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ROLE OF ENDOTHELIAL CONTRACTING AND RELAXING FACTORS IN THE MECHANISM OF ENDOTHELIAL DYSFUNCTION IN SUBCUTANEOUS ARTERIES (MICROVASCULATURE) OF DIABETIC PATIENTS.

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2015

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SCHOOL OF MEDICAL SCIENCES

UNIVERSITI SAINS MALAYSIA

UNIVERSITY RESEARCH GRANT
FINAL REPORT
Geran Penyelidikan Universiti
Laporan Akhir

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	PARTICULARS OF RESEARCH / *MAKLUMAT PENYELIDIKAN:*
i)	**Title of Research:** Role Of Endothelial Contracting And Relaxing Factors In The Mechanism Of Endothelial Dysfunction In Subcutaneous Arteries (Microvasculature) Of Diabetic Patients
i)	
	PERSONAL PARTICULARS OF RESEARCHER:
)	**Name of Research Leader:** Professor Aida Hanum Ghulam Rasool
	Name of Co-Researcher: Prof. Dr Imran Yusof Dr Rapeah Suppian Dr Wan Azman Wan Sulaiman Zulkifli Sanip Professor Paul M Vanhoutte (Hong Kong University) Dr Susan Leung (Hong Kong University)
i)	**School/Institute/Centre/Unit:** School of Medical Sciences
	Duration of this research: 4 years **From** : 01 Jan 2011 **To** : 31st Dec 2014

G. **COMPREHENSIVE TECHNICAL REPORT**

Applicants are required to prepare a comprehensive technical report explaining the project.
(This report must be attached separately)

See attachment 1 (Not for publication)

List the key words that reflect our research:

English	Bahasa Malaysia
Endothelial dysfunction	Disfungsi endotelium
Type 2 Diabetes	Diabetes taip 2
Microcirculation	Salur darah mikro
Nitric oxide	Nitrik oksida
Endothelium derived hyperpolarising factor (EDHF)	Penghiperkutuban-bergantung endotelium (EDH).
Prostacyclin	Prostasiklin

H. a) **Results/Benefits of this research**

No.	Category/Number:	Promised	Achieved (All promised achived)
1.	**Research Publications (Specify target journals)**	4	**4 (see list for details)** - 2 published (International – ISI, Scopus, Pubmed) - 1 undergoing minor revision (International - ISI) - 1 under review (international)
2.	**Human Capital Development**		
	a. Ph. D Students	1	1
	b. Masters Students	0	0
	c. Undergraduates (Final Year Project)	0	0
	d. Research Officers	0	0
	e. Research Assisstants	1	1
	f. Other: Please specify	0	1 international award from Asian Society of Vascular Biology
3.	**Patents** *Paten*	0	0
4.	**Specific / Potential Applications** *Spesifik/Potensi aplikasin*	1	1
5.	**Networking & Linkages** *Jaringan & Jalinan*	1	1 (Hong Kong University: - student attachment - staff exchange visits - contribution of chemicals, dissecting plates by HKU without charge
6.	**Possible External Research Grants to be Acquired**	1	1 (FRGS acquired 2014)

- Kindly provide copies/evidence for Category 1 to 6.

b) Equipment used for this research.

Items *Perkara*	Approved Equipment	Approved Requested Equipment	Location
Specialized Equipment	Dual Chamber Myograph		Pharmacology Vascular Laboratory
Facility Kemudahan	Dissecting microscope Power lab Facilities for western blot, immunohistochemistry		School of Medical Sciences (Pharmacology Dept, Central Research Lab)
Infrastructure Infrastruktur			

- Please attach appendix if necessary.

H.

COMMENTS OF PTJ'S RESEARCH COMMITTEE
KOMEN JAWATANKUASA PENYELIDIKAN PERINGKAT PTJ

General Comments:
Ulasan Umum:

Excellent research outcome. 2 nos publications in ISI. Journal with total impact factor 4.7. networking, PhD. ~~no~~ student

Recommended to be closed.

ROLE OF ENDOTHELIAL CONTRACTING AND RELAXING FACTORS IN THE MECHANISM OF ENDOTHELIAL DYSFUNCTION IN SUBCUTANEOUS ARTERIES (MICROVASCULATURE) OF DIABETIC PATIENTS

Study Aims:

This study aims to examine the signalling pathways underlying endothelium-dependent responses in subcutaneous arteries of humans with Type 2 diabetes mellitus (T2DM) by assessing the relative contributions of nitric oxide (NO), prostacyclin and endothelium derived hyperpolarising factor (EDH) to responses to endothelium-dependent and independent agonists.

Materials and methods

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 to 65 years old were recruited among those undergoing lower limb surgical procedures.

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-7H-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 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.

Subcutaneous tissues from lower limb surgical procedures were transported to Pharmacology Vascular laboratory in ice cold physiological salt solution. Subcutaneous arteries were dissected free of connective tissue and fat, and then cut into rings. Care was

taken during the dissecting procedure to protect the endothelium from damage. In some preparations, the endothelium was removed. 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 allowed to equilibrate, before being subjected to a normalisation process, which determines the passive tension characteristics of each individual preparation. The rings were then exposed to potassium chloride and phenylephrine. After steady state contraction to phenylephrine had been reached, acetylcholine was added to assess the presence [or absence] of endothelium. [14].

To study endothelium-dependent responses, the rings were contracted with phenylephrine. When the phenylephrine-induced contraction had reached steady state, acetylcholine was added in a cumulative manner. 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 plus TRAM-34 and UCL1684 (10^{-6} M) [15] ; b) EDH-type relaxations: the rings were incubated with indomethacin plus L-NAME (10^{-4} M) [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 before the administration of phenylephrine.

Endothelium-independent responses were determined in rings without endothelium. The rings were contracted with phenylephrine, and exposed to cumulative concentrations of sodium nitroprusside, salbutamol, or prostacyclin.

Western blotting and immunohistochemistry were performed as described [10]. 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}) and area under the curve (AUC) were 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 was used to analyse non-categorical data such as medications and underlying diseases. 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]. P values less than 0.05 were considered to indicate statistically significant differences.

Results

Patient characteristics, concurrent medical history and underlying diseases are summarized in Table 1.

