

# **SUPPLEMENTAL MATERIAL**

## **Supplemental Methods**

### **Experimental animals**

All experiments involving animals were conform to the guidelines for the Care and Use of Laboratory Animals published from Directive 2010/63/EU of the European Parliament and were approved by IRCCS INM Neuromed review board (ref. number 1070/2015 PR). Diabetes was induced by four intraperitoneal injection (i.p. – once a day) of streptozotocin (STZ, 40 mg/kg body weight and dissolved in 0.1 M citrate buffer, pH 4.5) in male wild-type C57BL/6 mice (weighing approximately 25g). Control mice received citrate buffer alone (vehicle). Three days after the last injection of STZ, blood glucose levels were measured using glucose test strips (Roche) and mice with blood glucose at least 3-fold higher than the pre-injection level were used as diabetic mice. Mice were housed in groups (4–6 mice per cage) in a specific pathogen-free controlled environment (inverted 12 h light cycle; lights off at 10:00 hours). All mice had free access to standard mice chow and water. The experiments were conducted after 4 weeks following the induction of diabetes. Animals that have not developed diabetes were excluded from the study. NSC23766 (Tocris Bioscience) was injected in mice i.p. at the dose of 5 mg/kg. This dose was chosen on the basis of a pilot study conducted in our laboratory and taking into account previously published data <sup>1, 2</sup>. Mice were euthanized by intraperitoneal injection of 150 mg/kg Ketamine, 20mg/Kg xylazine.

### **Human studies**

In our clinical study two population of patients were enrolled: 1) subjects without type-2 diabetes (CTRL, n=11) and 2) subjects with type-2 diabetes (Db, n=22). All the experiments used to evaluate platelets aggregation were carried in these two populations using the CTRL group as a reference. Table 1 describes the baseline characteristics of both populations. Subjects were enrolled at the Cardiology division of the University of Naples Federico II. The study protocol was approved by the institutional review board of the medical center, and each patient who accept to participate provided written informed consent. All Db patients enrolled in our study fulfilled the criteria of National

Diabetes Data Group for diabetes<sup>3</sup>. Diabetes developed in most of the patients after 40 years of age. None of the subject enrolled in both the two populations (CTRL and Db) had a history of coronary heart disease, recent myocardial infarction, unstable angina pectoris, heart failure, thyroid, renal, or hepatic disease. Pharmacological therapy is presented in Tale 1. A subgroup of 7 Db patients of the 22 enrolled were treated with ASA 100 mg daily and platelets aggregation were performed on blood samples 14 days after treatment as described before.

### **Vascular reactivity studies**

Vessels were placed in a wire myograph system filled with glucose-free Krebs solution. First, an analysis of vascular reactivity curves was performed. In particular, vasoconstriction was assessed with 80 mmol/L of KCl or with increasing doses of phenylephrine (from  $10^{-9}$  M to  $10^{-6}$  M) or U46619 ( $10^{-11}$  M to  $10^{-6}$  M), in control conditions. Vascular responses were then tested before and after glucose treatment (5-25 mM). Endothelium-dependent and -independent relaxation was assessed by measuring the dilatory responses of mesenteric arteries to cumulative concentrations of acetylcholine (from  $10^{-9}$  M to  $10^{-5}$  M) or nitroglycerine (from  $10^{-9}$  M to  $10^{-5}$  M), respectively, in vessels pre-contracted with phenylephrine at a dose necessary to obtain a similar level of pre-contraction in each ring (80% of initial KCl-evoked contraction). Caution was taken to avoid endothelial damage; functional integrity was reflected by the response to acetylcholine (from  $10^{-9}$  M to  $10^{-6}$  M). Some studies were performed treating the vessels with NSC23766 (30  $\mu$ mol/L) or with ML171 (0,5  $\mu$ mol/L) or with GTK137831 (1  $\mu$ mol/L) . Vascular relaxation is reported as percentage, considering basal tension before phenylephrine stimulus as -100% of vascular relaxation and phenylephrine-induced tension as 0% of vascular relaxation.

