	**149**
IL-1β	AGCTACGAATCTCCGACCAC CGTTATCCCATGTGTCGAAGAA	**NM_000576.2**	**186**	GAGGCTGACAGACCCCAAAAGAT GCACGAGGCATTTTTGTTGTTCA	**NM_031512.2**	**337**
IL-8	TGGCAGCCTTCCTGATTT CTTCTCCACAACCCTCTG	**NM_000584.3**	**245**	GAAGATAGATTGCACCGA CATAGCCTCTCACACATTC	**NM_030845.1**	**365**
ICAM-1	GCAAGAAGATAGCCAACCA TGCCAGTTCCACCCGTTC	**NM_000201.2**	**106**	GGGATGGTGAAGTCTGTCAA GGCGGTAATAGGTGTAAATGG	**NM_012967.1**	**199**
VCAM-1	GTTGAAGGATGCGGGAGT GCAGGTATTATTAAGGAGG	**NM_001078.3**	**159**	GCTATGAGGATGGAAGATTCT GCGTCAGTGTGGATGTAG	**NM_012889.1**	**271**
IL-6	GGTACATCCTCGACGGCATC CCAGTGCCTCTTTGCTGCTT	**NM_000600.4**	**84**	CGGAGAGGAGACTTCACA GCATCATCGCTGTTCATAC	**NM_012589.2**	**155**
GAPDH	GTCCACTGGCGTCTTCACCA GTGGCAGTGATGGCATGGAC	**NM_001289746.1**	**261**	CGGCAAGTTCAACGGCACAG AGACGCCAGTAGACTCCACGAC	**NM_017008.4**	**146**

Supplemental Table 3. The primers used for ChIP

Target gene	Homo sapiens			Wistar rats		
	Primer 5’ → 3’	Accession No.	Product (bp)	Primer 5’ → 3’	Accession No.	Product (bp)
P2Y12	GATCGCTTGTCTCCTAGCTCTT TTGTTGTGTAACAACAGTGCCT	NC_000003.12	258	GACTCAGCCTCATGTGGGAA GCAGCAAGCTGAAACGTGAC	NC_005101.4	172
