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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 ($10^{-9}$ to$10^{-5}$ 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

endothelium in the blood vessels was verified by the lack of relaxation to acetylcholine ($10^{-5}$ M) [16] and [17]. The rings were contracted with phenylephrine, and exposed to cumulative concentrations of sodium nitroprusside ($10^{-8}$ to $10^{-4}$ M), salbutamol [$\beta_2$-adrenergic agonist] ($10^{-7}$ to $10^{-3}$ M) or prostacyclin ($10^{-8}$ to $10^{-4}$ M).

### 2.5. Western blotting and immunohistochemistry

Western blotting was performed as described [10]. Briefly, the subcutaneous arteries were homogenized, and the total protein concentrations were quantified using the Bradford assay. 60 μg of homogenates protein were subjected to SDS-PAGE and Western blot analysis with primary antibodies (all 1:5000) against eNOS, COX-1, COX-2, PGIS and IP receptors. To normalize for the amount of protein presence in the sample, membranes were reprobed with β-actin (1:5000). All proteins were detected by enhanced chemiluminescence after incubation with HRP-conjugated secondary antibodies (1:5000). All protein bands were quantified by Image J software; the results are expressed as ratio of the loading control.

Immunohistochemistry were performed as described [10]. Briefly, paraffin sections of the subcutaneous arteries were immunolabeled with primary antibodies against eNOS (1:100), COX-1 (1:200), COX-2 (1:200), PGIS (1:100) or IP receptors (1:100). Immunostainings were visualized using 3, 3′diamino-benzidine-tetrahydrochloride substrate (DAB, Roche, Mannheim, Germany) after incubation with HRP-conjugated secondary antibody (1:200).

### 2.6. Statistical analysis

Statistical analyses were performed using SPSS statistical software (Version 20.0; SPSS, Chicago, IL, USA). Relaxation is expressed as a percentage relative to the maximal tension generated by phenylephrine. The maximal relaxation ($R_{max}$) in each protocol was the greatest relaxation achieved to the agonist studied. Sensitivity to agonists ($pEC_{50}$ = negative log of the concentration required to produce 50% of $R_{max}$) was calculated using the GraphPad Prism version 5 for windows (Graphpad Software, San Diego California, USA). Patient's characteristics were compared using independent t-test or Mann–Whitney test. Chi-square test or Fisher's exact test was used to analyse non-categorical data such as gender, medications and underlying diseases. In diabetic group, regression analysis was performed to examine the influence of concurrent medications on $R_{max}$ to acetylcholine, sodium nitroprusside, salbutamol and prostacyclin. There were no significant relationships between the $R_{max}$ to acetylcholine, sodium nitroprusside, salbutamol and prostacyclin to concurrent medications in diabetic group [supplementary Table 1]. Variables tested and subsequently used in the analysis of covariance (ANCOVA), when a significant difference from the control was found, included age [18], hypertension [19] and hyperlipidemia [20], because these factors have the potential to affect vascular responses. P values less than 0.05 were considered to indicate statistically significant differences.

## 3. Results

### 3.1. Patient characteristics

Patient characteristics, concurrent medical history and underlying diseases are summarized in Table 1. The average age of diabetic patients was higher than that of controls. Although systolic blood pressure and total cholesterol were higher in diabetic patients, these values were within the normal range in both groups. As expected, fasting blood glucose and glycated haemoglobin were significantly higher in the diabetic patients compared to controls. There were no significant differences among the two groups in gender, body mass index, diastolic blood pressure and serum creatinine level.

### 3.2. Contractions to KCl and phenylephrine

No significant differences in responses to KCl (60 mM) and phenylephrine ($10^{-4}$ M) were observed between the study groups (Tables 2 and 3).

### 3.3. Endothelium-dependent relaxations

#### 3.3.1. Control response

The maximal relaxation to acetylcholine was significantly attenuated in subcutaneous arteries from diabetics compared to controls. The $pEC_{50}$ for acetylcholine was not different between the two groups (Fig. 1A, Table 2).

#### 3.3.2. NO-mediated relaxation

In the presence of indomethacin, TRAM 34 and UCL 1684, the maximal relaxation to acetylcholine was significantly lower in subcutaneous arteries from diabetics compare to controls The $pEC_{50}$ for acetylcholine was not significantly different between the two groups of preparations (Fig. 1B, Table 2).

NO-mediated relaxation showed significant negative correlations with either fasting blood glucose ($r = -0.51$, $P = 0.003$) or glycated haemoglobin levels ($r = -0.59$, $P < 0.001$) (Fig. 2A and B).

#### 3.3.3. EDH-type relaxation

In the presence of indomethacin and L-NAME, the maximal relaxation to acetylcholine was significantly greater in subcutaneous arteries of diabetics compared to controls. The $pEC_{50}$ for acetylcholine was not significantly different between the two groups (Fig. 1C, Table 2).

#### 3.3.4. Prostacyclin-mediated relaxation

In the presence of L-NAME, TRAM 34 and UCL 1684, the maximal relaxation and the $pEC_{50}$ for acetylcholine were not significantly different in the preparations of the two groups (Fig. 1D, Table 2).