## **Cell culture**

Commercially available human umbilical veins endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD, USA) and grown in EBM-2 basal medium. Cells were used within the five passage and at 70% confluence for the following sets of experiments. The cells were treated with 5 mM or 25 mM of glucose for 24 hours, subsequently were treated with NSC23766 30 $\mu$ M or LY27632 30 $\mu$ M for 30 minutes. For the western blot experiments, cells were treated with acetylcholine 100  $\mu$ mol/L for the last 15 minutes.

## **Measurement of NADPH oxidase activity**

HUVECs was depleted of serum and prepared for the assay. Vessels were isolated and prepared for the assay. Cells were treated with 0 mM, 5 mM and 25 mM of glucose and then with NSC23766, 30 $\mu$ M for 30 minutes. At the end of treatment (1 hours) cells were resuspended at  $2 \times 10^6$  cells/ml confluency in aerated balanced salt solution.  $1 \times 10^6$  cells/ml were used for the assay. Cells from various treatments were added to a scintillation vial containing lucigenin (5  $\mu$ mol/L) in the aerated balanced salt solution. After a 5-minute dark adaptation, photon emission was measured for 10 min using the Beckman LS6500 Multipurpose Scintillation counter measuring single photon emission. First, photon emission was measured using a buffer blank and dark-adapted lucigenin. NADPH (100  $\mu$ mol/L) was added after measurement of background lucigenin chemiluminescence and measurements were performed for other 10 minutes.

## **Platelet experiments**

### *Platelet isolation*

Venous blood was drawn from peripheral antecubital vein in fasting without refrain from smoking volunteers at University Federico II of Naples, who were free from medication known to interfere with platelet function (CTRL group). Other blood samples were drawn from diabetic patients free or in treatment acetylsalicylic acid (ASA) 100 mg per day (Db and Db+ASA group). Platelet-rich plasma

(PRP) was prepared from blood (about 15 ml) that was drawn by venipuncture into 3 ml of 3.8% trisodium citrate (w/v). PRP was obtained by centrifugation of blood at 150 g at 25°C for 15 minutes and was used as source of platelets. Platelet-poor plasma (PPP) was prepared from the residual blood by centrifugation of the rest of the blood at 1400 g at 25°C for 10 minutes.

#### *Study of vascular reactivity evoked by platelets supernatant*

Platelet pellets were produced by centrifugation of PRP at 900 g for 7 minutes, and re-suspended in Tyrode's solution (132 mmol/L NaCl, 4 mmol/L KCl, 1.6 mmol/L CaCl<sub>2</sub>, 0.98 MgCl<sub>2</sub>, 23.8 mmol/L NaHCO<sub>3</sub>, 0.36 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 10 mmol/L glucose, 0.05 mmol/L Ca-Titriplex, and gassed with 95% O<sub>2</sub>, 5% CO<sub>2</sub> and pH 7.4 at 37 °C). After a further centrifugation step (900 g, 4 minutes), washed platelets were re-suspended in the same solution, allowed to equilibrate for 10 minutes at 37 °C and then stimulated with insulin (1 µmol/L) for 10 minutes. Some experiments were performed in platelets pre-treated with N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) (300 µmol/L, 30 minutes) for 30 minutes. After stimulation, the platelet suspension was centrifuged for 2 minutes at 900g and increasing doses of supernatant (0.1-0.2-0.4-0.8 ml) was added to phenyleprine-precontracted arteries mounted in an organ chamber (final volume, 15 ml). Total number and purity of platelets for each preparation was assessed by flow cytometry. Total protein from each preparation was also determined. Similar number of platelets ( $174 \pm 19 \times 10^6/\text{mL}$ ) between all samples was estimated.