GAPDH	ATGGTTGCCACTGGGGAT TGCCAAAGCCTAGGGGAAGA	NC_000012.12	174	CGTAGCTCAGGCCTCTGCGCCCTT CTGGCACTGCACAAGAAGATGCGGCTG	NC_005103.4	176

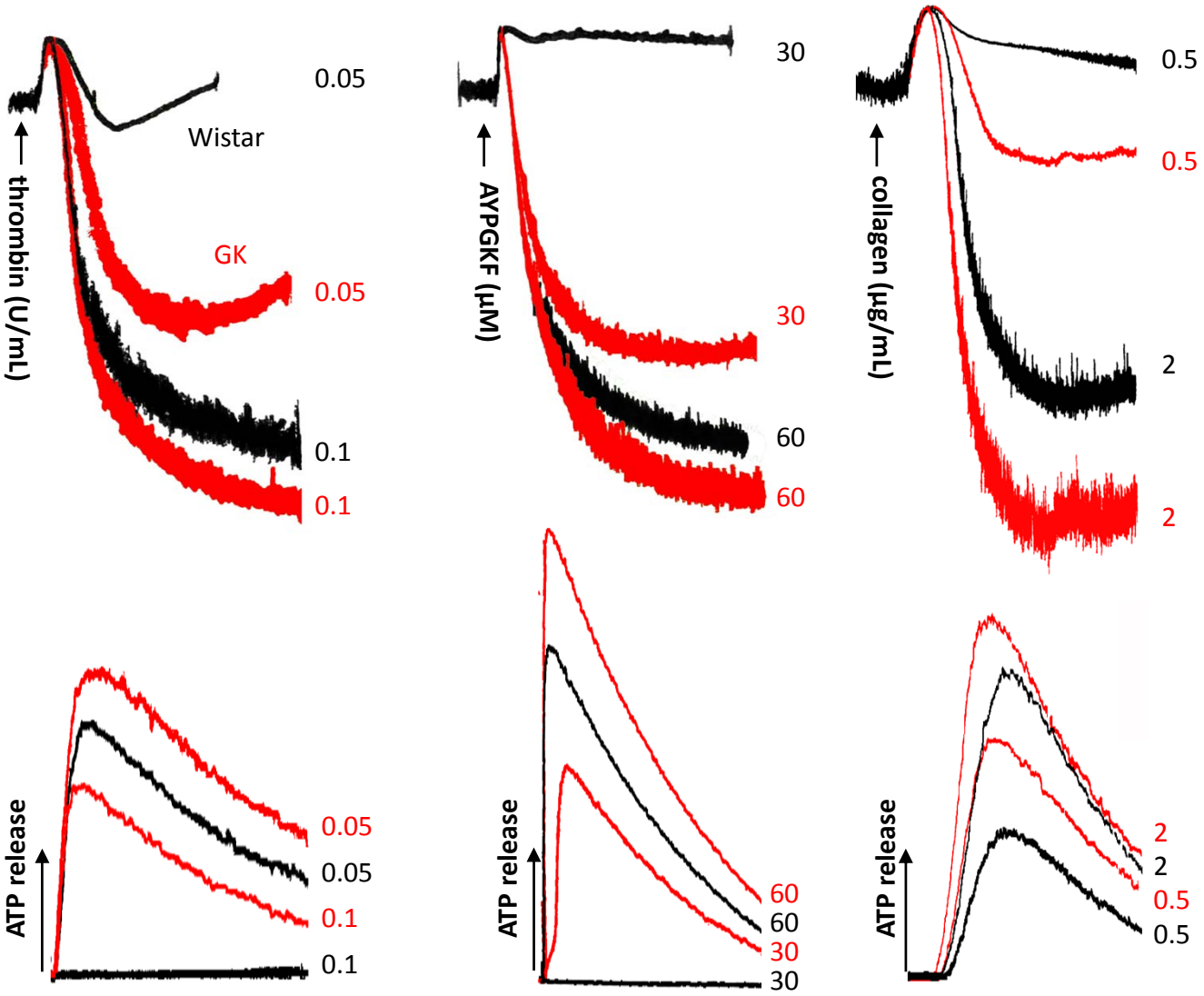
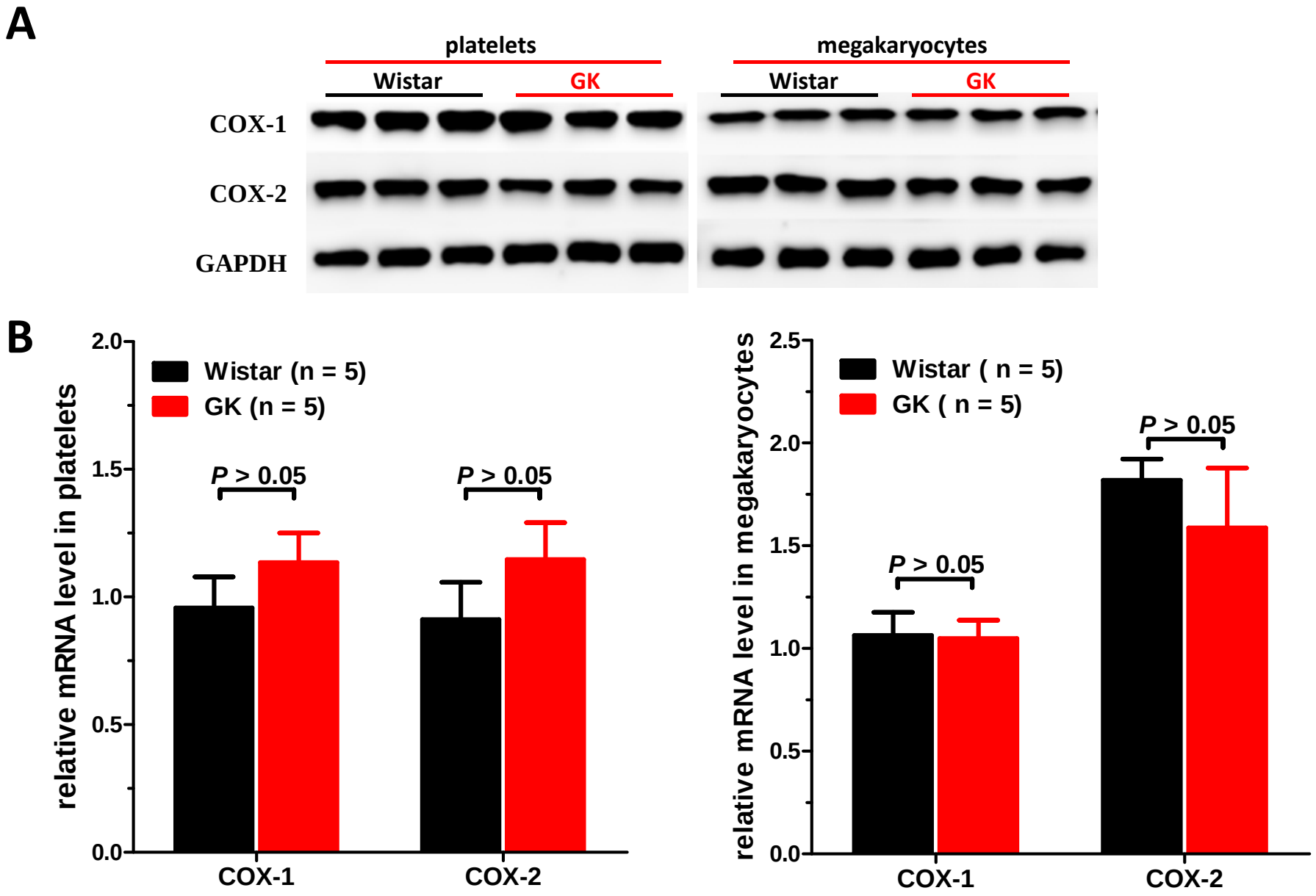
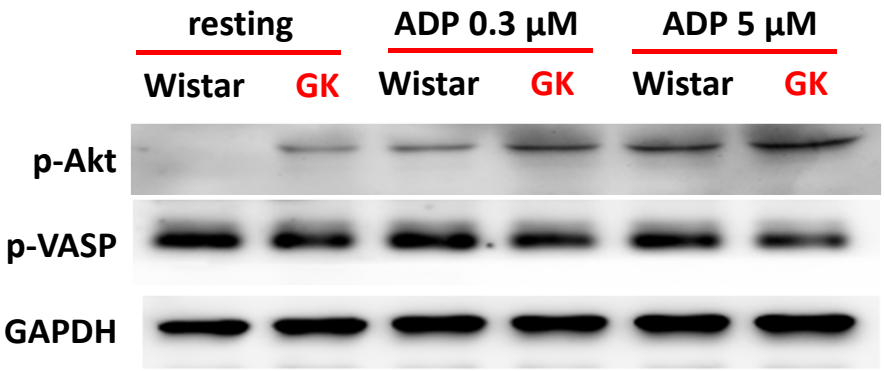


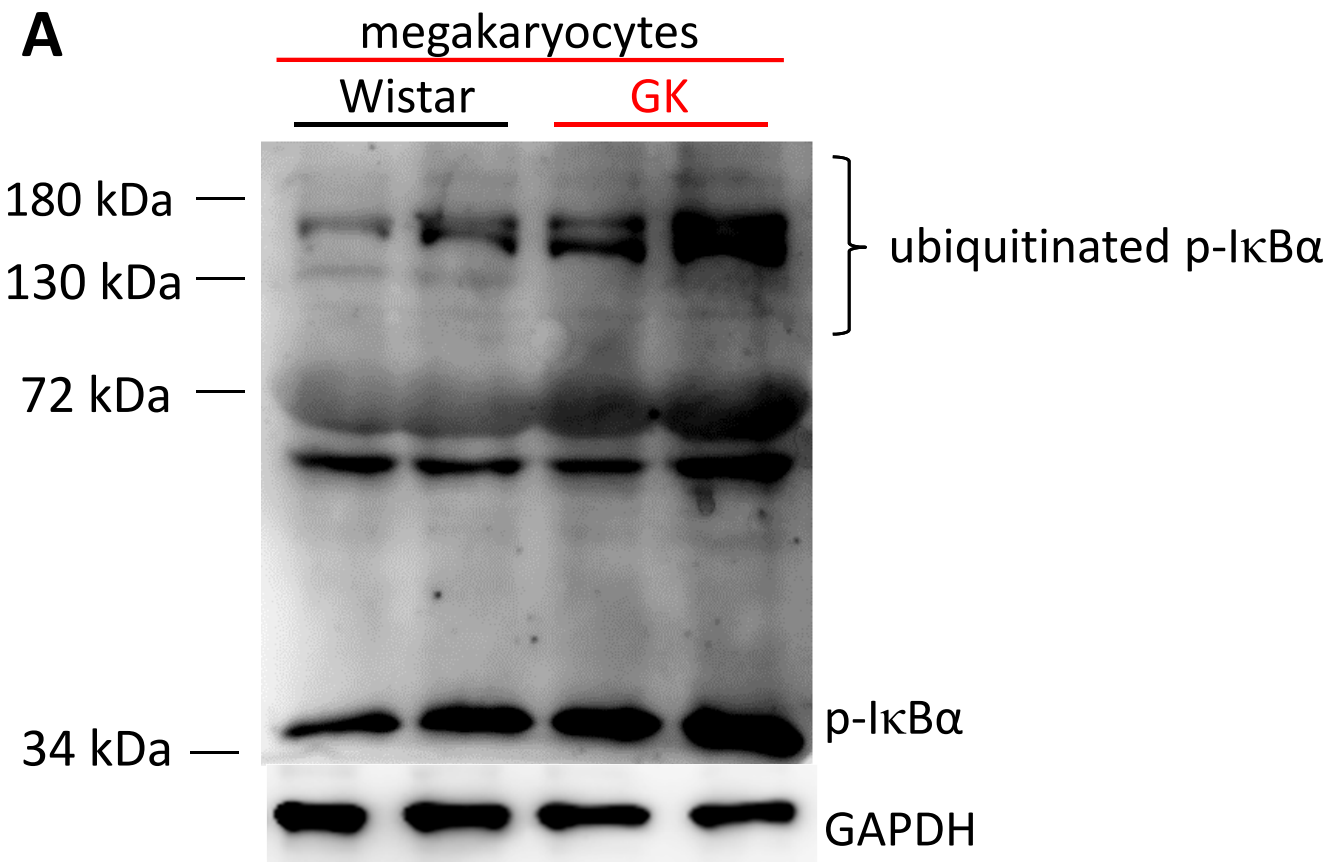
Figure S1. Increased platelet aggregation and ATP release in diabetic GK rats in response to thrombin, AYPGKF and collagen. ATP release from platelet dense granule was simultaneously recorded together with aggregation. Results shown are representative of 3 experiments using different rats.

