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DECREASED CONTRACTILE RESPONSE TO ENDOTHELIN-1 OF PERIPHERAL MICROVASCULATURE FROM DIABETIC PATIENTS

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Abstract

Objectives—We compared the contractile responses to ET-1, with and without the inhibition of ET-A receptors and protein kinase C- α (PKC- α) in the human peripheral microvasculature of diabetic and case-matched non-diabetic patients.

Methods—Chest wall skeletal muscle was harvested from patients with and without diabetics undergoing cardiac surgery. Peripheral arterioles (90-180 micrometer in diameter) were dissected from the harvested tissue. Microvascular constriction was assessed by videomicroscopy in response to ET-1, with and without an endothelin A (ET-A) receptor antagonist, or **an endothelin B (ET-B) antagonist** or a PKC- α inhibitor.

Results—ET-1 induced a dose-dependent contractile response of skeletal muscle arterioles from diabetes and non-diabetes. The contractile response of diabetic arterioles from both pre-bypass and post-bypass to ET-1 (10^{-9} mol/L) was significantly decreased compared with those of non-diabetics ($P < 0.05$), respectively. The contractile responses of microvessels of both diabetics and non-diabetics to ET-1 were significantly inhibited in the presence of either ET-A receptor antagonist BQ123 (10^{-7} mol/L, $P < 0.05$, respectively) or the PKC- α inhibitor safinol (2×10^{-5} mol/L), respectively. In contrast, the ET-1-induced vasoconstriction was not affected by the administration of the ET-B receptor antagonist BQ788 (10^{-7} mol/L). There were no significant differences in skeletal muscle levels of the ET-A and ET-B receptors between diabetic and non-diabetic groups.

Conclusion—Diabetic patients demonstrated a decreased contractile response to ET-1 in human peripheral microvasculature. The contractile response to ET-1 is via activation of ET-A receptors and PKC- α in diabetics. These results provide novel mechanisms of ET-1-induced contraction in vasomotor dysfunction in patients with diabetes.

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Keywords

Endothelin; Diabetics; Cardiopulmonary bypass; Microcirculation; Receptors; Skeletal Muscle; Protein Kinase C

Introduction

Several investigators have found that coronary effluent release of endothelin-1 (ET-1) is significantly enhanced in patients with diabetes indicating increased ET-1 production.¹⁻⁴ More recently, we found that cardioplegic ischemia/reperfusion is associated with a reduction in contractile responses of human coronary arterioles to ET-1.⁵ We also showed that the contractile response to ET-1 is via activation of ET-A receptors and protein kinase C- α (PKC- α) in the coronary microvasculature.⁵ However, the role of ET-1 in peripheral arteriolar vasomotor dysfunction in patients with diabetes and the related molecular mechanisms underlying ET-1-induced vasomotor dysfunction remain to be elucidated. Therefore, this study was designed to compare the skeletal muscle arteriolar responses to ET-1, with and without ET-receptor antagonists and a PKC- α blocker, in diabetic and case-matched non-diabetic patients. These responses were also related to possible alterations in the localization of ET receptors in human skeletal muscle tissue.

Methods

Human subjects and tissue harvesting

Samples of skeletal muscle from the intercostals muscle in the left internal mammary artery bed were harvested⁶ pre-CPB from patients. The pre-CPB specimen was taken after cannulation in the left internal mammary artery bed. Tissue for immunofluorescent staining was fixed in 10% formalin buffered solution for **24 hours** followed by paraffin mounting and sectioning into 5- μ m slices. Tissue for microvascular studies was placed in cold (5° to 10°C) Krebs buffer solution. Blood glucose levels were measured at the patients initial outpatient visit or upon initial admission to the hospital, and again during the operation at the time of tissue harvest. All procedures were approved by the Institutional Review Board of Beth Israel Deaconess Medical Center, Harvard Medical School, and informed consent was obtained from all enrolled patients as required by the Institutional Review Board.