### 3.4. Endothelium-independent relaxations

There was no significant difference in the maximal relaxation to either sodium nitroprusside or salbutamol in subcutaneous arteries of the two groups. However, the maximal relaxation to prostacyclin was significantly attenuated in subcutaneous arteries from diabetics compared to controls. The $pEC_{50}$ for sodium nitroprusside, but not those for salbutamol or prostacyclin, was significantly higher in preparations from control subjects (Fig. 3, Table 3).

### 3.5. Western blotting and immunohistochemistry

Western blot analysis demonstrated that the expression levels of eNOS, PGIS and IP receptors were significantly lower in subcutaneous arteries from diabetic patients compared to controls (Fig. 4). Likewise, immunostaining showed that the intensities of immunoreactive eNOS, PGIS and IP receptor proteins were lower in the subcutaneous arteries of diabetic patients compared to controls (Fig. 5). COX-2 expression was significantly higher in the subcutaneous arteries from diabetic patients compared to controls, as shown both by Western blotting and immunostaining. The presence of COX-1 protein was not significantly different in subcutaneous arteries of the two groups. These proteins were localized throughout the arterial walls, both in endothelial and smooth muscle layers.

**Table 1**
Background characteristics of control and diabetic patients.

| | Controls (n = 16) | Diabetics (n = 20) | P Value |
|---|---|---|---|
| Male/female ratio | 9/7 | 8/12 | 0.332 |
| Post-menopausal women (>50 year olds) | 2 | 9 | 0.074 |
| Age (years) | 31.5 ± 3.1 | 55.3 ± 2.2 | <0.001[a] |
| Weight (kg) | 63.9 ± 2.9 | 67.6 ± 3.6 | 0.437 |
| Height (cm) | 163.4 ± 2.4 | 159.0 ± 1.6 | 0.120 |
| BMI (kg/m$^2$) | 24.4 (5.2) | 25.5 (6.1) | 0.154 |
| SBP (mm/Hg) | 120.0 (5.5) | 122.0 (4.8) | 0.007[a] |
| DBP (mm/Hg) | 74.9 ± 1.7 | 78.4 ± 1.6 | 0.159 |
| Cholesterol (mmol/l) | 3.6 (0.7) | 4.8 (1.8) | 0.003[a] |
| FBG (mmol/l) | 4.7 (1.1) | 10.5 (3.9) | <0.001[a] |
| $HbA_{1C}$ (%) | 5.3 (1.5) | 9.6 (2.3) | <0.001[a] |
| Creatinine (μmol/l) | 82.3 ± 2.5 | 88.9 ± 5.4 | 0.296 |
| Underlying diseases; n(%) | | | |
| Hypertension | 0 (0) | 9 (45.0) | 0.002[a] |
| Hypercholesterolemia | 0 (0) | 5 (25.0) | 0.031[a] |
| Medications; n (%) | | | |
| ACE inhibitor | 0 (0) | 4 (20.0) | 0.113 |
| Aspirin | 0 (0) | 1 (5.0) | 1.000 |
| Calcium channel blocker | 0 (0) | 3 (15.0) | 0.238 |
| Insulin | 0 (0) | 16 (80.0) | <0.001[a] |
| Lipid lowering | 0 (0) | 6 (30.0) | 0.024[a] |
| NSAIDs | 2 (12.5) | 1 (5.0) | 0.574 |
| Oral antidiabetics | 0 (0) | 16 (80.0) | <0.001[a] |
| Paracetamol | 1 (6.3) | 7 (35.0) | 0.053 |

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; $HbA_{1C}$, glycosylated haemoglobin; n is the number of patients. Data were presented as means ± SEM or median (interquartile range) using independent t-test or Mann Whitney test. Tests of significance for categorical data were performed using the chi-square test or Fisher's exact test.
[a] Indicates a statistically significant difference between the two groups.

**Table 2**
Endothelium-dependent relaxations in subcutaneous arteries from control and diabetic patients.

| | Controls (n = 16) | Diabetics (n = 15) | P Value |
|---|---|---|---|
| Internal arterial diameter (μm) | 298.8 ± 2.4 | 301.3 ± 3.4 | 0.531 |
| Contraction to KCl, 60 mM (mN/mm) | 5.4 ± 0.5 | 4.8 ± 0.4 | 0.340 |
| Contraction to phenylephrine, $10^{-4}$ M (mN/mm) | 7.1 ± 0.5 | 7.2 ± 0.4 | 0.966 |
| Control solution | | | |
| $pEC_{50}$ | 7.1 ± 0.1 | 7.0 ± 0.2 | 0.693 |
| $R_{max}$ | 91.3 ± 1.2 | 81.2 ± 3.5 | 0.009[a] |
| | | | **0.012**[b] |
| Indomethacin +TRAM 34 + UCL 1684 | | | |
| $pEC_{50}$ | 6.8 ± 0.1 | 6.5 ± 0.2 | 0.154 |
| $R_{max}$ | 73.6 ± 3.5[c] | 31.6 ± 6.8[c] | <0.001[a] |
| | | | **0.003**[b] |
| L-NAME + Indomethacin | | | |
| $pEC_{50}$ | 6.8 ± 0.2 | 6.5 ± 0.2 | 0.411 |
| $R_{max}$ | 43.1 ± 4.3[c,d] | 63.5 ± 4.6[d] | 0.003[a] |
| | | | **0.029**[b] |
| L-NAME +TRAM 34 + UCL 1684 | | | |
| $pEC_{50}$ | 7.0 ± 0.2 | 6.7 ± 0.3 | 0.383 |
| $R_{max}$ | 19.6 ± 4.4[c,d,e] | 9.9 ± 3.2[c,d,e] | 0.088 |