Contractions to KCl and phenylephrine: No significant differences in developed isometric tension in response to KCl and phenylephrine were observed between the study groups (Table 2 and 3).

Endothelium-dependent relaxations: Control Response: The maximal relaxation to acetylcholine was significantly attenuated in subcutaneous arteries from diabetics compared to controls.

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

EDH-type Responses 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).

Prostacyclin-mediated Responses 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).

Endothelium-independent responses

There was no significant difference in the responses 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). 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 and diabetic patients.

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. (Upper panel) Representative blots showing the density of the protein bands in diabetic patient (n = 15) compared to controls (n = 16). (Lower panel)

Conclusion

The present study demonstrates that: (a) acetylcholine-induced endothelium-dependent responses 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. Responses of subcutaneous vascular smooth muscle to sodium nitroprusside and salbutamol are not affected in diabetic conditions; however, those to prostacyclin are reduced.

Acknowledgement

We thank Prof. Dr. Wan Mohd Zahiruddin for statistical consultations.

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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
Age (years)	31.5 ± 3.1	55.3 ± 2.2	< 0.001*
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*
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*
FBG (mmol/l)	4.7 (1.1)	10.5 (3.9)	< 0.001*
HbA_{1C} (%)	5.3 (1.5)	9.6 (2.3)	< 0.001*
Creatinine (μmol/l)	82.3 ± 2.5	88.9 ± 5.4	0.296
Underlying diseases; n(%)			
Hypertension	0 (0)	9 (45.0)	0.002*
Hypercholesterolemia	0 (0)	5 (25.0)	0.031*
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*
Lipid lowering	0 (0)	6 (30.0)	0.024*
NSAIDs	2 (12.5)	1 (5.0)	0.574
Oral antidiabetics	0 (0)	16 (80.0)	< 0.001*
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. * Indicates a statistically significant difference between the two groups.

ıble 2. Endothelium-dependent relaxations in subcutaneous arteries from control and diabetic ıtients

	Controls (n = 16)	Diabetics (n = 15)	P value
ternal arterial diameter (μm)	298.8 ± 2.4	301.3 ± 3.4	0.531
ean increase in tension to KCl (mN/mm)	5.4 ± 0.5	4.8 ± 0.4	0.340
ean increase in tension to phenylephrine iN/mm)	7.1 ± 0.5	7.2 ± 0.4	0.966
ntrol 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*
			0.012$^{\#}$
lomethacin			
RAM 34 + UCL 1684			
pEC_{50}	6.8 ± 0.1	6.5 ± 0.2	0.154
R_{max}	73.6 ± 3.5	31.6 ± 6.8	< 0.001*
			0.003$^{\#}$
NAME + Indomethacin			
pEC_{50}	6.8 ± 0.2	6.5 ± 0.2	0.411
R_{max}	43.1 ± 4.3	63.5 ± 4.6	0.003*
			0.029$^{\#}$
NAME			
TRAM 34 + UCL 1684			
pEC_{50}	7.0 ± 0.2	6.7 ± 0.3	0.383
R_{max}	19.6 ± 4.4	9.9 ± 3.2	0.088

C_{50}, sensitivity to agonists; R_{max}, maximal relaxation; n is the number of arteries from different subjects ed in the study.

lues were presented as means ± SEM.

ndicates a statistically significant difference between two groups using the independent t-test.

NCOVA controlling for age, hypertension and hypercholesterolemia.

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

	Controls (n = 16)	Diabetics (n = 15)	P value
Mean increase in tension to KCl (mN/mm)	4.1 ± 0.4	4.3 ± 0.3	0.716
Mean increase in tension to phenylephrine (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* **0.134**[#]
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* **0.018**[#]

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.
* Indicates a statistically significant difference between two groups using independent t-test.
[#] ANCOVA controlling for age, hypertension and hypercholesterolemia.

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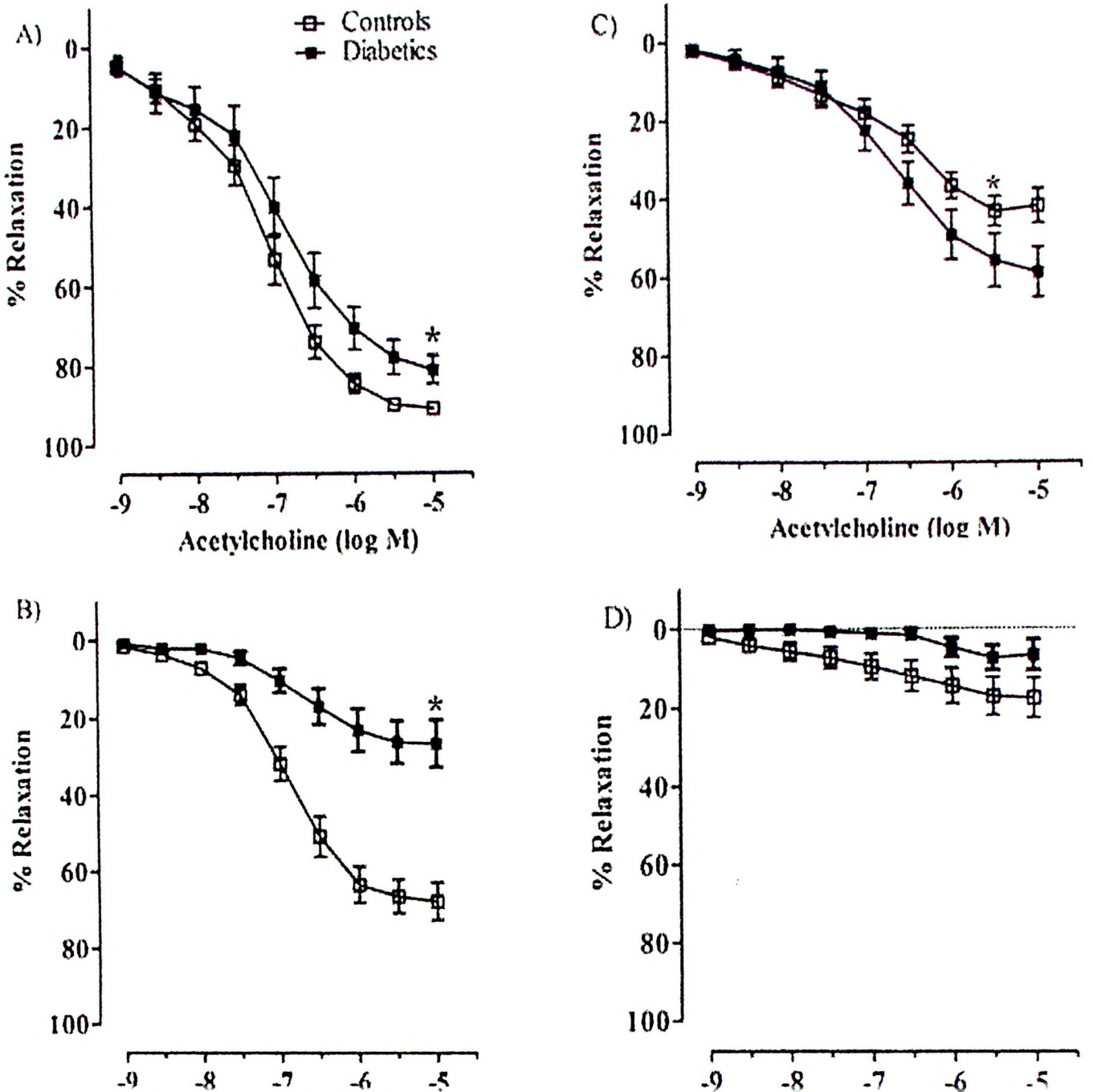