Microvessel reactivity

Skeletal muscle arterioles (90-180 μ m internal diameters) from the left internal mammary artery bed were dissected from pre- and post-CPB tissue samples. Microvessel studies were performed by *in vitro* organ bath videomicroscopy as described previously.⁵⁻⁷ Microvessel studies were performed on pre- and post-CPB skeletal muscle microvessels as follows: (1) measurement of contraction to escalating doses of ET-1 (10^{-12} - 10^{-7} mol/L), (2) measurement of contraction to ET-1 with and without ET-A receptor antagonist BQ123 (10^{-7} mol/L) or ET-B antagonist BQ788 (10^{-7} mol/L) (Sigma-Aldrich, St. Louis, MO) and (3) measurement of contraction to ET-1 with and without a PKC- α safingol (2.5×10^{-5} mol/L) (Avanti Polar Lipids, Alabaster, AL.) pretreatment for 20 minutes. Baseline diameter was defined as the diameter measured after cannulation of the vessel and equilibration in the buffer solution. Internal diameters measured after treatment with ET-1 were normalized to the baseline diameter. The microvessels were washed with a Krebs buffer solution and allowed to equilibrate 15-30 minutes between interventions.

Confocal immunofluorescence microscopy

Skeletal-muscle tissue sections from five patients were deparaffinized in xylene, rehydrated in graded ethanol and phosphate-buffered saline solution (PBS), and antigen-unmasked with sodium citrate (10 mmol/L, pH = 6.0), followed by PBS wash and blocking with 2% bovine serum albumin in PBS at room temperature for 2 h.⁵ After PBS wash, sections were incubated overnight with ET-A and ET-B receptor antibodies (each used at 1:200) (Santa Cruz Biotechnology) at 4°C. Anti-mouse, α -smooth muscle actin (1:1000) (Sigma-Aldrich) was used to detect microvascular smooth muscle. Sections were then washed in PBS and incubated with the appropriate Alexa-fluor secondary antibody and mounted using fluorescent mounting medium (Vector Labs, Burlingame, Calif.). Tissue was visualized using a Zeiss LSM510 confocal microscope system (Carl Zeiss MicroImaging, Inc. Thornwood, NY). Tissue labeling with secondary antibodies (ET-A or ET-B) alone, and primary and secondary antibodies for α -smooth muscle actin served as negative controls.

Chemicals

ET-1, BQ123, and BQ788 were obtained from Sigma-Aldrich. Safingol was purchased from Avanti Polar Lipids (Alabaster, AL). ET-1 was dissolved in ultrapure distilled water and prepared on the day of the study. BQ123, BQ788 and safingol were dissolved in dimethylsulfoxide to make a stock solution. All stock solutions were stored at 4°C or -20°C. All dilutions were prepared daily.

Data analysis

Data are presented as the mean and standard error of the mean (SEM). Microvessel responses are expressed as the percentage of contraction of baseline. Repeated-measures ANOVA and Student's *t*-test were used to compare variables among or between vessels. The concentration effects (EC₅₀) were assessed using nonlinear regression (sigmoidal dose response). *P* values < 0.05 were considered significant.

3. Results

Patient characteristics

Tissue samples from 14 each from nondiabetic and diabetic patients undergoing coronary artery bypass graft (CABG) were studied. The patient characteristics are summarized in table 1. All patients with preoperative hypertension were on medication (β -blocker, aspirin, calcium channel blocker, or angiotensin-converting enzyme inhibitor), and received perioperative β -blockade.

Microvascular reactivity

ET-1 induced a dose-dependent contractile response of skeletal muscle arterioles from diabetics and non-diabetics with EC₅₀ values of 1.5×10^{-8} M and 6.6×10^{-9} M, respectively (Fig 1). Endothelin-1-mediated vasoconstriction in vessels was greater in diabetic arterioles than that of non-diabetic patients (Fig 1). Pretreatment of pre-CPB skeletal muscle arterioles with ET-A receptor antagonist BQ123 significantly inhibited ET-1-induced vasoconstriction in both non-diabetics and diabetics (Fig 2 A, B), respectively. In contrast, inclusion of the ET-B receptor antagonist BQ788 in both non-diabetic and diabetic arterioles failed to affect ET-1-induced vasoconstriction, (Fig 3 A, B). Pretreatment of peripheral arterioles with the PKC- α inhibitor safingol significantly prevented ET-1-induced vasoconstriction in both non-diabetics and diabetics. (Fig 4 A, B).