$pEC_{50}$, sensitivity to agonists; $R_{max}$, maximal relaxation; n is the number of arteries from different subjects used in the study.
Values were presented as means ± SEM.
[a] Indicates a statistically significant difference between two groups using the independent t-test.
[b] ANCOVA controlling for age, hypertension and hypercholesterolemia.
[c] Indicates a statistically significant difference compared to control solution.
[d] compared to indomethacin + TRAM 34 + UCL1684.
[e] compared to LNAME + indomethacin within the same group, using ANOVA.

## 4. Discussion

The present study demonstrates that: (a) acetylcholine-induced endothelium-dependent relaxations in isolated subcutaneous arteries from healthy humans is dependent on NO release and EDH, whereas prostacyclin appears to play a very minor role; (b) endothelial dysfunction is evident in subcutaneous arteries of diabetic patients and this is predominantly caused by a reduced bioavailability of NO, which in turns, leads to a compensatory increase in EDH-type response; and (c) subcutaneous arteries of diabetic patients have reduced protein expressions of eNOS, PGIS and IP receptor, but augmented COX-2 protein expression. Relaxations of subcutaneous vascular smooth muscle to sodium nitroprusside and salbutamol are not affected in diabetic conditions; however, those to prostacyclin are reduced.

The present observations are consistent with other studies demonstrating impaired relaxations to acetylcholine and bradykinin in subcutaneous small arteries of patients with Type 1 [21] and Type 2 diabetes [13]; however, the relative contribution of NO, EDH and prostacyclin to endothelium-dependent relaxations in arteries

**Table 3**
Endothelium-independent relaxations in subcutaneous arteries from control and diabetic patients.

| | Controls (n = 16) | Diabetics (n = 15) | P Value |
|---|---|---|---|
| Contraction to KCl, 60 mM (mN/mm) | 4.1 ± 0.4 | 4.3 ± 0.3 | 0.716 |
| Contraction to phenylephrine, $10^{-4}$ M (mN/mm) | 6.9 ± 0.5 | 7.1 ± 0.7 | 0.749 |
| Sodium nitroprusside | | | |
| $pEC_{50}$ | 7.0 ± 0.2 | 6.5 ± 0.2 | 0.048[a] **0.134**[b] |
| $R_{max}$ | 90.5 ± 3.9 | 85.6 ± 3.2 | 0.345 |
| Salbutamol | | | |
| $pEC_{50}$ | 4.3 ± 0.3 | 4.8 ± 0.2 | 0.145 |
| $R_{max}$ | 74.0 ± 5.1 | 72.3 ± 5.6 | 0.793 |
| Prostacyclin | | | |
| $pEC_{50}$ | 3.6 ± 0.3 | 3.8 ± 0.4 | 0.304 |
| $R_{max}$ | 70.8 ± 5.9 | 45.8 ± 8.0 | 0.017[a] **0.018**[b] |

$pEC_{50}$, sensitivity to agonists; $R_{max}$, maximal relaxation; n is the number of arteries for different subjects used in the study.
Values were presented as means ± SEM.
[a] Indicates a statistically significant difference between two groups using independent t-test.
[b] ANCOVA controlling for age, hypertension and hypercholesterolemia.