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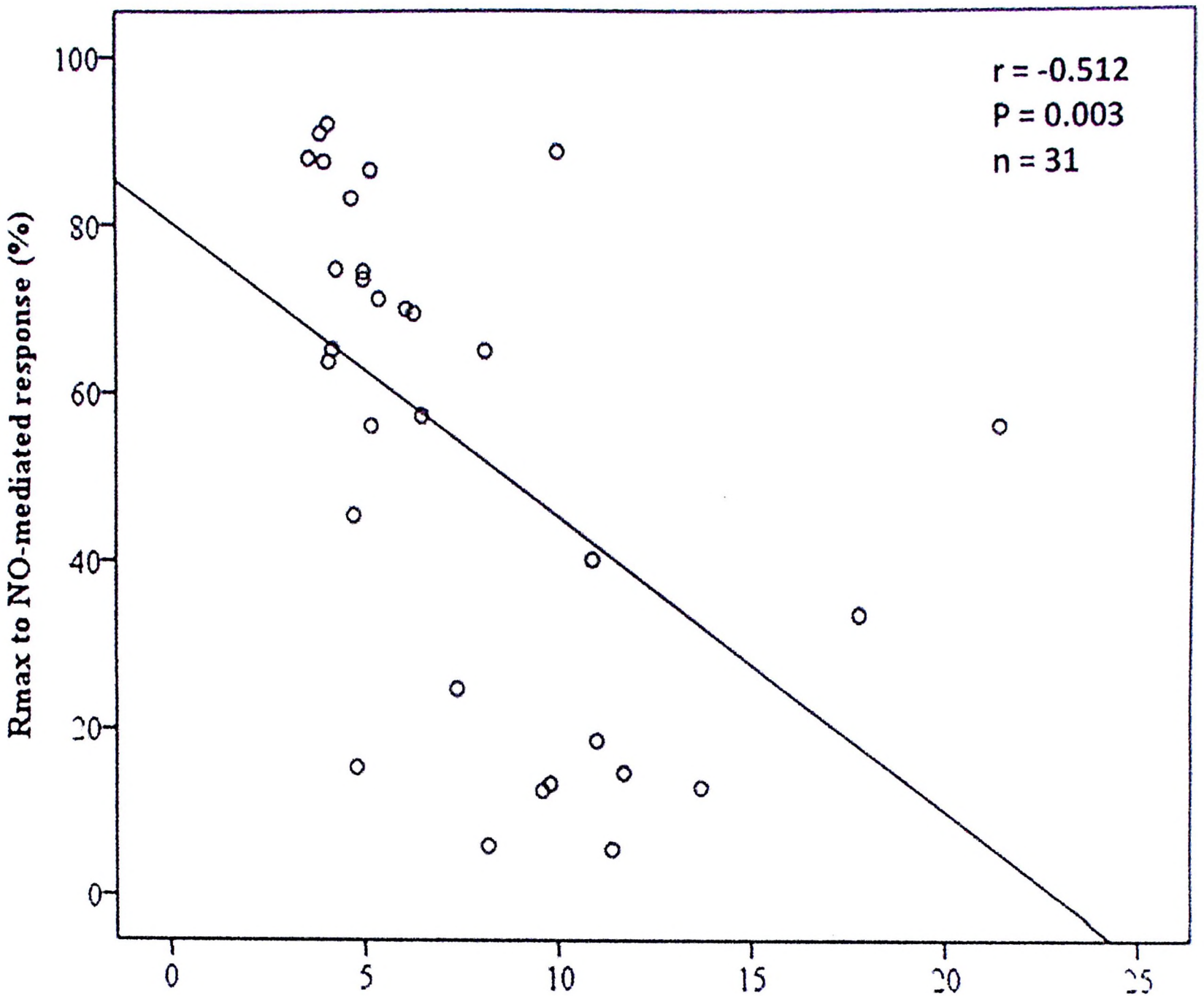