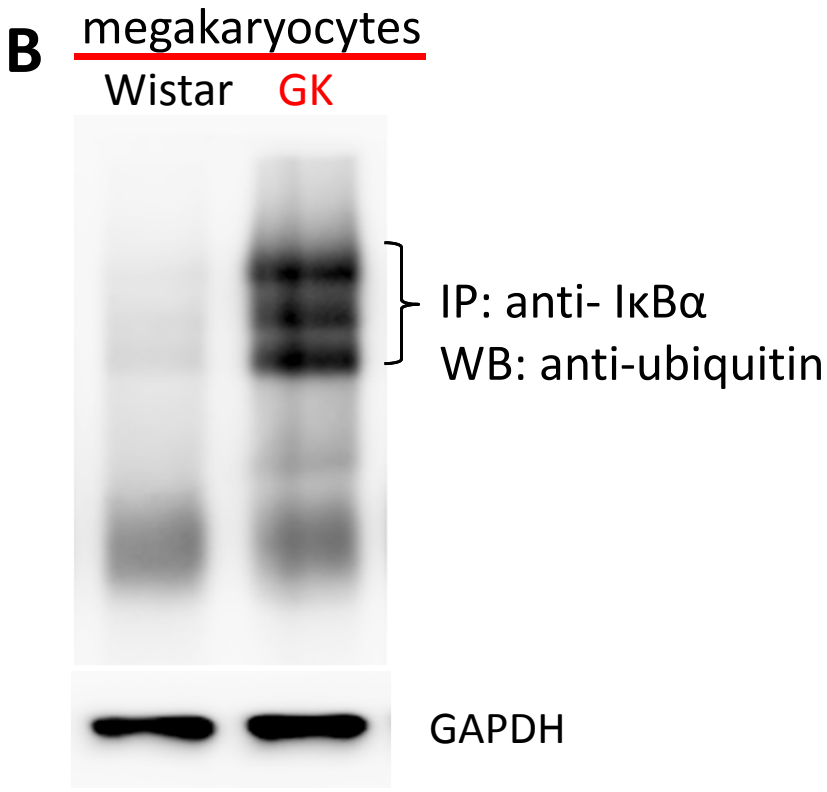


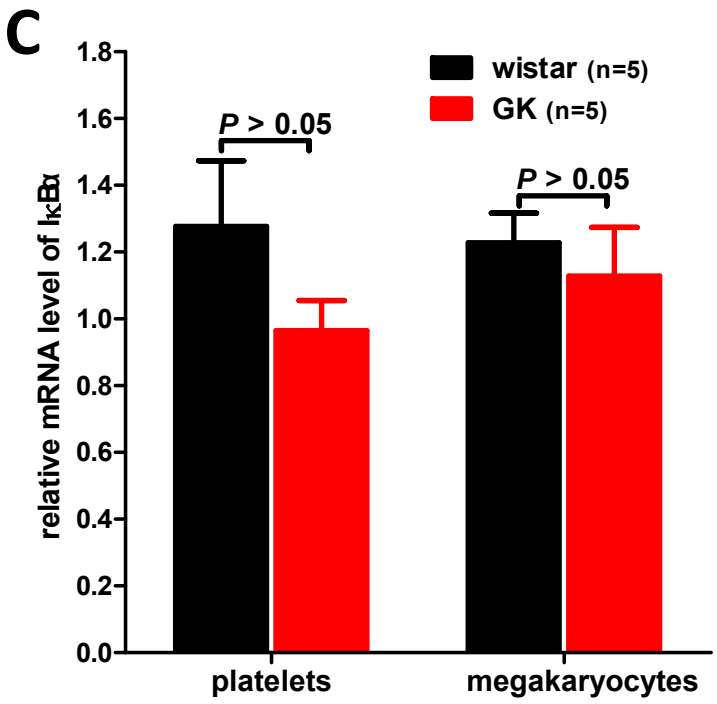
Supplemental Figure 2. Similar expression of COX-1 and COX-2 in platelets and megakaryocytes from diabetic GK rats and healthy Wistar rats. A: Western blot detection of COX1 and COX2 representing 3 different experiments. **B**: Real time PCR detection of COX1 and COX2. Unpaired t-test is used except for COX2 expression in megakaryocytes which is analyzed by unpaired t-test with Welch's correction was used.