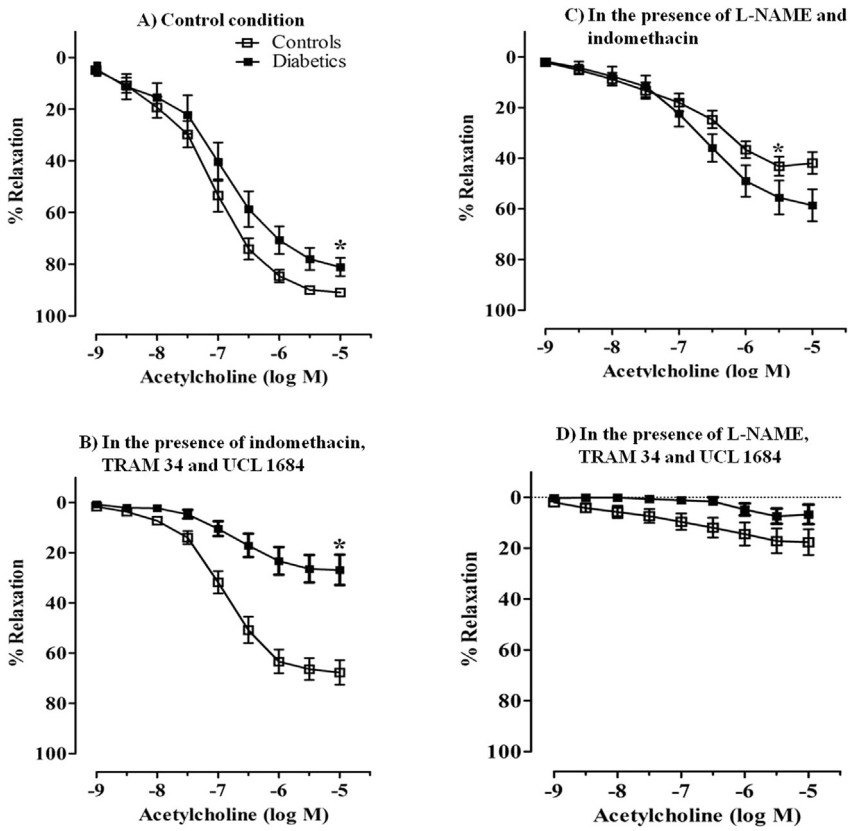


**Fig. 1.** Endothelium-dependent relaxations of subcutaneous arteries from control (n = 16) and diabetic patients (n = 15). Concentration-response curves to acetylcholine ($10^{-9}$ to $10^{-5}$ M) in the (A) absence of pharmacological inhibitor or in the presence of (B) indomethacin ($10^{-5}$ M) plus TRAM-34 ($10^{-6}$ M) and UCL 1684 ($10^{-6}$ M) (NO-mediated response), (C) L-NAME ($10^{-4}$ M) plus indomethacin (EDH-type response) or (D) L-NAME plus TRAM-34 and UCL 1684 (prostacyclin-mediated response). Relaxations are expressed as a percentage of the contraction induced by phenylephrine ($10^{-4}$ M). *Indicates a statistically significant difference in maximal relaxation between the two groups ($P < 0.05$). Values are means ± SEM.

of Type 2 diabetes patients had not been reported.

### 4.1. NO-mediated relaxation

In order to evaluate NO-mediated relaxation, the EDH-type and prostacyclin-mediated responses were inhibited by blockers of endothelial calcium-activated potassium channels and COX, respectively [15]. An impaired NO-mediated response was observed in subcutaneous arteries of diabetics compared to controls. This phenomenon may be due to reduction of NO bioavailability and/or of the sensitivity of smooth muscle cells to NO. The present study shows that the maximal relaxation to the NO donor sodium nitroprusside was comparable between the two study groups; however, the sensitivity of smooth muscle cells to exogenous NO appears to be reduced in diabetics compared to controls, as suggested by the potency (pEC50) of sodium nitroprusside being significantly smaller in the diabetic group. On the other hand, after adjustment of confounders (age and concurrent

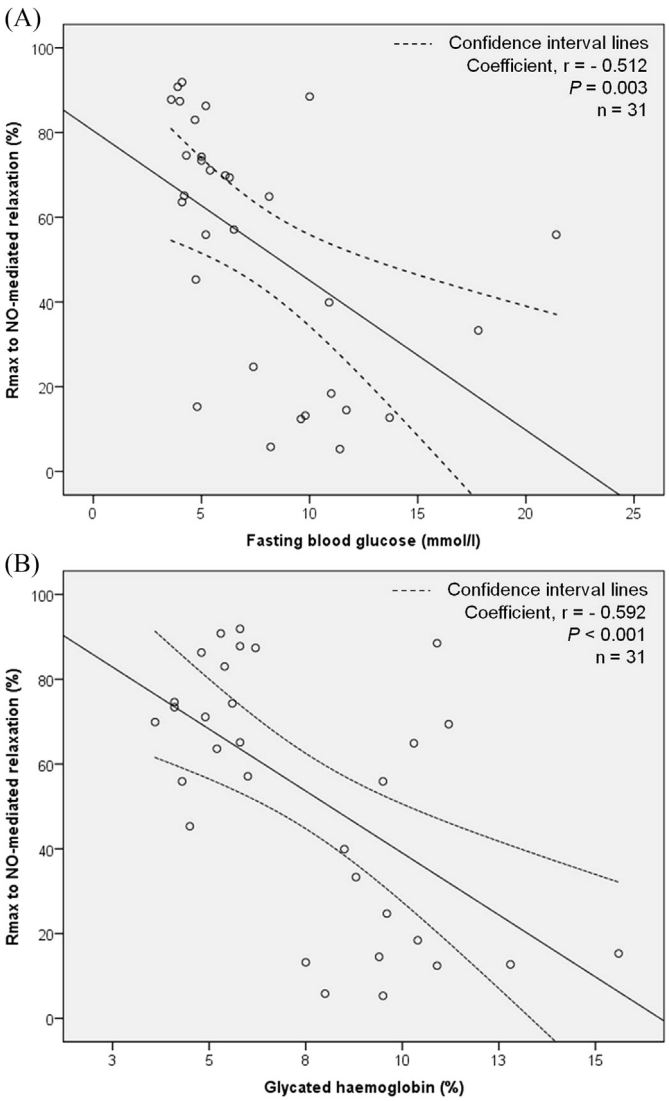


**Fig. 2.** Correlation between NO-mediated relaxations and (A) fasting blood glucose and (B) glycated haemoglobin levels. Statistically significant negative correlations exist between NO-mediated relaxations and both fasting blood glucose and glycated haemoglobin levels in human subcutaneous arteries (n = 31).