To test the vasorelaxant effect evoked by platelet supernatants, studies of vascular reactivity on isolated vessels from C57BL/6 mice were carried out. Four ring segments (3 mm width) of thoracic aorta from each mouse were mounted between stainless steel triangles in a water-jacketed organ bath (37°C) for measurement of tension development as previously described<sup>4</sup>. Preliminary experiments demonstrated that the optimal resting tension for development of active contraction was 1g. Vessels were gradually stretched over one-hour period to this tension. The presence of functional endothelium and smooth muscle layer were assessed in all preparations by the ability respectively of acetylcholine

and nitroglycerine ( $10^{-9}$  to  $10^{-5}$  mol/L) to induce the relaxation of vessels precontracted with phenylephrine ( $10^{-9}$  to  $10^{-6}$  mol/L) to obtain a similar level of precontraction in each ring (80% of initial KCl-induced contraction). Responses to vasoconstrictors were examined at this resting tension and related to maximal vasoconstriction elicited by depolarization with 80 mmol/L KCl. Responses to vasodilator supernatants obtained from stimulation of platelets were examined after achieving a precontracted tone with increasing doses of phenylephrine ( $10^{-9}$  to  $10^{-6}$  mol/L) to obtain a similar level of precontraction in each ring (80% of initial KCl-induced contraction).

#### *Measurement of platelet aggregation*

Isolated platelets were centrifuged at 1500 g for 10 minutes and platelets were washed twice with a 6:1 mixture of Hank's balanced salt solution (HBSS) and Acid citrate dextrose (ACD).

Platelet-rich plasma (PRP) were incubated with 5, 15, 20, 25 or 50 mmol/L glucose or mannitol in the presence or absence of NSC23766 (15, 30, 50, 60, 75, 150 and 300  $\mu$ M), EHT 1864 (Tocris Bioscience; 50 and 100  $\mu$ M) or LY27632 for 30 minutes at 37°C. Platelet aggregation was monitored at 37°C with constant stirring (1200 rpm) in a dualchannel lumi-aggregometer (model 700; Chrono-Log). The maximum aggregation was expressed as a percentage of maximum light transmission, with unstimulated PPP being 0% and stimulated PRP 100%. Platelet aggregation was measured as the increase in light transmission for 5 minutes, with the addition of 0.8  $\mu$ g/ml collagen, arachidonic acid (AA: 0.5 mM), adenosine diphosphate (ADP: 50 mM) or thrombin receptor-activating peptide (TRAP: 25 $\mu$ M) (Chrono-Log) as a proaggregatory stimuli.

#### *Measurement of nitric oxide production in platelets*

Platelet were resuspended (100  $\mu$ L) in HBSS at room temperature and stimulated for 10 minutes with 1  $\mu$ mol/L insulin. After incubation, the measurement of nitric oxide in platelets-supernatants were performed using NOAi Sievers 280 as described elsewhere.<sup>5</sup>

### **Rac1-GTP pull-down experiments.**

Pooled mice mesenteric arteries, HUVECs, and human platelets were isolated as previously described. After glucose-exposure (5 or 25 mmol/L) or mannitol (5 or 25 mmol/L) or NSC23766 (30  $\mu$ mol/L) or LY27632 (30  $\mu$ mol/L) at 37°C, tissues or cells were lysed in a buffer containing NP-40 equipped by kit. In some platelets samples, after pre-incubation with glucose, mannitol, NSC23766 or LY27632, platelets were stimulated with 1  $\mu$ g/ml collagen for 5 min. The reaction was terminated by the addition of an ice-cold EDTA (10 mmol/L) solution, followed by centrifugation at 10000g at 4°C for 2 minutes<sup>6</sup>. P21-binding domain (PBD) of p21-activated protein kinase (PAK) bound to agarose beads was added, and active Rac1, binding PAK1, was separated by repetitive centrifugation and washing. After, the specimens were boiled in Laemmli buffer, subjected to SDS-PAGE, and Rac was quantified by western blot analysis. In details, Rac1-GTP was detected with the monoclonal antibodies anti-Rac1-GTP $\gamma$  (1:800; STA-401-1, Cells Biolab Inc.), and total Rac1 with monoclonal anti-Rac1 (1:1000; Abcam). Densitometry analysis was performed using Quantity One software (Bio-Rad Laboratories). The amount of Rac1-GTP was normalized to the total amount of Rac1 or GAPDH in mesenteric arteries or in cell lysates for the comparison of Rac1 activity (GTP-bound Rac1) among different samples.