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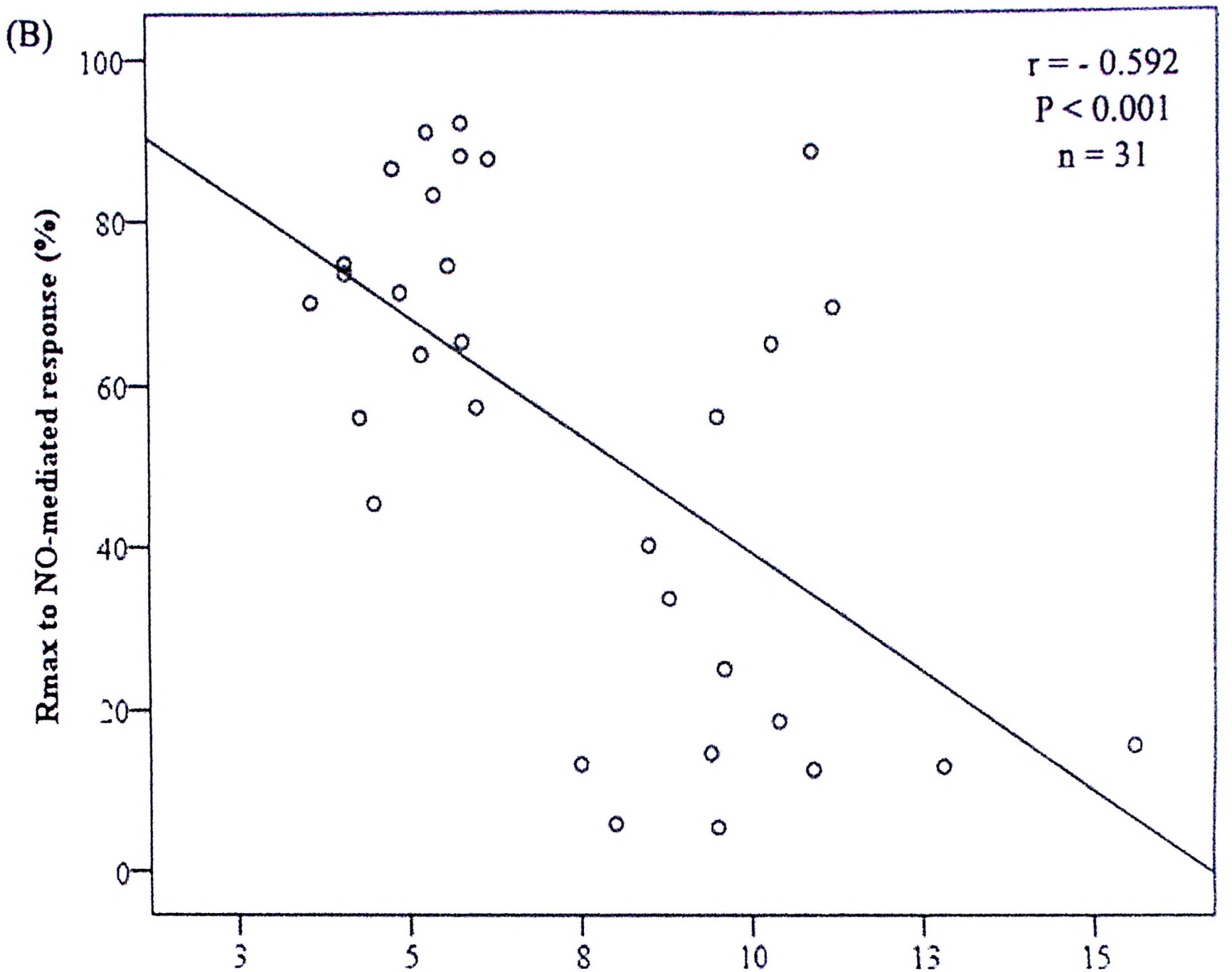

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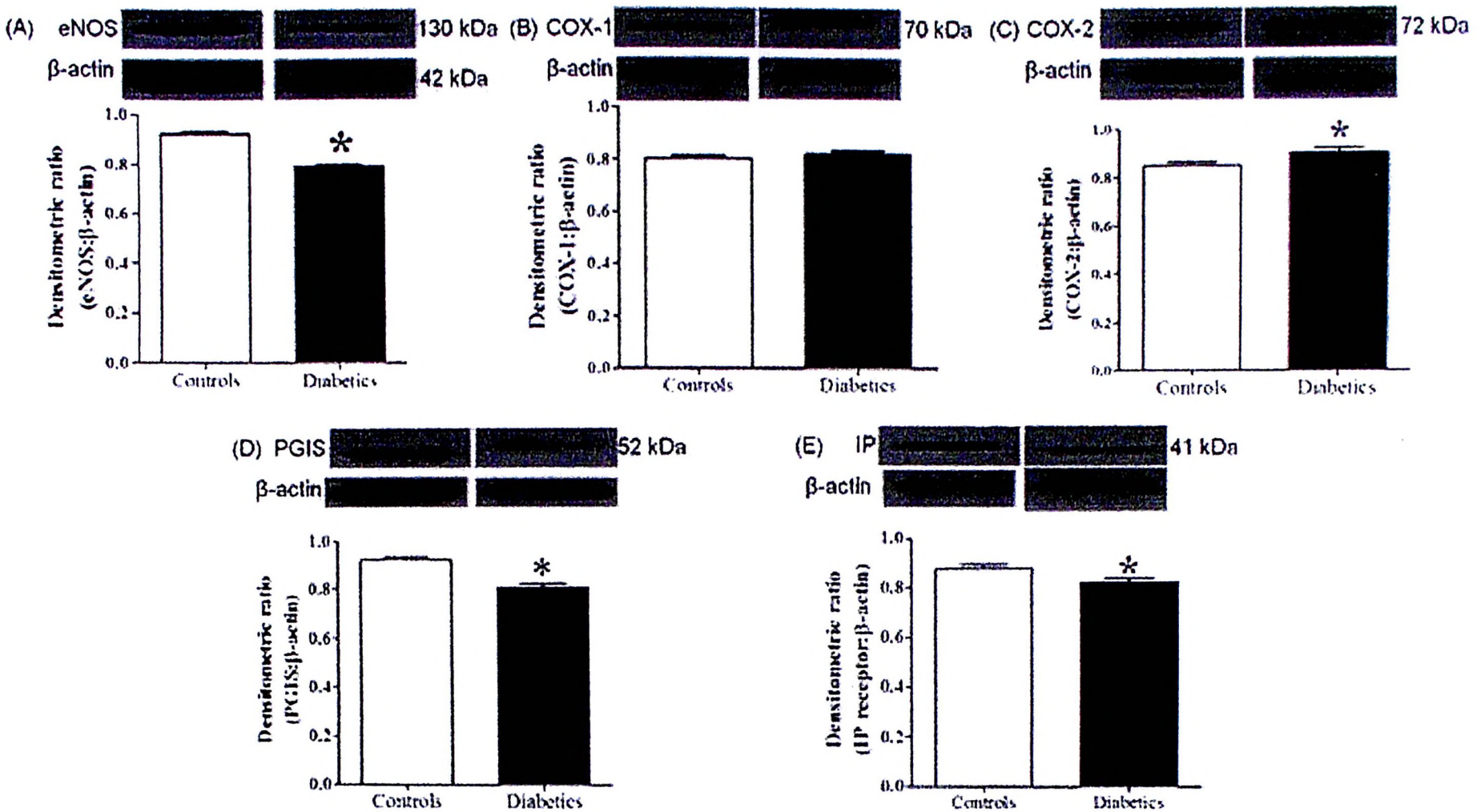