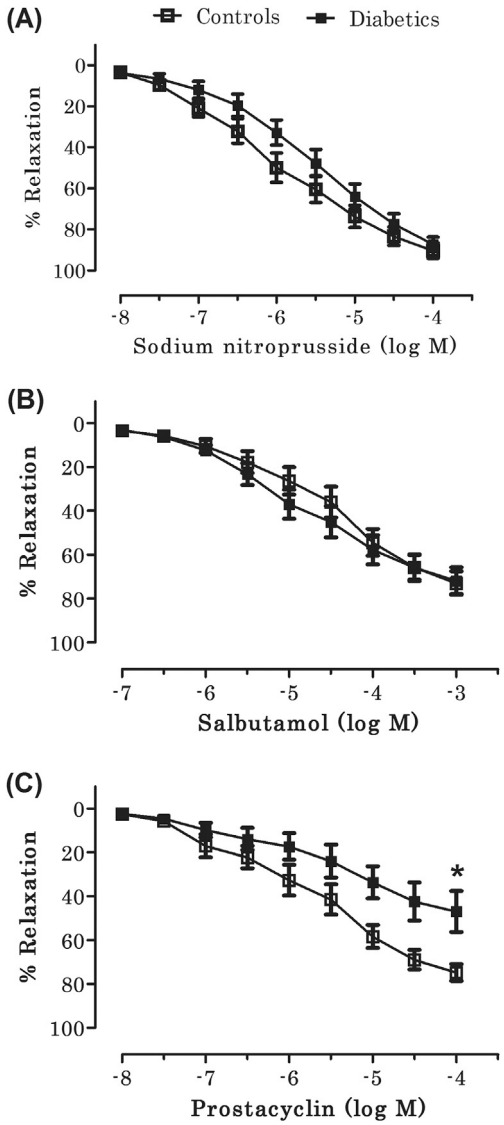


**Fig. 3.** Endothelium-independent relaxations of subcutaneous arteries from control and diabetic patients. Concentration-response curves to (A) sodium nitroprusside ($10^{-8}$ to $10^{-4}$ M), (B) salbutamol ($10^{-7}$ to $10^{-3}$ M) and (C) prostacyclin ($10^{-8}$ to $10^{-4}$ M) in subcutaneous arteries of control (n = 16) and diabetic patients (n = 15). Relaxations are expressed as a percentage of the contraction induced by phenylephrine ($10^{-4}$ M). *Indicates a statistically significant difference in maximal relaxation between the two groups ($P < 0.05$). Data are shown as means ± SEM.

diseases) with ANCOVA analysis, the pEC50 were not significantly different between the two groups. This finding is different to that with the maximal NO-mediated relaxation to acetylcholine, in which statistical significant difference between diabetic and non-diabetic groups can still be observed after adjusting for age and other concurrent diseases with ANCOVA analysis. Moreover, the impairment of endothelium-derived NO-mediated relaxation in diabetic patients was greater than that of endothelium-independent sodium nitroprusside-induced relaxation, thus suggesting that a reduction in smooth muscle sensitivity to NO is unlikely the major contributor to the reduced NO-mediated relaxation observed in diabetic patients.

Changes in NO bioavailability can be caused by a reduction in NO production or an increased in NO breakdown. The present findings confirm a previous observation [10] of the reduced protein presence of eNOS in subcutaneous arteries from diabetic patients; this likely accounts for the impairment in NO-mediated relaxation. The present experiments are also in line with a previous study reporting that NO-mediated relaxation is impaired in internal mammary arteries of Type 2 diabetes patients [22], an impairment attributed to a reduction in eNOS mRNA and protein expressions [22]. Impaired NO-mediated relaxation in diabetes can be due to the hyperglycaemic condition associated with diabetes. The present study shows that a negative correlation exist between NO-mediated relaxation and fasting blood glucose, and glycated haemoglobin levels in humans. These results suggest that elevated blood glucose concentrations and poor glycemic control may induce impairment of NO-mediated relaxation in human subcutaneous arteries. Hyperglycemia induces oxidative stress by increasing superoxide anion production [23] and [24]. When superoxide anions are present at high concentration, they rapidly react with vascular NO to form peroxynitrite, which exerts its effect through oxidation of proteins, initiation of lipid production and nitration of amino acids. Hyperglycemia-induced synthesis of superoxide anions also leads to the uncoupling of eNOS and hence