### **Platelet fractionation**

Blood was collected from the heart through cardiac puncture of isoflurane-anesthetized mice. PRP was prepared by adding 1-2 ml of saline to pooled blood and centrifuging the blood at 150 g for 10 min at ambient temperature. Platelets were quickly resuspended at 4° C in 1 mL of hypotonic buffer (5mM Tris-HCl/5mM EDTA, pH 7.5) and frozen in liquid nitrogen. Five cycles of rapid freezing and thawing lysed the cells, and the homogenate was centrifuged at 160000 g for 15 min. The resulting supernatant, representing the platelets cytosolic fraction, was rapidly removed and stored at -20° C. At this point, the pellets were resuspended in 300  $\mu$ L of Tris/EDTA buffer, and 50  $\mu$ L of this mixture was layered on top of 100  $\mu$ L of a solution contained 27% (wt/vol) sucrose, 10mM

This-HCl, 1 mM EDTA and phenylmethylsulfonyl fluoride (45 µg/mL) in each microcentrifuge tubes. After centrifugation at 160000 g for 2 min, the membrane fractions were observed as two faint bands at the top of the sucrose layer. Membrane fractions were carefully aspirated and diluted in buffer Tris/EDTA. Finally, cytosolic and membrane fractions were use to immunoblotting analyses.

### **Immunoblotting**

Platelets, pooled vessels or cells were solubilized in lysis buffer containing: 20 mmol/L Tris-HCl, 150 mmol/L NaCl, 20 mmol/L NaF, 2 mmol/L sodium orthovanadate, 1% Nonidet, 100 µg/ml leupeptin, 100 µg/ml aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride. Then, samples were left on ice for 30 minutes and centrifuged at 10621 g for 20 minutes, and the supernatants were used to perform immunoblot analysis. Total protein levels were determined using the Bradford method. Rac1 activity was determined using a commercially available kit (Cell BioLabs Inc. STA-401-1). 50 micrograms of proteins were resolved on 12% SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with anti-Rac1-GTP (1:1000, Cell BioLabs) or with anti-Rac1 (1:1000, Abcam); anti-ROCK1 (1:800, Abcam); anti-p-eNOS or anti-eNOS (1:800, Abcam); anti-p-PI3K (1:800, Thermo Fisher); anti-PI3K (1:1000, Thermo Fisher); anti-PAK1 (1:800, Thermo Fisher); anti-p-Akt (1:800, Santa Cruz); anti-Akt (1:1000, Santa Cruz); anti-RhoA-GTP (NewEast Biosciences); anti-RhoA (Thermo Fisher) and anti-β-Actin (1:1000, Cell signaling) anti CD62 (1:800, Abcam), anti Na/K-ATPase (1:1000, Cell signaling). HRP-conjugated secondary antibodies were used at 1:3000 dilution (Bio-Rad Laboratories). Protein bands were detected by ECL Prime (Amersham Biosciences) and densitometry analysis was performed using Quantity One software (Bio-Rad Laboratories).

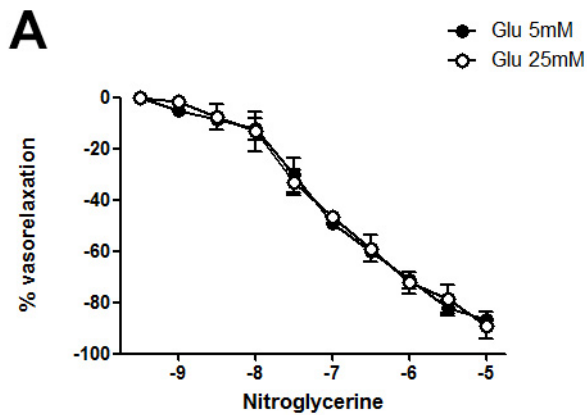
### **RNA extraction and quantitative real-time PCR**

Total RNA from tissue samples was extracted using TRIzol reagent (Invitrogen). Quantitative real-time PCR (qRT-PCR) assays were carried out to detect mRNA expression using the PrimeScript RT Reagent Kit (TaKaRa) and SYBR Premix Ex Taq (TaKaRa) according to the manufacturer's