Effect of CPB on levels of ET-A and ET-B receptor polypeptides

Both ET-A and ET-B receptors are presented in skeletal muscle microvasculature of diabetic and non-diabetics. ET-A was predominately present in smooth muscle (Fig 5A), whereas, the ET-B receptor was largely present in endothelium (Fig 5B). There were no significant differences in skeletal muscle levels of the ET-A and ET-B receptors between diabetic and non-diabetic groups. (Fig 5A, B).

Discussion

There are several novel observations in the present study. First, ET-1 induced dose-dependent vasoconstriction of human skeletal muscle arterioles in diabetics, and the contractile response of pre-CPB vessels to ET-1 in diabetics was significantly reduced compared with non-diabetics. Second, the responses to ET-1 were significantly inhibited in the presence of ET-A receptor antagonist BQ123, but unchanged in the ET-B receptor antagonist BQ788 pretreatment in both diabetic and non-diabetic groups. Third, both ET-A and ETB receptors were expressed in skeletal muscle microvasculature of diabetic and non-diabetics. ET-A was predominately present in smooth muscle, whereas the ET-B receptor stained the endothelium. There were no significant differences in ET-A and ET-B receptor expression between the two groups. Finally, the contractile responses to ET-1 were significantly prevented in the presence of PKC- α inhibitor safinol in both diabetic and non-diabetics.

The molecular mechanisms responsible for phenylephrine and vasopressin-induced vasomotor dysfunction have been extensively investigated in our laboratory. Several protein kinases, such as mitogen-activated protein kinases (MAPK), extracellular signal regulated kinases 1/2 (ERK1/2), p38 kinase, and protein kinase C (PKC) have been suggested to be involved in vasomotor dysfunction.⁸⁻¹² Recently, we observed that the contractile response to ET-1 is via activation of ET-A receptors and PKC- α in the human coronary microvasculature.⁵ The present work demonstrates that the ET-A receptors are the predominant subtype found within the human peripheral microvasculature. Vasoconstriction was inhibited in the presence of the ET-A receptor antagonist, and not the ET-B antagonist, further suggesting that ET-A, not ET-B receptors are responsible for ET-1-induced vasoconstriction in both diabetic and non-diabetic microvasculature.

The present study shows a significantly reduced contractile response of human peripheral arterioles to ET-1 in patients with diabetes compared with non-diabetes. The mechanism responsible for this dysfunction may be related in part to the ET-1 and ET receptor responses to diabetes. The endogenous stress response to diabetes leads to enhanced release of vaso-active **peptide** ET-1, which acts predominately on ET-A receptors.¹⁻⁴ The sustained increase in circulating levels of ET-1 in diabetics *in vivo*, or prolonged exposure to ET-1 *in vitro*, may cause subsequent loss of ET-1-mediated vascular smooth muscle cell contraction. In addition, diabetes may significantly enhance activation or release of oxygen free radicals, prostaglandins, nitric oxide, complement, and pro-inflammatory cytokines and chemokines, all of which can contribute to vasomotor dysfunction through vasodilatation/vasoconstriction, leading to increased vascular permeability and worsened tissue edema.^{8,13-16} Recently, we have also found that several genes, such as vascular endothelial growth factor (VEGF), which are related to increased vascular permeability from diabetic patients are differentially regulated in diabetic and non-diabetic patients, supporting the notion that diabetes is associated with worsened vasomotor dysfunction and subsequent tissue edema.^{17,18} Peripheral edema in diabetic patients is associated with numerous adverse consequences.