ISI Impact factor = 1.283

Tohoku J. Exp. Med., 2013, 231, 217-222

Reduced Expression of Prostacyclin Synthase and Nitric Oxide Synthase in Subcutaneous Arteries of Type 2 Diabetic Patients

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Diabetic endothelial dysfunction is characterized by impaired endothelium-dependent relaxation. In this study, we measured the expression of endothelial nitric oxide synthase (eNOS), cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), prostacyclin synthase (PGIS), and prostacyclin receptor (IP) in subcutaneous arteries of type-2 diabetic and non-diabetic patients. Subcutaneous arteries were dissected from tissues from seven diabetics (4 males and 3 females) and seven non-diabetics (5 males and 2 females) aged between 18 to 65 years, who underwent lower limb surgical procedures. Diabetics had higher fasting blood glucose compared to non-diabetics, but there were no differences in blood pressure, body mass index and age. Patients were excluded if they had uncontrolled hypertension, previous myocardial infarction, coronary heart disease, renal or hepatic failure and tumor. The relative expression levels of eNOS, COX-1, COX-2, PGIS and IP receptor were determined by Western blotting analysis, normalized with the β-actin level. Increased expression of COX-2 was observed in subcutaneous arteries of diabetics compared to non-diabetics, whereas the expression levels of eNOS and PGIS were significantly lower in diabetics. There were no significant differences in expression levels of COX-1 and IP receptor between the two groups. Immunohistochemical study of subcutaneous arteries showed that the intensities of eNOS and PGIS staining were lower in diabetics, with higher COX-2 staining. In conclusion, type-2 diabetes is associated with higher COX-2 expression, but lower eNOS and PGIS expression in subcutaneous arteries. These alterations may lead to impaired endothelium-dependent vasodilatation, and thus these proteins may be potential targets for protection against the microvascular complications of diabetes.

Keywords: diabetes; endothelial dysfunction; microcirculation; nitric oxide synthase; prostacyclin synthase
Tohoku J. Exp. Med., 2013 November, 231 (3), 217-222.

Introduction

It has been estimated that 285 million (6.4%) people orldwide were afflicted with diabetes mellitus in 2010, d that diabetes would affect 439 million (7.7%) adults by 30 (Shaw et al. 2010). Micro and macrovasculopathy are e major complications of diabetes and thus cardiovascular sorders (CVD) account for up to 80% of premature mor-lities due to diabetes (Winer and Sowers 2004).

The endothelium, a monolayer of cells lining and covering the internal surface of blood vessels, plays a crucial role in regulating vascular tone by releasing endothelium-derived contracting and relaxing factors. Imbalance in the production of these factors is a characteristic of endothelial dysfunction, one key event in the development of microvascular complications in human and animal models of diabetes (Pieper 1998; De Vriese et al. 2000; Matsumoto et al. 2005). Indeed, endothelium-dependent vasodilatation is impaired in arterioles of humans with type-2 diabetes (Georgescu et al. 2011; Kizhakekuttu et al. 2012).

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Nitric oxide (NO) and prostacyclin (PGI_2) are two major endothelium-derived relaxing factors. NO is synthesized from L-arginine, in the presence of oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) in a reaction catalyzed by NO synthase (NOS). Three NOS isoforms are expressed in mammalian cells: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Felaco et al. 2001). Of those, eNOS is expressed constitutively in endothelial cells; NO produced by eNOS is essential for the regulation of vascular tone (Duda et al. 2004). Blunted NO-mediated relaxations have been reported in coronary (Belmadani et al. 2008; Gao et al. 2008) and mesenteric arteries (Lagaud et al. 2001) from diabetic (db/db) mice. These observations may be due to the decreased expression of eNOS in the small arteries of diabetics.

Cyclooxygenase (COX) transforms arachidonic acid into prostaglandin endoperoxides (PGH_2). PGH_2 is converted further into PGI_2 by prostacyclin synthase (PGIS), into thromboxane A_2 (TXA_2) by TXA_2 synthase and into other prostaglandins by the respective synthases (Zou et al. 2004). PGI_2 stimulates prostacyclin receptors (IP receptor) in vascular smooth muscles causing relaxation under physiological conditions (Linton and Fazio 2008). Both PGH_2 and TXA_2 oppose the action of PGI_2 by activation of the thromboxane receptors (TP receptor) in smooth muscles causing vasoconstriction (Vanhoutte 2011).

Two isoforms of COX have been identified in blood vessels. COX-1 is constitutively expressed in a wide variety of tissues and participates in physiological responses, whereas COX-2 is an inducible enzyme. In coronary arterioles of humans with diabetes mellitus the expression of vascular COX-2 protein is increased compared to non-diabetics (Szerafin et al. 2006). COX-2 is also widely expressed in atherosclerotic plaques and arterial walls of patients with atherosclerosis (Baker et al. 1999). The expression levels of both COX-1 and COX-2 proteins are increased in the femoral arteries of diabetic rats (Shi and Vanhoutte 2008).

There is a lack of information on the expression levels of COX-1, PGIS and IP receptor proteins in the microcirculation of diabetic patients. Thus, the present study aims to determine the expression levels of eNOS, COX-1, COX-2, PGIS and IP receptor proteins in subcutaneous arteries of diabetic patients compared to non-diabetic controls. Information gained may help to elucidate the roles of these proteins in the development of endothelial dysfunction in the human diabetic microcirculation (Szerafin et al. 2006; Georgescu et al. 2011).

