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Endothelium dependent hyperpolarization-type relaxation compensates for attenuated nitric oxide-mediated responses in subcutaneous arteries of diabetic patients

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ABSTRACT

Diabetes impairs endothelium-dependent relaxations. The present study evaluated the contribution of different endothelium-dependent relaxing mechanisms to the regulation of vascular tone in subcutaneous blood vessels of humans with Type 2 diabetes mellitus. Subcutaneous arteries were isolated from tissues of healthy controls and diabetics. Vascular function was determined using wire myography. Expressions of proteins were measured by Western blotting and immunostaining. Endothelium-dependent relaxations to acetylcholine were impaired in arteries from diabetics compared to controls ($P = 0.009$). Acetylcholine-induced nitric oxide (NO)-mediated relaxations [in the presence of an inhibitor of cyclooxygenases (COX; indomethacin) and small and intermediate conductance calcium-activated potassium channel blockers (UCL1684 and TRAM 34, respectively)] were attenuated in arteries from diabetics compared to controls ($P < 0.001$). However, endothelium-dependent hyperpolarization (EDH)-type relaxations [in the presence of indomethacin and the NO synthase blocker, L-NAME] were augmented in arteries from diabetics compared to controls ($P = 0.003$). Endothelium-independent relaxations to sodium nitroprusside (NO donor) and salbutamol (β-adrenoceptor agonist) were preserved, but those to prostacyclin were attenuated in diabetics compared to controls ($P = 0.017$). In arteries of diabetics, protein expressions of endothelial NO synthase, prostacyclin synthase and prostacyclin receptors were decreased, but those of COX-2 were increased. These findings suggest that in human diabetes, the impairment of endothelium-dependent relaxations is caused by a diminished NO bioavailability; however, EDH appears to compensate, at least in part, for this dysfunction.

1. Introduction

Small arteries play an important role in the regulation of blood flow to the peripheral organs and play a crucial role in the control of systemic blood pressure. The endothelium, a monolayer of cells lining the internal surface of blood vessels, contributes to the regulation of vascular tone by releasing relaxing and contracting factors. Endothelium-dependent relaxations are mediated by different signals including release of nitric oxide (NO) and/or prostacyclin and initiation of endothelium-dependent hyperpolarizations (EDH) [1].

Abbreviations: NO, nitric oxide; EDH, endothelium-dependent hyperpolarization; eNOS, endothelial nitric oxide synthase; T2DM, type 2 diabetes mellitus; TRAM 34, 1-[(2- Chlorophenyl) diphenylmethyl]; 1H, pyrazole; UCL 1684, 6,12,19,20,25,26-hexahydro-5,27:13,18:21,24-trietheno-11,7-metheno-7*H*-dibenzo [*b*,*n*] [1,5,12,16]tetraazacyclotricosine-5,13-diium dibromide; LNAME hydrocloride, L-NG-Nitroarginine methyl ester; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; KCl, potassium chloride; PGIS, prostacyclin synthase; IP, prostacyclin receptor; EET, arachidonic acid; 14, 15-epoxyeicosatrienoic acid; H_2O_2, hydrogen peroxide.

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Diabetes mellitus has significant adverse effects on the quality of life of the patients as a result of its microvascular and macrovascular complications. A hallmark and precursor of these vascular complications is the development of endothelial dysfunction, characterized by impaired endothelium-dependent relaxations in both conduit and resistance arteries in human and animal models of the disease [2–6].

Both NO and EDH contribute to endothelium-dependent relaxation in small arteries of healthy individuals [7–9]. NO is synthesized in a reaction catalyzed by endothelial NO synthase (eNOS). The protein expression of eNOS is reduced in the subcutaneous arteries of Type 2 diabetic patients [10]. We hypothesized that reduction in eNOS protein expression may lead to the blunted NO-mediated responses in subcutaneous arteries of diabetic humans as demonstrated in mesenteric [5] and coronary arteries [11] of diabetic animals.

Although impairment of endothelium-dependent relaxations has been documented in small and large arteries of diabetic patients [2–5], the relative contributions of the individual endothelium-dependent signals to these dysfunctional endothelium-dependent relaxations remain poorly understood. Therefore, the present study was designed to examine the signalling pathways underlying endothelium-dependent relaxations in subcutaneous arteries of human with Type 2 diabetes mellitus (T2DM) by assessing the relative contributions of NO, prostacyclin and EDH to relaxations in response to endothelium-dependent vasodilators.

2. Materials and methods

2.1. Subjects

This study was approved by the Human Ethical Committee of Universiti Sains Malaysia (USM); work conducted in this study conformed to the provisions of the Declaration of Helsinki. Written informed consent was obtained from patients undergoing lower limb surgical procedures. Sixteen healthy controls and twenty diabetic patients between the ages of 18–75 years old were recruited among those undergoing lower limb surgical procedures such as wound debridement, amputations, fracture stabilization and skin grafting. Patients were excluded if they had uncontrolled hypertension, previous myocardial infarction, coronary heart disease or renal or hepatic failure.