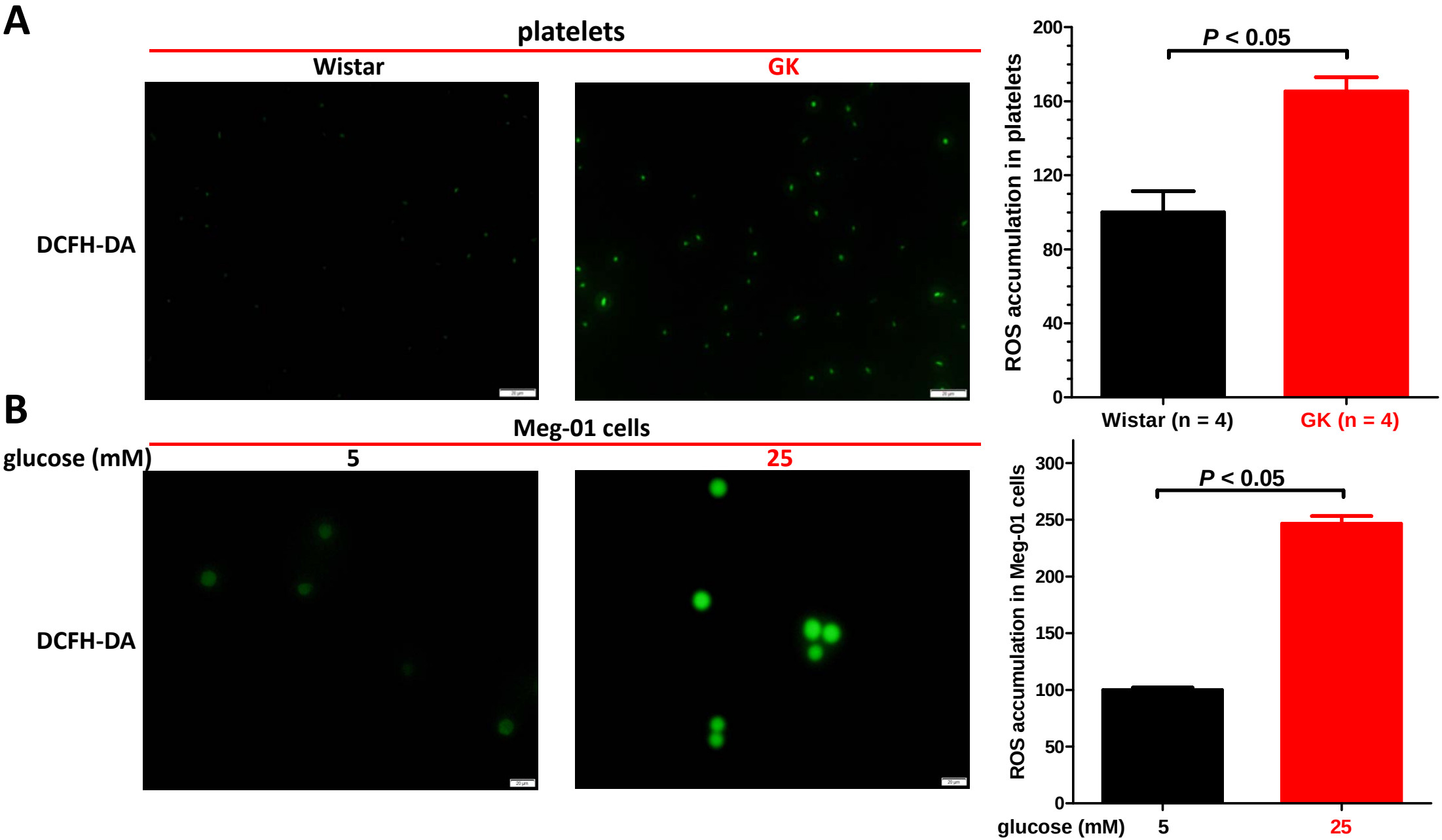
Supplemental Figure 3. Enhanced pAkt and decreased pVASP downstream of $P2Y_{12}$ in platelets of diabetic GK rats compared with Wistar rats. Results shown are representative of at least 3 experiments using platelets from different rats.



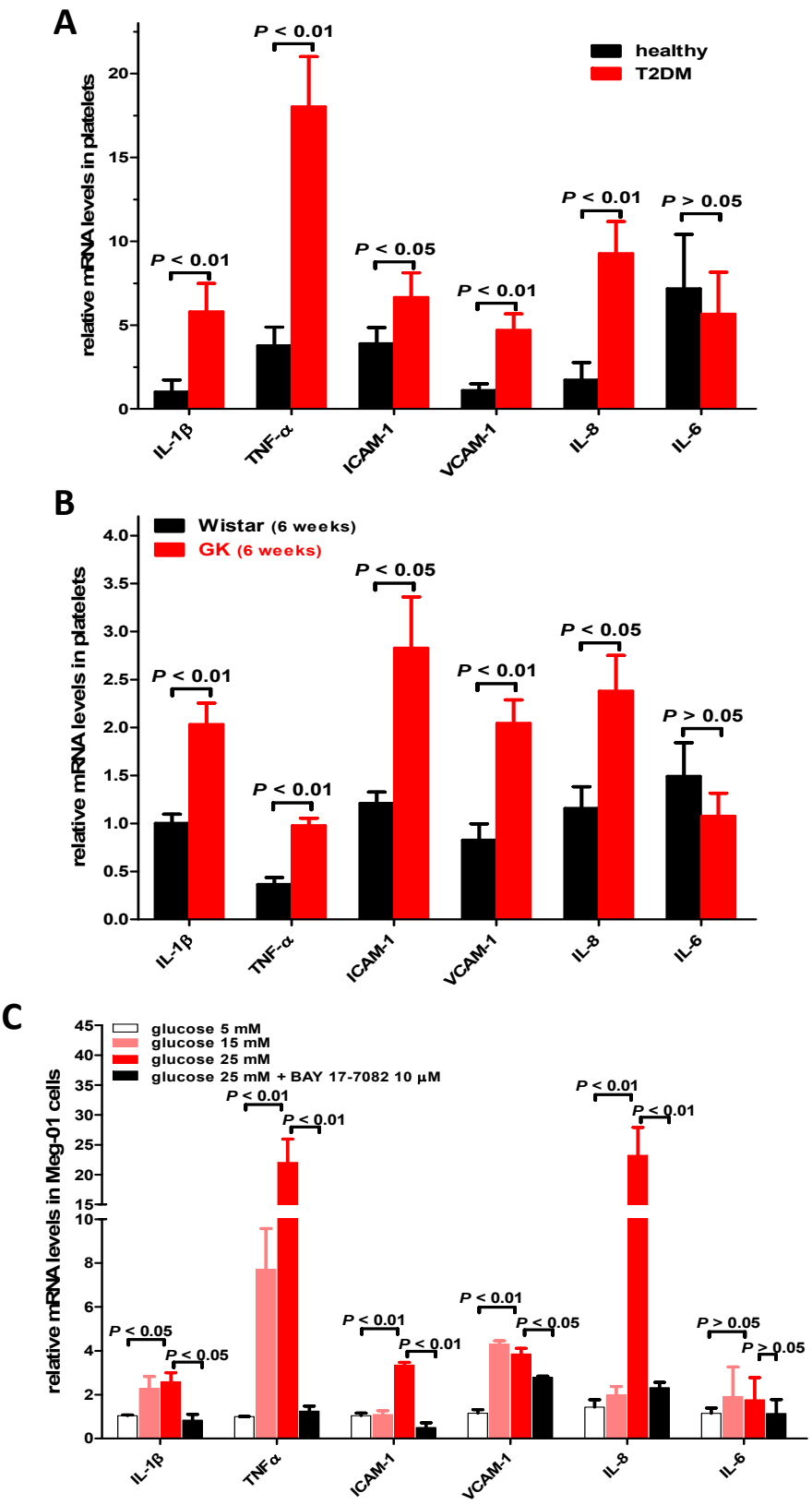




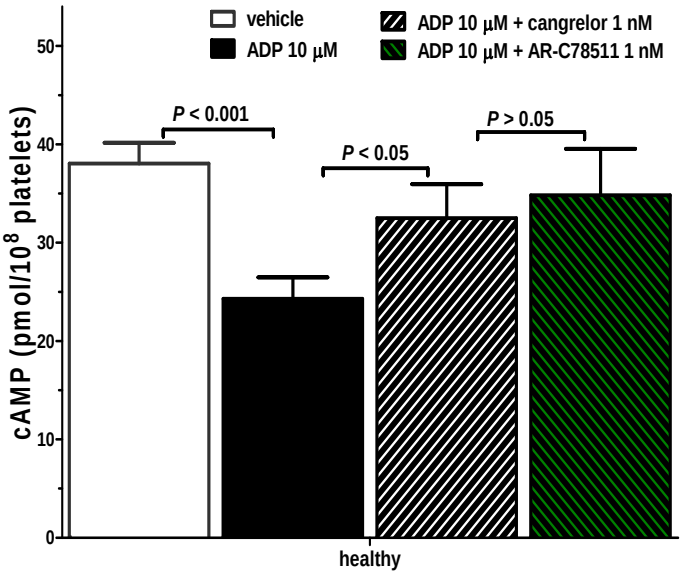
Supplemental Figure 4. Increased IκBα degradation via the ubiquitin pathway in megakaryocytes from diabetic GK rats. A: Increased high molecular weight ubiquitinated p-IκBα in megakaryocytes from diabetic GK rats than Wistar rats. Western blot analysis with antibody against phosphorylated IκBα (prolonged exposure). **B:** Increased ubiquitination of IκBα in megakaryocytes from diabetic GK rats than Wistar rats. IκBα was immunoprecipitated from cell lysates and immunoblotted with antibody against ubiquitin. **C:** Similar expression of IκBα in platelets and megakaryocytes from diabetic GK rats compared with Wistar rats detected by real time PCR.