Materials and Methods

Patient Characteristics

This study was approved by the Human Ethical Committee of Universiti Sains Malaysia; work conducted in this study conformed to the provisions of the Declaration of Helsinki. All of the patients gave their informed consent to participate in this study, which was conducted at the Universiti Sains Malaysia Hospital. Subcutaneous tissues were obtained from patients who underwent lower limb surgical procedures such as wound debridement, amputations, fracture stabilization and skin grafting. Specimens from patients who were operated on for any tumors were excluded. Subjects consisted of seven patients with type-2 diabetes mellitus (4 males and 3 females) while the controls consisted of seven non-diabetic subjects (5 males and 2 females). All patients were between 18 to 65 years of age. Patients were excluded if they had uncontrolled hypertension, previous myocardial infarction, coronary heart disease and renal or hepatic failure. Evaluation of patients consisted of a physical examination, determination of body mass index (BMI) and measurements of systolic (SBP) and diastolic (DBP) blood pressures. Blood was collected to analyze the fasting blood glucose (FBG).

Protein preparation and Western blot analysis

Subcutaneous tissues were transported to the laboratory in ice cold physiological saline solution (PSS). They were dissected and homogenized in the lysis buffer (NaCl 150 mM, Tris 50 mM, Triton-X 1%, sodium dioxycholate 10%, sodium dodecyl sulfate (SDS) 0.1%, ethylenediaminetetraacetic acid (1 mM) and a protease inhibitor cocktail 0.05% (Sigma Chemical Co., St Louis, MO, USA). Samples were then centrifuged at 3,000 g for 20 minutes at 4°C, and the supernatants were collected. Protein concentrations were determined using the Bradford assay. In all immunoblot experiments, the same amount of protein was loaded in each lane of 10% SDS-polyacrylamide gel. After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride (PVDF) Immobilon membranes (Millipore Corp., Billerica, MA, USA) and incubated for two hours at room temperature with primary antibodies against eNOS (1:15,000 Abcam, Cambridge, UK), COX-1 (1:10,000; Abcam), COX-2 (1:10,000; Abcam), PGIS (1:10,000; Abcam) and IP receptors (1:10,000; Abcam). The same blot was stripped and then reprobed with other proteins. To normalize for the amount of proteins, β-actin was used as a loading control (1:10,000; Sigma Chemical Co.). Membranes were then incubated in horseradish peroxidase (HRP)-conjugated polyclonal secondary antibody, (1:10,000; Abcam) in blocking buffer for one hour at room temperature. Membranes were incubated with an Immobilon Western chemiluminescent HRP substrate (Millipore Corp.) for five minutes and exposed to CL-Xposure films (Thermo Fisher Scientific, Rockford, IL, USA). The intensity of protein bands representing the amount of proteins was measured with Image J software (http://rsb.info.nih.gov/ij/). The relative protein presence of eNOS, COX-1, COX-2, PGIS or IP receptors was expressed as percentage of the total amount of protein (indicated by the intensity of protein band for β-actin) in the same patient sample (Sugimura et al. 2010; Lee and Lee 2011).

Immunohistochemistry

Subcutaneous skin samples were fixed and processed for 18 hours with 4% paraformaldehyde solution in PBS at pH 7.4 and embedded in paraffin. Samples were cut to 5 μm thick slices, and collected on poly-L-lysine slides; they underwent de-paraffinization in xylene and rehydration in graded alcohol solutions. Endogenous peroxidases were blocked by 0.3% hydrogen peroxide and antigen retrieval was performed in citrate buffer, pH 6.0. Primary antibodies against eNOS (1:100), COX-1 (1:200), COX-2 (1:200), PGIS (1:100) and the IP receptor (1:100) were diluted with PBS-T and incubated on slides for two hours at room temperature. After having been rinsed

wo times for five minutes with PBS-T solution, the slides were incuated with secondary antibody conjugated to HRP (1:200) for one our at room temperature. The immunoreactions were visualized sing 3, 3'diamino-benzidine-tetrahydrochloride substrate (DAB) Roche, Mannheim, Germany).

)ata Analysis

The Statistical Package for Social Sciences software for Windows, Version 20.0 (SPSS Inc. 2011) was used to analyze the ata. In all cases, *n* refers to the number of patients. Differences etween the two groups were assessed by independent *t*-test. All data ere expressed as the mean ± standard deviation. Differences were onsidered significant at $p < 0.05$.

Results

'atient characteristics

The diabetic patients were slightly older than the non-iabetic controls; however the difference was not statistically significant (Table 1). The FBG concentration was significantly higher in the diabetic patients (Table 1). BMI and BP did not differ between the groups. One patient in the iabetic group was treated for hypertension and hyperlipidmia. All patients in the diabetic group took hypoglycemic rugs. None of the patients in the non-diabetic control roup was taking any regular medication.

Vestern blotting

Western blot analysis demonstrated that the expression evels of eNOS and PGIS were significantly lower in the ubcutaneous arteries from diabetic patients compared to on-diabetic controls (Fig. 1A and B): eNOS expression, iabetic 13.7 ± 3.8% vs. non-diabetic 26.9 ± 1.8% ($p <$ .001); and PGIS, diabetic 14.2 ± 4.3% vs. non-diabetic 1.6 ± 5.1% ($p = 0.013$). COX-2 expression was significantly higher in the subcutaneous arteries from diabetic atients compared to non-diabetic controls (diabetic 23.5 ± 7% vs. non-diabetic 11.4 ± 2.9%, $p < 0.001$). The expreson levels of COX-1 and IP receptor proteins were not sigficantly different in the subcutaneous arteries from both roups: COX-1, non-diabetic 12.6 ± 2.6% vs. diabetic 14.2 ± 2.6% ($p = 0.264$); and IP receptor, non-diabetic 8.5 ± 1.6% vs. diabetic 7.66 ± 1.3% ($p = 0.308$).

Immunohistochemistry

Immunohistochemical analysis demonstrated that the intensities of immunoreactive eNOS and PGIS protein were higher in the subcutaneous arteries of the non-diabetic controls compared to the diabetic patients (Fig. 2). The intensity of immunoreactive COX-2 protein was higher in the arteries of diabetic patients compared to the non-diabetic controls. There were no significant differences in the COX-1 and IP proteins staining between the groups.