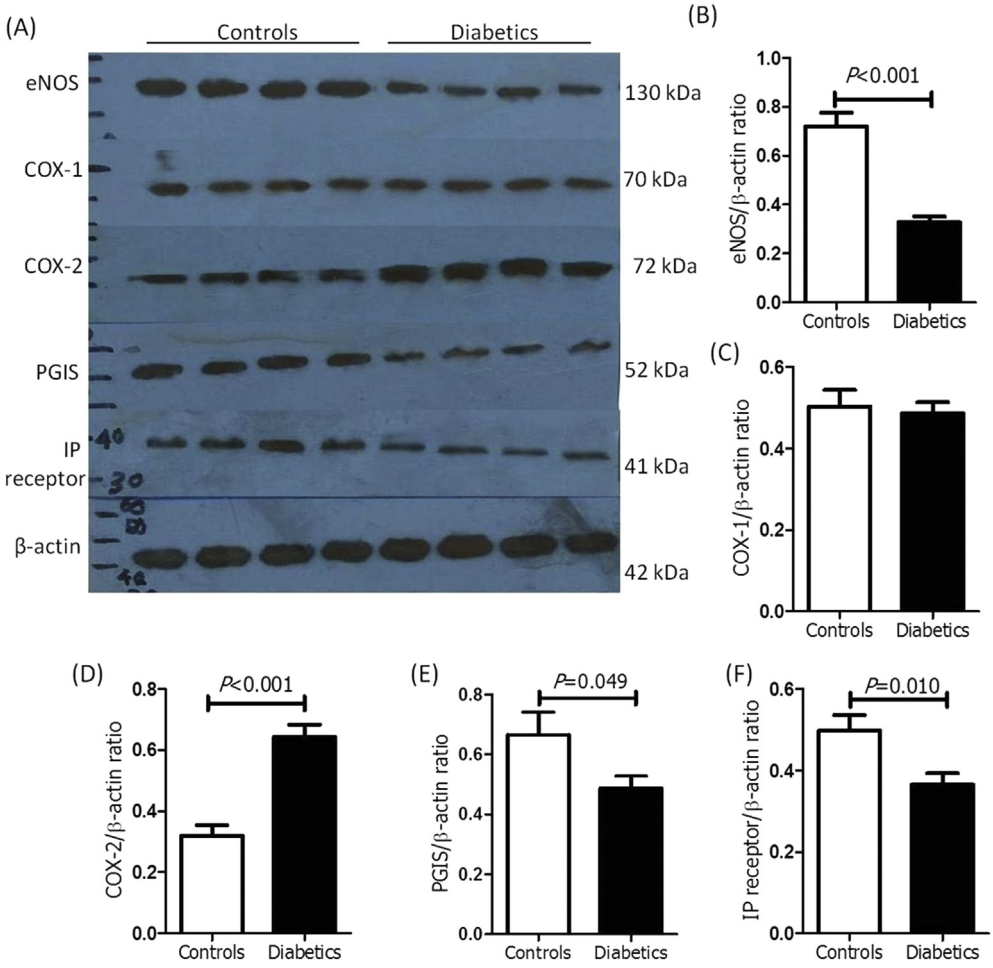


**Fig. 4.** Protein expressions of eNOS, COX-1, COX-2, PGIS and IP receptors in human subcutaneous arteries. (A) Representative blots showing the density of the protein bands in diabetic patient (n = 15) compared to controls (n = 16); (B–F) Graphical representation of the data, normalized to β-actin. Values are means ± SEM. *Indicates a statistically significant difference between the two groups ($P < 0.05$).

dysfunction [25]. Uncoupled eNOS starts making peroxynitrite itself, and eventually becomes an enzyme that generates only superoxide anions instead of NO [26].

### 4.2. EDH-type relaxation

EDH causes relaxation of vascular smooth muscle by hyperpolarizing their cell membrane and closing voltage-operated calcium channels, thus resulting in reduced intracellular free calcium level [27]. EDH-type responses differ among human vascular beds and more than one endothelium-derived mediator/signal may be involved in individual blood vessels. Candidates for EDH include cytochrome P450 metabolites of arachidonic acid, 14, 15-epoxyeicosatrienoic acid (EET) [28], and hydrogen peroxide ($H_2O_2$) in coronary arteries, EET in subcutaneous arteries [29], potassium ion in renal arteries [8], EET in internal mammary arteries [9] and $H_2O_2$ in mesenteric arteries [30]. EDH-type responses in human blood vessels are associated with activation of various types of potassium channels, in particular the $IK_{Ca}$ and $SK_{Ca}$ channels in the endothelium [27]. In this study acetylcholine-mediated relaxation was almost abolished by the combination of L-NAME, TRAM-34 and UCL 1684, thus suggesting that the involvement of other EDH signals that are not mediated through activation of $IK_{Ca}$ and $SK_{Ca}$ channels, such as cytochrome P450 products, if any, would be modest.

EDH, like NO, contributes to endothelium-dependent relaxation [7–9]; it can function in parallel and/or synergistically with NO [31,32]. In view of the complex interactions between NO and EDH, the role of EDH *per se* in endothelium-dependent relaxations were assessed after inhibition of COX and eNOS. Under these conditions, the maximal relaxation to acetylcholine was augmented in arteries from diabetics compared to controls. Possibly, such augmentation could be due to the pharmacological treatments in the diabetic patients [33–36]. However, using univariate analysis, the influence of antidiabetic and antihypertensive drugs on maximal EDH-type relaxation was examined and a correlation between these parameters was not obtained (supplementary Table 1). Therefore, the present findings suggest that in arteries of diabetics with their reduced NO bioavailability, there is a compensatory increase in EDH-type responses. This finding is consistent with a previous study in hypertensive patients in which dilatation of the peripheral microcirculation is mediated by an alternative compensatory mechanism when NO activity was impaired [19,37]. The fact that the EDH-type response becomes apparent only in the presence of impaired NO availability is in line with experimental evidence in the animal indicating that endothelial NO dampens EDH-type responses under physiological conditions and that the latter become more prominent when the production of NO is curtailed [38–41].