Alpha-adrenergic-induced vasoconstriction in the human peripheral microvasculature is partially mediated by PKC- α .¹² The present study indicates that ET-1-induced vasoconstriction in the peripheral arterioles of patients with diabetes also acts via PKC- α activation. The upstream molecular mechanism contributing to this dysfunction may be diminished inositol 1,4,5-triphosphate turnover in diabetic smooth muscle, which results in decreased concentrations of diacylglycerol and calcium, both of which are required for conventional PKC activation. This effect of diminished inositol phospholipid metabolism in diabetics and blunted vascular smooth muscle cell contraction in response to ET-receptor stimulation can also be worsened with prolonged exposure to phorbol ester, a highly potent PKC activator.^{5,12,19,20}

The present data have also shown that circulating glycosylated hemoglobin was significantly elevated, indicating that advanced glycosylation end products (AGEs) may have been significantly accumulated in the diabetic patients. Elevated circulating and tissue AGEs can induce complex vascular dysfunction.²¹ Experimental studies have shown that exogenous AGEs can modify basement membrane and attenuate ET-1-induced calcium signaling and contraction in retinal microvascular pericytes.²² In the present study, we did not measure circulating and tissue AGEs. But, it can be assumed that the accumulated AGEs in the diabetic vessels may contribute to the decreased responses to ET-1. It will be very interesting, in the future to investigate endogenous and exogenous AGEs induce microvascular dysfunction in the setting of diabetes. Also, in the present investigation, it is difficult to separate the effects of hyperglycemia and other hyperglycemia-independent effects of diabetes. Although, in the present investigation, glucose held constant during the ex vivo experiment and which well controlled during surgery, argues against acute effects of hyperglycemia. However, as mentioned above chronic hyperglycemia can have numerous multi-faceted deleterious effects on vascular reactivity.

In conclusion, diabetes decreases myogenic contractile function of human peripheral arterioles in response to ET-1 in patients with diabetes. The contractile responses to ET-1 in diabetics are through activation of ET-A receptors and PKC- α . These findings support the notion that diabetes is associated with worsened vasomotor dysfunction. These results provide novel mechanisms of ET-1-induced contraction in vasomotor dysfunction in patients with diabetes.

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Fig 1. Skeletal microvascular vasoconstriction in response to endothelin-1 (ET-1) (A) Endothelin-1 (ET-1) induced pre-CPB arterioles contraction, * $P < 0.05$ diabetics versus non-diabetics.

Fig 2. Skeletal microvascular vasoconstriction in response to Endothelin-1 (ET-1) in the presence of ET-A antagonist BQ123. **(A)** * $P < 0.05$ Non-diabetics vs. BQ123 + Non-diabetics; **(B)** * $P < 0.05$ Diabetics vs. BQ123 + diabetics.

Fig 3. Skeletal microvascular vasoconstriction in response to Endothelin-1 (ET-1) in the presence of ET-B antagonist BQ788. **(A)** Non-diabetics vs. BQ 788 + Non-diabetics; **(B)** Diabetics vs. BQ788 + diabetics.

Fig 4. Skeletal microvascular vasoconstriction in response to Endothelin-1 (ET-1) in the presence of PKC α inhibitor Safingol. **(A)** *P < 0.05, Non-diabetics vs. safingol + non-diabetics; **(B)** *P < 0.05, Diabetics vs. safingol + diabetics.

Fig 5. Immunolocalization of ET-A and ET-B receptor polypeptides in human skeletal muscle microvessels. Vessels were co-stained for smooth muscle actin and either (A) ET-AR. or (B) ET-BR. Matched negative controls are displayed below the rows of primary antibodies.

Table 1

HbA1C, hemoglobin A1C; BMI, body mass index; NS, no significance

Patient Characteristics	Non-diabetes	Diabetes	P values
Age (y)	69 ± 3.5	65 ± 3.0	0.5
Male/Female	11/3	12/2	NS
Out-patient Blood Glucose (mg/dL)	110 ± 9.0	219.3 ± 15.0	0.001
HbA1C (%)	4.9 ± 0.2	7.7 ± 0.4	0.01
Operative Blood Glucose (mg/dL)	110 ± 7	109 ± 19.0	0.45
Obesity (BMI>30)	2	2	NS
Hypertension (n)	3	3	NS
Atrial fibrillation (n)	2	2	NS
Hypercholesterolemia	5	6	NS