Discussion

The results from this study show that the expression of eNOS and PGIS proteins is decreased, but that of the COX-2 protein is increased in the subcutaneous arteries of the diabetic patients compared to the non-diabetic controls.

The decreased eNOS protein expression observed in the subcutaneous arteries of diabetic patients may be related to the high plasma glucose levels, often associated with diabetes. The eNOS protein expression in cultured human coronary arterial and aortic endothelial cells incubated in media containing a high glucose concentration (25 mM) is reduced compared to that in the lower glucose concentration (5.5 mM) (Ding et al. 2000; Srinivasan et al. 2004). In cultured human aortic endothelial cells, hyperglycemia increased mitochondrial production of reactive oxygen species (ROS). Mitochondrial ROS, in turn, activate the transcription factor activator protein (AP-1) to bind to DNA. This binding inhibits the transcription of the eNOS gene, thus resulting in the reduction of eNOS protein expression (Srinivasan et al. 2004). Reduction in eNOS protein expression in diabetes may lead to the loss of vasodilator responses. This conclusion is supported by the reduced endothelium-dependent vasodilatation observed in subcutaneous arterioles of patients with type-2 diabetes (Georgescu et al. 2011). This reduction was associated with decreased eNOS protein expression and reduced endothelial NO pro-

Table 1. Patient Characteristics.

Characteristics	Non-Diabetic ($n = 7$)	Diabetic ($n = 7$)	p value
Sex (Male/Female)	5/2	4/3	
Age (year)	39 ± 12.7	49 ± 4.8	0.110
SBP (mmHg)	129 ± 5.5	129 ± 8.5	0.520
DBP (mmHg)	81 ± 4.0	75 ± 10.9	0.224
Weight (kg)	67 ± 6.4	68 ± 7.6	0.748
Height (cm)	162 ± 7.6	159 ± 5.5	0.367
BMI (kg/m^2)	25 ± 1.5	26 ± 1.6	0.277
FBG (mmol/l)	4.8 ± 0.5	9.7 ± 2.5*	0.002

Data are expressed as mean ± S.D. *$p < 0.05$, diabetic patients compared to non-diabetic controls. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass Index; FBG, fasting blood glucose.

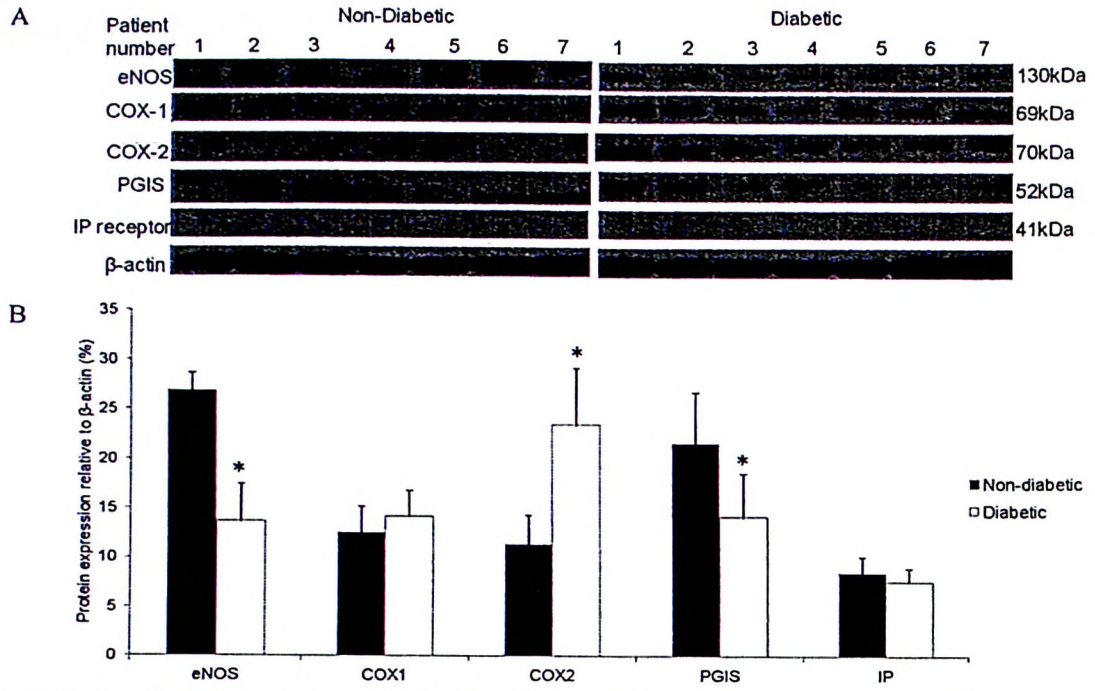

Fig. 1. Western blot analysis of proteins in the subcutaneous arteries of non-diabetic controls and diabetic patients. (A) Western blot demonstrated the expression of eNOS, COX-1, COX-2, PGIS and IP receptor proteins from seven diabetic and seven non-diabetic controls. (B) Graphical representation of the data, normalized to β-actin, is shown as the mean percentage ± S.D. ($n = 7$).
*$P < 0.05$ diabetic patients compared to non-diabetic controls.

