Materials and Methods

Reagents

Insulin (#I9278), L-NAME (L-N^G-nitroarginine methyl ester, #N5751), A23187 (#C7522) and Glucose (#G8769) were from Sigma, SP600125 (#420119), DAF-2 DA (#251505), Box5 (#681673) from Calbiochem, Dihydroethidium (DHE) was from Invitrogen (#D-23107), H_2O_2 was from Fisher Scientific (#H325) and recombinant human Wnt5a protein (#645-WN) was obtained from R&D Systems.

Study subjects

Adult patients with Type 2 diabetes mellitus and non-diabetic control volunteers were enrolled from the clinical practices at Boston Medical Center. Diabetes was defined as fasting glucose ≥126 mg/dL or ongoing pharmacological treatment for Type 2 diabetes. Control individuals without diabetes were defined as fasting glucose <100mg/dL. Fasting glucose and lipids were measured in the Boston Medical Center Clinical Laboratory. The study protocol was approved by the Boston Medical Center Institutional review Board, and all participants provided written informed consent.

Vascular function testing

We measured brachial artery flow-mediated dilation in each patient as previously described [1, 2]. Briefly, high-resolution ultrasound was used to measure brachial artery diameter before and 1 minute after induction of hyperemic response by 5-minute cuff occlusion of the upper arm. Doppler flow signals were recorded from the brachial artery before and after cuff occlusion to measure reactive hyperemia [3].

Fresh isolation of human endothelial cells

Peripheral venous endothelial cells biopsy was performed as previously described [4, 5]. Briefly, a 20-gauge intravenous catheter was placed into a superficial forearm vein using aseptic technique. Endothelial cells were collected by gentle abrasion of the vessel wall with a 0.018-in J-wire introduced through the catheter. Endothelial cells were recovered from the wire by centrifugation in a dissociation buffer and plated on poly-L-lysine coated microscope slides. Once plated, cells were either directly fixed with 4% paraformaldehyde immediately, or fixed after insulin, A23187, or rWnt5a stimulation. Slides were then washed with PBS, fixed, and stored at -80°C until further processing. We either fix the freshly isolated endothelial cells immediately or after acute treatments up to 1 hour to preserve in vivo phenotype.

Endothelial cell culture

Human aortic endothelial cells (HAECs) were purchased from Lonza and maintained with EGM-2 media containing 5 mmol/L glucose in a standard incubator (37°C, 5% CO_2). Cells from passages 4 to 7 and 90% confluence were used for the experiments after being starved for 3h in serum free-medium.

Western blot analysis

After drug treatment, endothelial cells were washed in PBS, trypsinized, and the pellet was lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 150 Na_2EDTA, 1% Triton) and protease/phosphatase inhibitor cocktail (following the instructions provided by the supplier). Protein content in samples was quantified by BCA protein assay (Pierce) and 20-35 μg protein was subjected to electrophoresis in 10-12% SDS-polyacrylamide gels under reducing and non-reducing conditions and then transferred to a polyvinylidene difluoride membrane using the Bio-Rad Transblot Turbo Transfer System. Membranes were blocked with 3% bovine serum albumin (BSA) for 1 hour, and incubated overnight with the respective primary antibody at 4ºC (1:1000-1:5000). Blots were probed with anti-eNOS (BD Transduction, #610296); anti phospho-eNOS (Ser1177, #9571), anti phospho-JNK (Thr183/Tyr185, #4688), anti JNK (#9258), anti phospho-Akt (Ser473, #4060) and Akt (#9272) from Cell Signaling; anti- Wnt5a (R&D Systems, #MAB3045).

Immunoblots were normalized to β-actin antibody (Sigma, #A1978)) or GAPDH antibody (Santa Cruz Biotechnology, #sc-32233). Horseradish peroxidase-conjugated secondary antibodies were incubated for 1 hour at room temperature (anti-rabbit #HAF008, anti-mouse #HAF007, or anti-rat #HAF005, R&D Systems). Immune complexes were visualized with Amersham ECL Western Blotting Detection Reagents. Membranes were stripped with restore western blot stripping buffer (ThermoFisher) for 10 minutes at 37°C, and reprobed with either total protein or housekeeping proteins to verify equal loading. Resulting bands were quantified by densitometry.

Quantitative immunofluorescence staining

Fixed samples were thawed and rehydrated with 50 mM Glycine in PBS. Cells were permeabilized in 0.1% Triton X-100, and nonspecific binding sites were blocked in 0.5% BSA. Slides were incubated overnight at 4°C with primary antibodies (1:100) against the following targets: phospho-eNOS (Ser1177, Abcam #ab75639), eNOS (BD Transduction, #610296); phospho-JNK (Thr183/Tyr185, Cell Signaling #9255), JNK (Santa Cruz Biotechnology #sc7345), Wnt5a (ThermoFisher #MA5-15502). Slides were incubated against Nitrotyrosine (Millopore, #05-233) for 2 hours at 37ºC. All cells were also stained with anti-von Willebrand Factor (vWF) antibody (1:300) for endothelial cell identification. Slides were incubated with corresponding Alexa Fluor-488 and Alexa-Fluor-594 antibodies (1:200) for 45 minutes at 37°C. Slides were mounted with Vectasheild containing DAPI for nuclear identification. Slides were imaged with a confocal microscope (Leica SP5) at x63 magnification. All images were captured at the same exposure time and corrected for background fluorescence. Fluorescent intensity was quantified with NIS Element AR Software. Fluorescence intensity of 20 cells from each patient and protein of interest, was averaged and normalized to the intensity of HAEC simultaneously stained. Intensity quantification was performed blinded to subject identify and diabetic status.

Nitric oxide detection

Endothelial nitric oxide levels were visualized and quantified using DAF-2 DA staining, a membrane-permeable fluorescent indicator for nitric oxide. Endothelial cells were loaded with DAF-2 DA solution in HBSS (5 μM, 30 min) at 37°C in darkness, and treated with Wnt5a recombinant protein or vehicle followed by insulin or A23187 stimulation. Where indicated, L-NAME (300 μM, 1 hour) or SP600125 (10 μM, 30 minutes) were added before loading the cells with DAF-2 DA probe, and incubated together with insulin or A23187 treatment. Cells were then washed once in PBS and imaged with a fluorescence microscope (Nikon Eclipse Ti-E) at x20 magnification, and digital images were captured by a Photometric CoolSnap HQ2 Camera. All images were captured at the same exposure time and corrected for background fluorescence. Fluorescent intensity was quantified with NIS Element AR Software.

ROS detection.

For superoxide radical anion detection, HAECs were incubated for 30 minutes at 37ºC in humidified air with 5% CO_2 with 5 μM DHE after exposure to either H_2O_2 (500 μM) or rWnt5a (100 ng/ml) for 1 hour. After incubation, DHE fluorescence was detected with a fluorescent plate reader using an emission wavelength of 606 nm and an excitation of 518 nm.

Statistical analysis

Statistical analyses were performed using SPSS version 20.00 (IBM Corp., Armonk, NY). Data are expressed as mean±standard deviation unless otherwise noted. Variables were evaluated for normality by the Shapiro-Wilk test. Variables with normal distribution were compared using unpaired t-test, paired t-tests, or chi-square tests for two-group comparisons or repeated-measures ANOVA for multi-treatment comparisons. Variables that were not normally distributed were compared with the

Mann-Whitney U test to compare diabetics and controls or the Wilcoxon Signed Rank test for paired samples. Spearman correlation coefficients were used to correlate measures of vascular function, and expression of proteins of interest. A two-tailed $P<0.05$ was considered to be statistically significant.

References

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