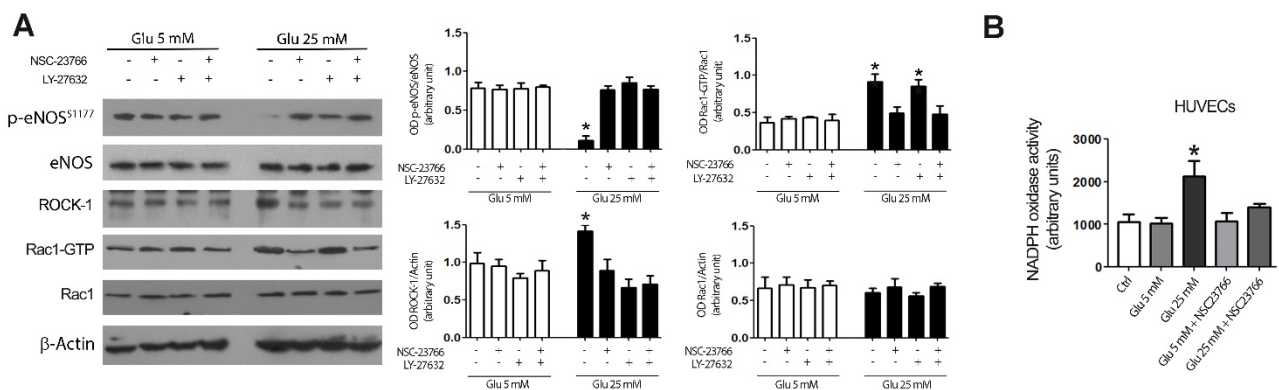


instructions. The levels of *ROCK1* transcript were measured by forward primer: TCTCATTGTGCCTTCCTTAC, and reverse primer: ACTGGTGCTACAGTGTCT.  $\beta$ -Actin was used as an internal control.