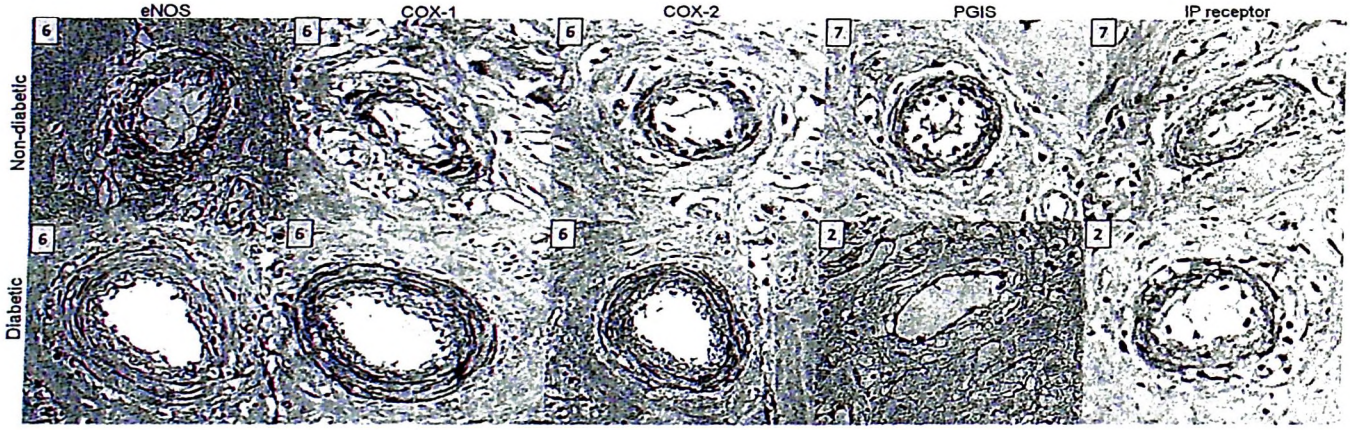

Fig. 2. Immunohistochemical analysis of proteins in subcutaneous arteries of non-diabetic controls and diabetic patients. Immunohistochemical staining of eNOS, COX-1, COX-2, PGIS and IP receptor proteins in the subcutaneous arteries of representative diabetic patients and non-diabetic controls. The number shown in the box at the top left side of each tissue section indentifies the patient from which the subcutaneous arteries were obtained (which corresponds to patient numbers on the top of the Western blot in Fig. 1A). Brown staining indicates expression of respective proteins (red arrows). Magnification 20 ×.

duction (Georgescu et al. 2011) and altered mitochondrial function (Kizhakekuttu et al. 2012).

The present immunohistochemical study and Western blotting analysis demonstrates increased COX-2 protein expression in the small arteries of diabetic patients. These findings are in accordance with an immunohistochemical study performed on coronary arterioles of diabetic patients compared to healthy controls (Szerafin et al. 2006). Increased COX-2 protein expression may result from the increased production of oxidative stress in diabetes. In human aortic endothelial cells cultured in high glucose medium, the production of superoxide anions was increased and this was associated with increased COX-2 protein expression (Cosentino et al. 2003). Superoxide anion pro-

uction was also increased in the coronary arterioles of dia-etic patients (Guzik et al. 2002). Increased expression of OX-2 protein (Bagi et al. 2005; Guo et al. 2005; Shi et al. 007) and increased production of superoxide anions Winer and Sowers 2004; Shi et al. 2007; Shi and Vanhoutte 008) also occur in vascular tissues of different animal odels of diabetes mellitus. Increased COX-2 protein xpression may also result from low-grade inflammation ssociated with diabetes (Szerafin et al. 2006). Pro-nflammatory cytokines such as tumor necrosis factor-α and nterleukin-1α/β can enhance the transcription of the OX-2 gene and increases the protein level of COX-2 Mitchell et al. 1995; Bagi et al. 2006).

In the coronary arterioles of diabetic patients, up-regu-tion of COX-2 protein is associated with increased vaso-latation most likely due to an increased production of lator prostaglandins (Szerafin et al. 2006). This may erve as a compensatory mechanism to maintain an ade-uate blood supply to the cardiac tissue of diabetic patients hose coronary blood vessels may be narrowed or dis-rbed by the disease process (Szerafin et al. 2006). While similar compensatory up-regulation of COX-2 protein xpression and activity opposing endothelial dysfunction as observed in the mesenteric vascular bed of mice with pe-1 diabetes (Nacci et al. 2009), in mice with type-2 dia-etes, the up-regulation of COX-2 enhances the synthesis f the vasoconstrictors PGH_2 and TXA_2 in the aorta (Guo et . 2005) and skeletal muscle resistance arteries (Bagi et al. 005).

Although PGI_2 as a rule is a vasodilator and an anti-ggregating agent, depending on the circumstances, PGI_2 n also act as an endothelium-derived contracting factor eletou et al. 2011; Vanhoutte 2011). In the aorta of spon-neously hypertensive rats (SHR), endothelium-dependent ntractions most likely involve the action of PGI_2 stimu-ing TP receptors (Gluais et al. 2005, 2006; Feletou et al. 09). The absence of relaxation in response to PGI_2 has en attributed to an early dysfunction of IP receptors in scular smooth muscles in this hypertensive rat model umaguchi et al. 1999; Feletou et al. 2011). The present udy shows the availability of IP receptors for the action of I_2 in subcutaneous arteries of both diabetic patients and n-diabetic controls. Indeed, no difference was found in e IP receptor protein presence between the two groups. though there were no changes in the expression of the IP eptor protein in diabetic patients, the present results do rule out differences in responsiveness of these receptors the action of PGI_2 at the level of the microcirculation.

No information seems available as regards to the pression of PGIS protein in human subcutaneous arteries. the present study, this expression was higher in non-dia-c controls compared to diabetic patients. One possible chanism, by which PGIS protein expression is decreased diabetes mellitus, is through the action of peroxynitrite uza et al. 2000). Indeed, exposure of endothelial cells to n glucose concentration increases the release of superox-ide anions ($O_2^{\cdot -}$) (Zou et al. 2002). Peroxynitrite is a potent oxidant formed by the reaction of $O_2^{\cdot -}$ and NO. It reduces the expression of PGIS protein in endothelial cells (Cooke and Davidge 2002). It also inactivates PGIS at concentrations as low as 50 nM through a tyrosine-nitration dependent mechanism (Zou et al. 2002, 2004). Nitration of tyrosine residues in certain proteins such as PGIS results in proteolytic degradation of the protein (Souza et al. 2000). Reduction of PGIS protein activity and/or expression results in the accumulation of its precursor, PGH_2, which activates their receptors (TP receptor) in vascular smooth muscles causing vasoconstriction (Zou et al. 2002; Feletou et al. 2011; Vanhoutte 2011).

In conclusion, type-2 diabetes mellitus is associated with higher COX-2 protein expression but lower eNOS and PGIS protein expression in human subcutaneous arteries.

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Conflict of Interest

The authors declare no conflict of interest in this study.

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