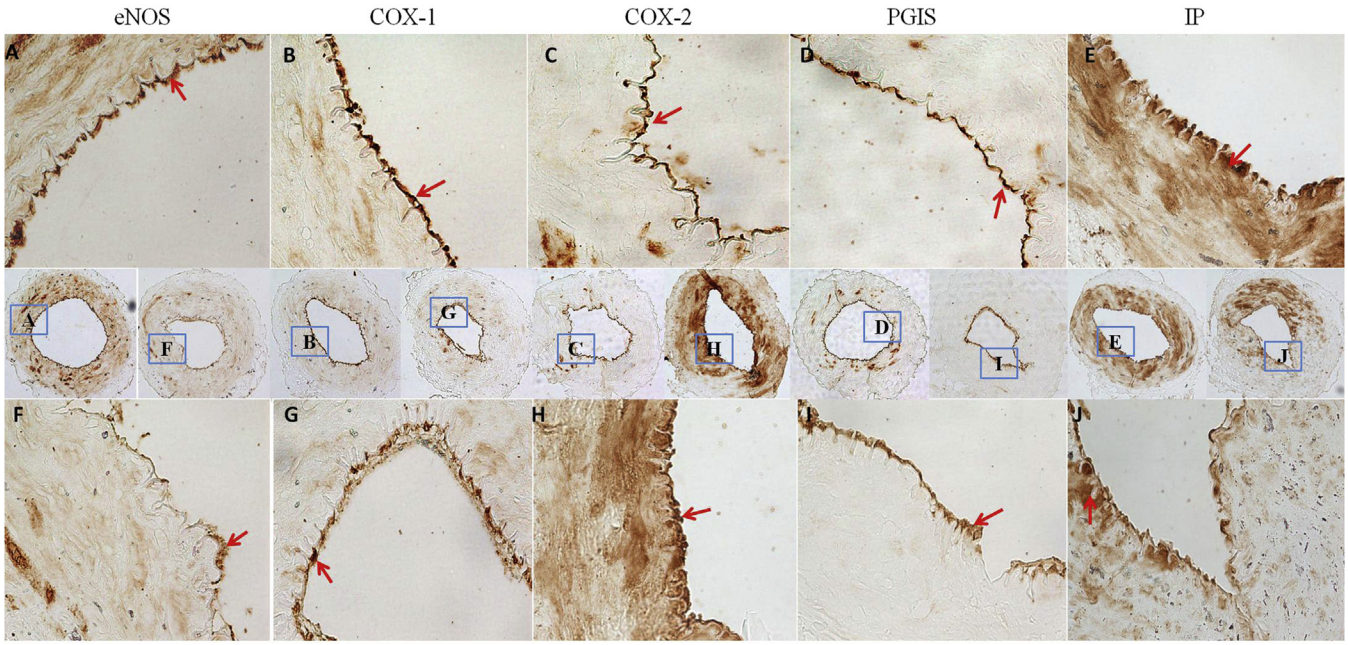


**Fig. 5.** Representative immunostaining of eNOS, COX-1, COX-2, PGIS and IP receptor proteins in human subcutaneous arteries. Light microscopic aspect of subcutaneous arteries from controls (A, B, C, D, E) and diabetic patients (F, G, H, I, J). Brown staining indicated expression of respective proteins (red arrows). Magnifications 20X and 40X. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 4.3. Prostacyclin-mediated relaxation

In the present study, prostacyclin appears to play a minimal role in acetylcholine-induced response in normal subcutaneous arteries. This limited response is preserved in the arteries of diabetic patients. The results from the present Western blotting and immunostaining show an increased expression of COX-2 protein, but a reduced PGIS protein in diabetes. In the coronary arterioles of diabetic patients, increased COX-2 protein expression may contribute to an increased synthesis/release of dilator prostaglandins, most likely prostacyclin [42]. Although there is an increased expression of COX-2 protein in subcutaneous arteries of diabetic patients in the present study, an increased non–NO–mediated and non-EDH-type relaxation was not observed. COX-2 synthesizes prostaglandin $H_2$ which then can be transformed not only to prostacyclin but also to vasoconstrictor prostaglandins, in particular thromboxane $A_2$. However, this is not likely the case in the present study as there was no secondary increase in the tension with higher concentration of acetylcholine in rings treated with L-NAME plus TRAM 34 and UCL 1684 [15,43,44]. The increased abundance of COX-2 may rather reflect the interruption of a negative feedback exerted normally by IP receptor activation [45], an interpretation which is consistent with the reduced response to prostacyclin and the decreased protein presence of IP receptors observed in the arteries of the diabetic patients.

The present study shows an impaired relaxation to prostacyclin in subcutaneous arteries without endothelium of diabetic patients. The role of prostacyclin in mediating vasodilatation not only depends on the amount of prostacyclin, but also on the number of functional IP receptors present in vascular smooth muscle cells and the postreceptor signalling mechanisms [46]. Prostacyclin and salbutamol exert their role as vasodilator through activation of adenylyl cyclase and induce cyclic adenosine 3′,5′-monophosphate (cAMP) signalling. In the present study, the relaxation of subcutaneous arteries to salbutamol was comparable in preparations of the diabetic and control groups. This observation probably rules out the possibility of impaired cAMP signalling and suggests that the impaired relaxation to prostacyclin in subcutaneous arteries of diabetes is due to the reduced number of IP receptors in the vascular smooth muscle cells. This finding is consistent with the Western blotting observations demonstrating that the expression of IP receptor protein is reduced in subcutaneous arteries of diabetic patients.