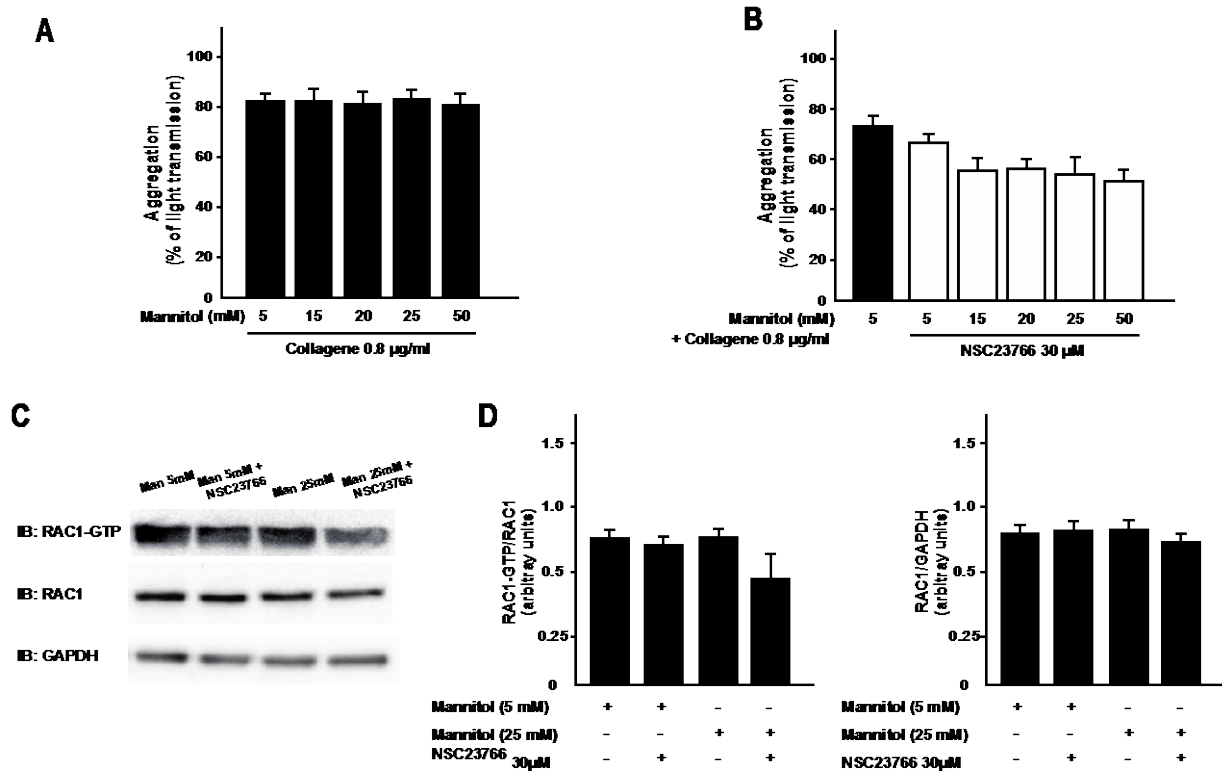
## SUPPLEMENTAL FIGURES



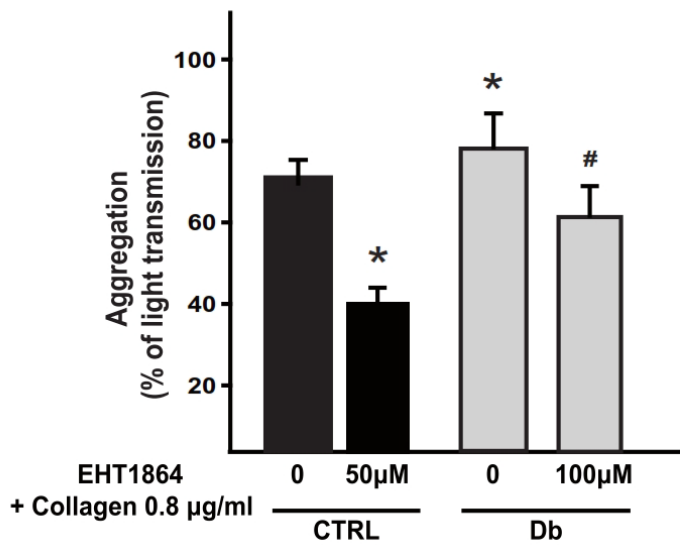
**Supplemental Figure 1. A)** Nitroglycerine-evoked vasorelaxation in pre-constricted mesenteric arteries (n=4 for each group) treated with low (Glu 5mM, full circles) and high glucose levels (Glu 25 mM, empty circles).



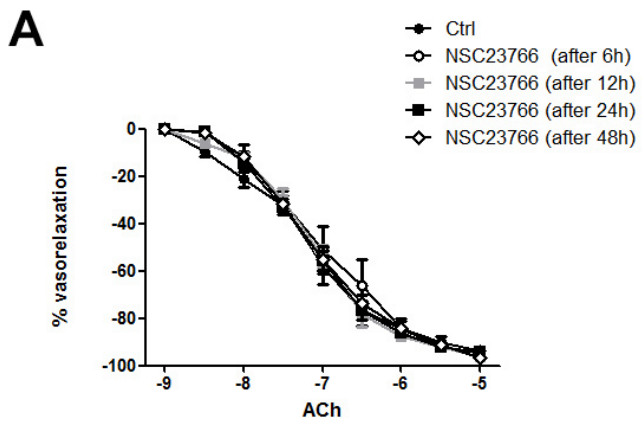
**Supplemental Figure 2. A)** Representative immunoblot (left) and densitometric analysis (right) of p-eNOS, eNOS, ROCK1, Rac1-GTP, Rac1 and  $\beta$ -actin in serum-starved HUVECs treated with 5mM or 25mM of glucose, stimulated with acetylcholine. Cells have been treated with NSC23766 alone, with LY27632 alone or concomitantly with both compound. The data are presented as mean  $\pm$ SEM from three independent experiments. \*  $p < 0.05$  vs. all. **B)** Effects of glucose and Rac1 inhibitor treatment on NADPH oxidase activity in HUVECs. Levels of NADPH activity was measured by lucigenin-enhanced chemiluminescence. 4 independent experiments. \*  $p < 0.05$  vs all.