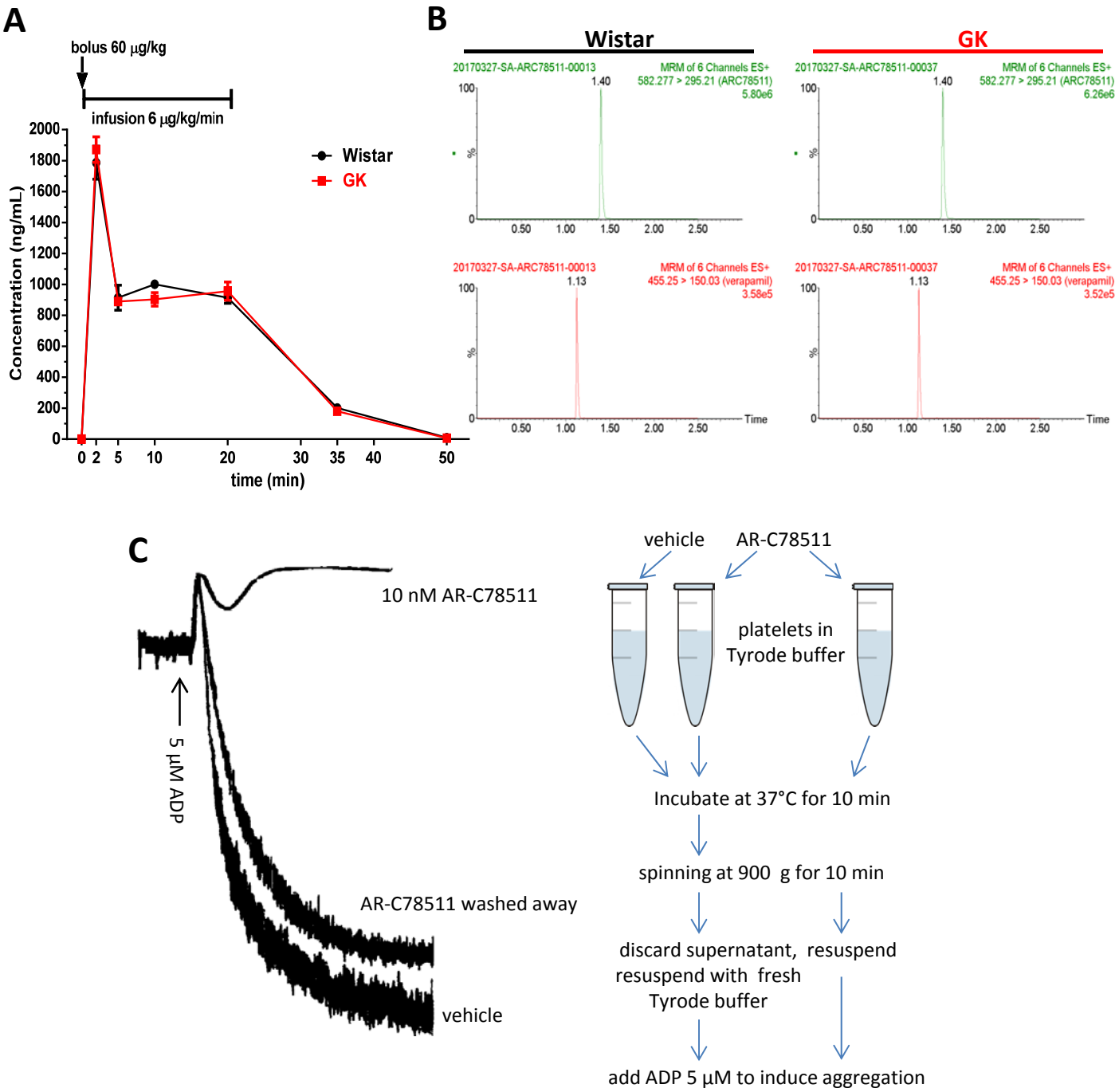
Supplemental Figure 5. Increased intracellular ROS in platelets from diabetic GK rats and megakaryocytic Meg-01 cells treated with high glucose. A: Intracellular ROS in platelets of diabetic GK rats measured by fluorescence microscopy (left panel) and microplate reader (right side). **B**: High glucose increases intracellular ROS in megakaryocytic Meg-01 cells measured by fluorescence microscopy (left side) and microplate reader (right panel). Fluorescence micrograph was taken by camera and fluorescence density was also measured by fluorescence microplate reader. Typical fluorescence micrographs shown are representative of 4 experiments run on different days. ROS accumulation measured by fluorescence microplate reader was expressed as percentage of control (platelets from Wistar rats or glucose 5 mM, taken as 100 %). Unpaired t-test with Welch's correction was used for ROS generation in megakaryocytes Meg-01 cells.



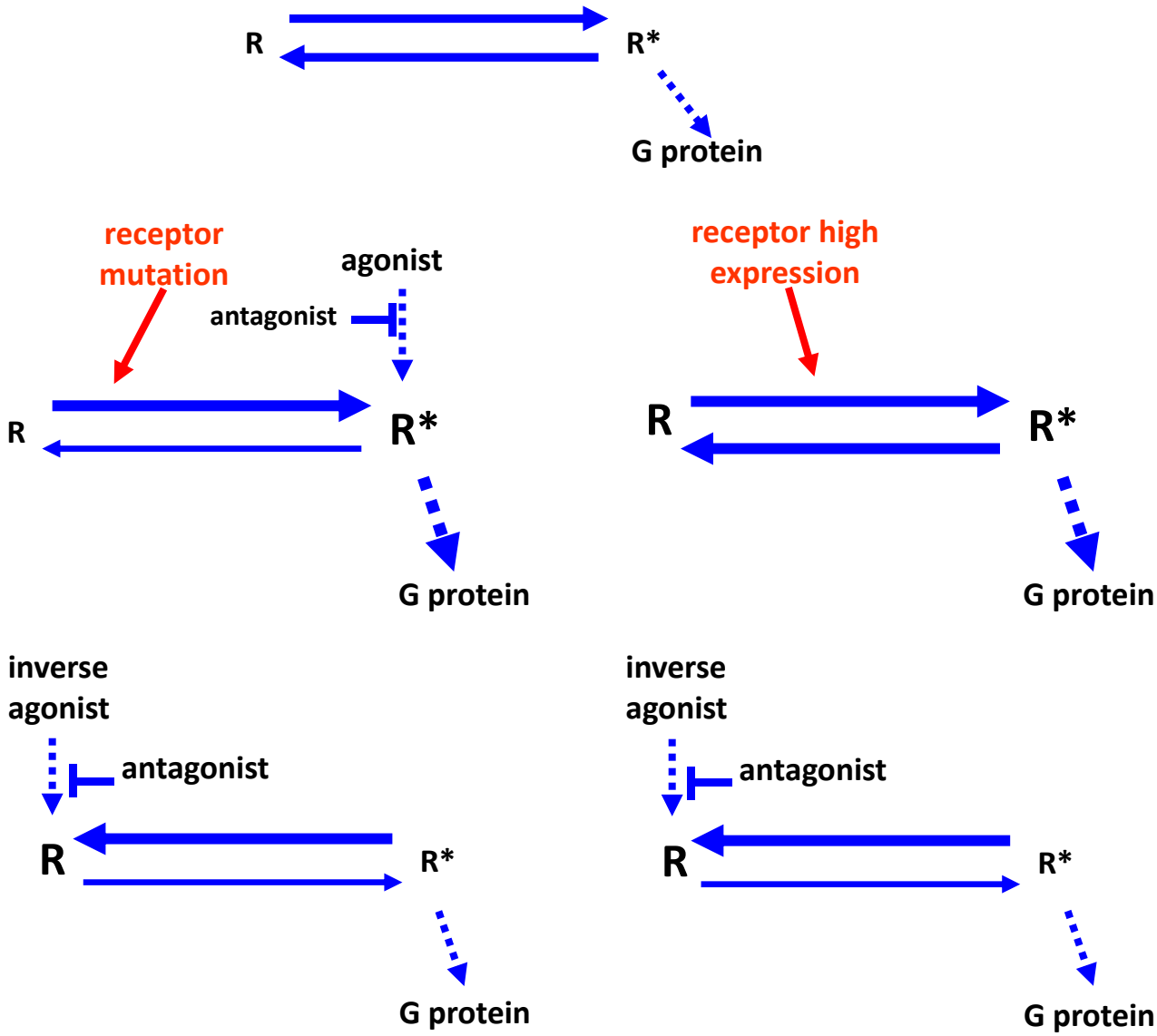
Supplemental Figure 6. Hyperglycemia upregulates proinflammatory cytokines via activation of NFκB signal pathway. **A:** Increased inflammatory cytokines in platelets of T2DM patients (n = 14 - 20) compared with healthy subjects (n = 12). **B:** Increased inflammatory cytokines in platelets of diabetic GK rats (n = 10 - 15) compared with Wistar rats (n = 9 - 15). **C:** Upregulation of inflammatory cytokines in Meg-01 cells stimulated by high glucose was reversed by NFκB inhibitor BAY 17-7082. Data are expressed as mean ± SEM of 3 - 4 independent experiments.



Supplemental Figure 7. Both cangrelor and AR-C78511 effectively antagonized ADP-induced cAMP decreased in platelets of healthy subjects. Data are expressed as mean ± SEM, n = 15; one-way ANOVA for repeated measures followed by Newman-Keuls test were used.