2.2. Drugs

Acetylcholine hydrochloride, phenylephrine and sodium nitroprusside were purchased from Sigma Chemical Co. (St. Louis, MO). 1-[(2- Chlorophenyl) diphenylmethyl]-1H-pyrazole (TRAM 34), 6,12,19,20,25,26-hexahydro-5,27:13,18:21,24-trietheno-11,7-metheno-7*H*-dibenzo [*b*,*n*] [1,5,12,16] tetraazacyclotricosine-5,13-diium dibromide (UCL 1684) and salbutamol were purchased from Tocris Bioscience (Bristol, UK). Indomethacin, L-NAME hydrochloride and prostacyclin were obtained from Cayman Chemical Company (Ann Arbor, MI). Distilled water was used to prepare the drug solutions, except for indomethacin, TRAM-34 and UCL 1684, which were dissolved in dimethyl sulphoxide (DMSO). Concentrations are given as final molar concentration in the bath solution.

Primary antibodies against endothelial nitric oxide synthase (eNOS; AB5589), cyclooxygenase-1 (COX-1; AB53766), cyclooxygenase-2 (COX-2; AB15191), prostacyclin synthase (PGIS; AB23668), prostacyclin (IP; AB123419) receptor and horseradish peroxidase (HRP)-conjugated secondary antibodies (AB 6721) were purchased from Abcam (Cambridge, UK). A rabbit polyclonal antibody to β-actin was purchased from Sigma Chemical Co.

2.3. Wire myography

Subcutaneous fat tissues were taken from lower limb surgical procedures and transported to the laboratory in ice cold physiological salt solution (control solution) of the following composition: 118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 5.5 mM D-glucose and 2 mM $CaCl_2$. Subcutaneous arteries were dissected free of connective tissue and fat, and then cut into rings (approximately 2 mm length). Care was taken during the dissecting procedure to protect the endothelium from damage. In some preparations, the endothelium was removed by rubbing the lumen of arteries with human hair. The rings were suspended in myograph chambers (410A, JP Trading) by treading onto two stainless steel wires (40 μm in diameter). Once suspended, they were warmed to 37 °C in control solution and allowed to equilibrate for 30 min, before being subjected to a normalisation process, which determines the passive tension characteristics of each individual preparation. To do so, they were then stretched to a normalized internal circumference of L_0, at which the active response is nearly maximal, whereby $L_0 = 0.9_{L100}$ and L_{100} is the internal circumference the blood vessel would have when relaxed at a transmural pressure of 100 mmHg [12] and [13]. From L_0 the normalized internal diameter was calculated [13]. After this normalization procedure, the rings were allowed to equilibrate for 60 min, and they were then exposed twice to potassium chloride (KCl, 60 mM) to check for the viability of vascular smooth muscle cells. After KCl was removed and the arteries had returned to basal tension, phenylephrine (10^{-4} M) was added into the chamber. When the steady state contraction to phenylephrine had been reached, acetylcholine (10^{-5} M) was added to assess the presence [or absence] of endothelium. Preparations that failed to contract to KCl or phenylephrine or that failed to relax at least by 70% to acetylcholine were discarded [14]. Among the total of 96 and 90 arteries isolated respectively from non-diabetic and diabetic patients, six (two in non-diabetic; four in diabetic) did not contract significantly to KCl and phenylephrine, and four (two in non-diabetic; two in diabetic) did not dilate to acetylcholine by more than 70% (the actual relaxation in these arteries were less than 10%). Therefore, there was no difference in the percentage of rejected vessels per patient group. These arteries were discarded and were not used in the subsequent pharmacological studies.

2.4. Pharmacological studies

To study endothelium-dependent relaxations, the rings were contracted with phenylephrine (10^{-4} M). When the phenylephrine-induced contraction had reached steady state, acetylcholine was added in a cumulative manner (10^{-9} to10^{-5} M). To investigate the contribution of NO, EDH and prostacyclin, acetylcholine-induced relaxations were compared in the presence of various inhibitors, as follows: a) NO-mediated relaxations: the rings were incubated with the combination of indomethacin (10^{-5} M), non-selective inhibitor of cyclooxygenase to prevent the production of prostacyclin) plus TRAM-34 (10^{-6} M) and UCL1684 (10^{-6} M) [blockers of intermediate (IK_{Ca})- and small (SK_{Ca})- conductance calcium-activated potassium channels, respectively, to inhibit EDH-type relaxations] [15]; b) EDH-type relaxations: the rings were incubated with indomethacin plus L-NAME (10^{-4} M) [inhibitor of endothelial nitric oxide synthase (eNOS)] [14]; and c) prostacyclin-mediated relaxations: the rings were incubated with L-NAME plus TRAM-34 and UCL1684. The preparations were incubated with the appropriate inhibitors for 30 min before the administration of phenylephrine.

Endothelium-independent relaxations were determined in rings without endothelium. The successful removal of the