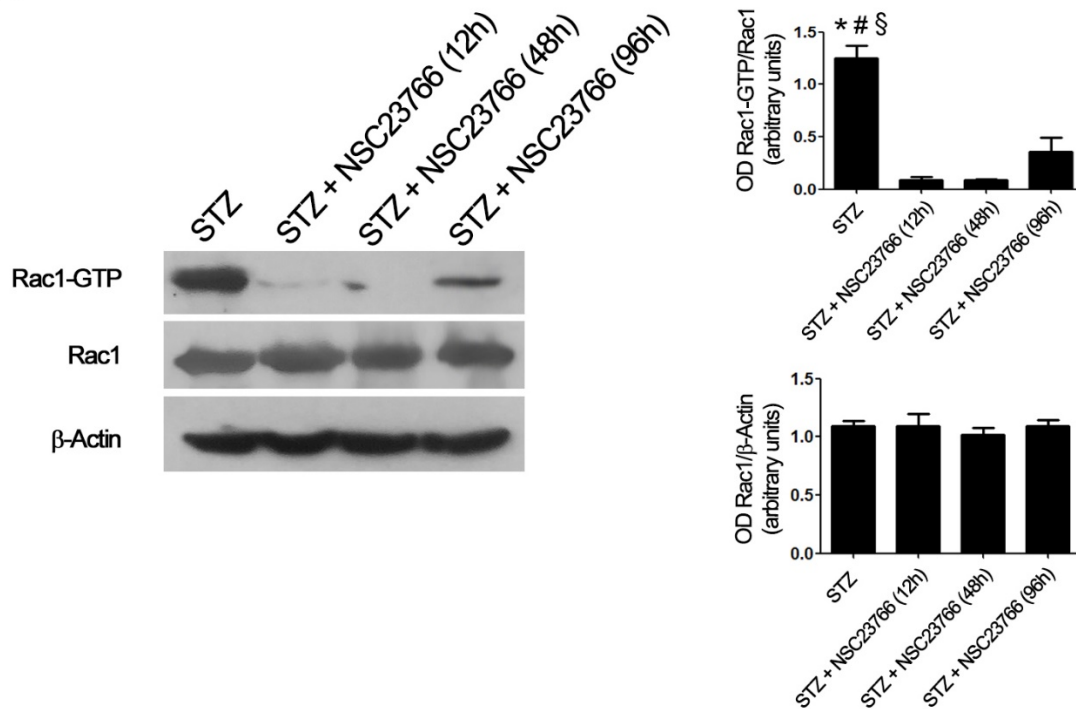
**Supplemental Figure 3.** A) Quantification of platelets aggregation presented as percentage of light transmission of platelets from control subjects treated with increasing concentrations of mannitol. Data are expressed as mean  $\pm$  SEM. n= 4 independent experiments from individual subjects B) Quantification of platelets aggregation presented as percentage of light transmission of platelets from control subjects treated with manitol plus increasing concentrations of NSC23766. Data are presented as mean  $\pm$  SEM. n= 4 independent experiments from individual subjects C) Representative immunoblot of Rac1 and Rac1-GTP levels in platelets from control subjects (CTRL) treated with mannitol 5mM and 25 mM plus NSC23766, (D) and relative densitometric analysis. n= 4 independent experiments from individual subjects