Supplemental Figure 8. Similar plasma concentrations in Wistar and diabetic GK rats and the reversible antiplatelet effects of AR-C78511. **A:** AR-C78511 administered intravenously has similar plasma concentrations in both healthy Wistar rats and diabetic GK rats. Plasma concentration of AR-C78511 is measured by UPLC system coupled to a Xevo TQ-S mass spectrometer (See the Expanded Materials and Methods). **B:** Typical LC-MS/MS chromatograms of AR-C78511 and I.S. verapamil. Plasma sample of Wistar and diabetic GK rats spiked with I.S. working solution. **C:** AR-C78511 is a reversible antiplatelet agent. Prewash as shown on the right panel prevented the inhibitory role of ARC-78511 on ADP-induced platelet aggregation of Wistar rats. Results shown are representative of 2 experiments using platelets from different rats.



Supplemental Figure 9. Two-state model of GPCR activation, constitutive activation of receptor and the therapeutic advantage of inverse agonists over pure antagonists. There is a balance between resting state (R) and active state (R*) of GPCR. Only active state couples to G protein leading to downstream signaling while resting state does not. Basal activity of GPCR can be observed because of the existence of R*. Increased active state R* can be achieved by 1) balance right-shift caused by agonist binding to R*; 2) balance right-shift caused by receptor gain of function mutation; 3) receptor high expression which increases both resting and active states of receptor. Under the 2nd and 3rd conditions, the receptors are activated in the absence of agonists, such activation is called constitutive activation. Inverse agonist, which is antagonist with inverse agonist activity, left-shifts the balance and hence inhibits receptor constitutive activation caused by receptor high expression or gain of function mutation. Antagonist binds the resting and active state equally and does not shift the balance. Neutral antagonist, exerts its role by blocking agonist binding to receptor, does not left-shift the balance due to lack of inverse agonist activity, and thus cannot inhibit the constitutive activation caused by receptor high expression or gain of function mutation.