The present study was performed using wire myograph technique, which measures the isometric tension (constant length myography) of small arteries; this is different to the measurement of isobaric tension (constant pressure myography) which is typically conducted using a pressure myograph. Both wire and pressure myograph systems have been used extensively to study the reactivity and pharmacological responses of small vessels [47,48]. The pressure myograph allows smaller vessels to be examined, down to a diameter of 12 μm [49], whereas wire myograph can be used on vessel with a diameter >100 μm up to 1000 μm [50]. The internal diameter of subcutaneous arteries used in this present study ranged between 280 and 320 μm, thus wire myography is an appropriate method to be used. It has been suggested that *in vivo* situations are more easily reproduced *in vitro* via pressurized vessels than in wire-mounted small vessels [51,52]. Nevertheless, in our isometric experiments, normalization procedure was performed to all vessels to standardize the experimental settings; this procedure also serves, in part, to take into account of the relationship between isometric tension and transmural pressure thereby allowing the arteries to be maintained in the setting that mimic more closely the physiological condition. Furthermore, the passive pressure-internal diameter relationship of the vessels on wire myograph was similar to that obtained using the pressure myograph (this was obtained using Laplace equation to convert wall tension-internal circumference data from wire myograph to effective pressure-diameter characteristics) [51]. Wire myography method requires the vessels to be stretched and flattened rather than to be in a cylindrical shape as in isobaric myography. Although there is a risk of the endothelium being damaged by inserting the

wires through the lumen during the mounting procedure, the risk is rather low with careful handling of the preparation; moreover, the endothelial (and smooth muscle) function can be tested at the start of experiments with the viability test (which was also performed in the present study).

The present experiments have some limitations. First, the experimental protocol was limited by the fact that only small pieces of human subcutaneous arteries could be obtained from the surgical procedures. Thus, it was not possible to obtain sufficient proteins from only the endothelial layer for Western blotting experiments. Therefore, the whole blood vessels were used and the blots detected the presence of COX in both smooth muscle and endothelial cells, although only COX protein expression in endothelial cells is relevant for endothelium-dependent relaxation. On the other hand, eNOS and PGIS proteins are likely to be present mainly in the endothelial cells, while IP receptor are expressed mainly in vascular smooth muscle cells. Second, the present study only reported the contraction to a single concentration of KCl and phenylephrine, thus demonstrating that arteries from non-diabetic and diabetic patients were contracted to a comparable level before the examination of their responses to different relaxing agents. Therefore, the data do not provide any additional information on whether or not diabetic would alter the contractile responses in subcutaneous arteries, which can only be studied after the construction of a complete concentration–response curve of KCl and phenylephrine. Third, the EDH-type relaxation was taken as the remaining relaxation to acetylcholine after inhibition of the activity of eNOS and COX. Using this approach, the EDH responses are unmasked from the inhibitory effect of endothelial NO [38,39], and from the opposing effect of COX-dependent contracting factors [43–45]. However, this approach cannot unmask the EDH-type relaxation due to $H_2O_2$ generated by the uncoupled eNOS [6,30]. Since eNOS uncoupling occurs mainly under pathological conditions, such as diabetes [6,25,26,30], the degree of EDH-type relaxation that was masked by the inhibition of the uncoupled eNOS, if any, would likely be greater in diabetic blood vessels than in non-diabetic ones. Therefore, the present finding that diabetes is associated with a compensatory increase in EDH-mediated responses would still be valid. Further experiments would be needed to address the role of eNOS uncoupling in the changes in vascular responses in diabetic conditions. Lastly, it is not possible to have complete match for the characteristics of the patients in the two study groups, in particular as regards to age and other concurrent disorders (hypertension and hypercholesterolemia). Aging has been linked to the deterioration of endothelial function resulting in the alteration of the release of endothelium-derived vasoactive substances [18,53]. In hypertension, blood vessels are exposed to increased sheer stress and this chronic hypertensive state causes progressive damage to the endothelial layer [54,55]. There is a reduced bioavailability of NO and prostacyclin in the endothelial dysfunction of aging animals [6,54,56,57]. EDH-type responses, however, remains present in aging or hypertensive animals [53,58,59]. Likewise, hypercholesterolemia is associated with endothelial dysfunction, mainly with a reduced NO bioavailability [60–63]. Thus, results for vascular responses in this study were controlled for age and presence of hypertension and hypercholesterolemia using ANCOVA analysis [38,64,65]. The impairment observed in endothelium-dependent and -independent responses in diabetic patients persisted even after controlling for these covariates. In the present study, there was not statistically significant gender difference between the two study groups. In view of the effect of gender on endothelial function [66–68], univariate analysis was performed and did not suggest a correlation between genders and NO-mediated or EDH-type relaxation in human subcutaneous arteries.

## 5. Conclusion

In conclusion, the present study demonstrates that subcutaneous arteries of diabetic patients exhibit impaired endothelium-dependent relaxations. The impairment is due predominantly to a diminished NO bioavailability, but is counteracted in part by an increased contribution of EDH-type responses.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.niox.2015.12.007.

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