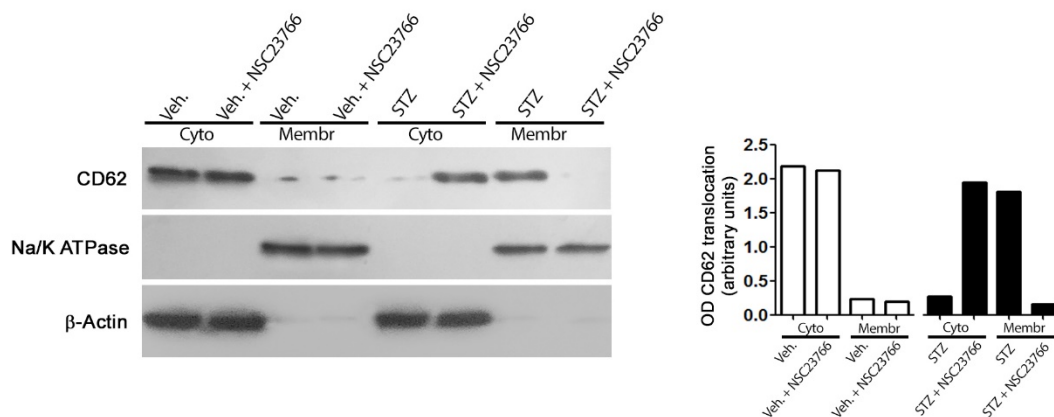
**Supplemental Figure 4.** Quantification of platelets aggregation presented as percentage of light transmission of platelets from control (CTRL) and diabetic (Db) subjects treated with two different dose of EHT1864. n= 4 independent experiments from individual subjects. \*p<0.05 vs. CTRL 0 (without EHT1864), #p<0.05 vs. Db 0 (without NSC23766).



**Supplemental Figure 5. A)** Acetylcholine (ACh) vasorelaxation in precontracted mice mesenteric arteries in basal condition (Ctrl, full circle) and after i.p. treatment with Rac1 inhibitor, NSC23766 5mg/Kg, at different timepoints (6-12-24-48 hours) (n=4 for each group).

**A**

**Supplemental Figure 6. A)** Representative immunoblot (*left*) and densitometric analysis (*right*) of Rac1-GTP, total Rac1 and  $\beta$ -Actin levels in mesenteric arteries from streptozotocin-treated mice injected i.p. with NSC23766 (5mg/Kg) and evaluated at different timepoints (12,48,96 hours). n= 3 independent experiments. \* p<0.05 vs STZ + NSC23766 (12h); # p<0.05 vs STZ + NSC23766 (48h); § p<0.05 vs STZ + NSC23766 (96h).

**A**

**Supplemental Figure 7. A)** Representative immunoblots (*left*) and densitometric analysis (*right*) showing protein levels of CD62, Na/K ATPase, and  $\beta$ -Actin in subcellular fractions (cytosol and membranes) of platelets isolated from STZ- and vehicle- treated mice in presence or absence of NSC23766. Na/K ATPase and  $\beta$ -Actin were used as membrane and cytoplasmic markers, respectively.

## SUPPLEMENTAL TABLES

**Supplemental Table 1.**

	<b>DIABETICS WITHOUT ASA (n=15)</b>	<b>DIABETICS WITH ASA (n=7)</b>
<b>HbA1c ≥ 10%</b>	4	0
<b>7% ≤ HbA1c &lt; 10%</b>	7	5
<b>HbA1c &lt; 7%.</b>	4	2

**Abbreviations used:** ASA= acetylsalicylic acid; HbA1c = glycohemoglobin.

**Supplemental Table 2.**

<i>Animal groups [n]</i>	<i>Blood glucose [mg/dL]</i>	<i>Body weight [grams]</i>
<i>Control [8]</i>	<i>125 ± 11</i>	<i>27.6 ± 0.9</i>
<i>STZ [10]</i>	<i>453 ± 18*</i>	<i>24.1 ± 0.5</i>
<i>STZ + NSC23766 [8]</i>	<i>464 ± 13*</i>	<i>24.3 ± 0.4</i>
<i>Vehicle [8]</i>	<i>128 ± 9</i>	<i>28.1 ± 0.7</i>
<i>Vehicle + NSC23766 [8]</i>	<i>122 ± 14</i>	<i>27.8 ± 0.5</i>

**Characteristics of diabetic mice used in the study.** Abbreviations: [n]=numbers; Mice received STZ dissolved in 0.1 M citrate buffer (STZ) or a solution of 0.1 M citrate buffer alone (vehicle). Control, untreated mice; STZ, streptozotocin-treated mice; STZ + NSC23766, streptozotocin-treated mice plus NSC23766; Vehicle, mice treated with sodium citrate buffer alone; Vehicle + NSC23766, mice treated with sodium citrate buffer alone plus NSC23766. \*p<0.05 vs. vehicle and vehicle plus NSC23766.

## Supplemental References

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