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Systemic hypertension augments, whereas insulin-dependent diabetes down-regulates, endothelin A receptor expression in the mammary artery in coronary artery disease patients

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Abstract

Background: *Endothelin (ET) A receptor antagonism causes decreased vasodilation in hypertensive coronary arteries and decreased effects on coronary artery compliance in diabetic patients.*

Methods: *We investigate the mRNA expression of ET-1, ET_A and ET_B receptors, using real time RT-PCR, in biopsies from the internal mammary artery obtained from 49 patients, 18 diabetics and 34 hypertensives, all undergoing coronary artery bypass grafting.*

Results: *Hypertensive patients had higher ET-1 mRNA expression (16438 [8417, 23917]), than normotensive patients (2974 [2283, 18055], p=0.008). Diabetic patients had significantly lower ET_A receptor levels than non-diabetic patients (455 [167, 1496] vs. 1660 [700, 3190], respectively, p = 0.003).*

Conclusions: *Multivariate analysis demonstrated that the presence of systemic hypertension was the only independent predictor of log ET_A receptor expression and log ET-1 expression, while insulin-dependent diabetes was negatively correlated with ET_A receptor expression. ET_B receptor expression was not correlated with any predictor. Systemic hypertension is associated with increased ET-1 and ET_A receptor mRNA expression, whereas insulin-dependent diabetes down-regulates ET_A receptor mRNA expression in the internal mammary artery in patients with coronary artery disease undergoing bypass grafting.* (Cardiol J 2009; 16, 4: 348–354)

Key words: endotelin, hypertension, diabetes

Introduction

Endogenous production of endothelin-1 (ET-1) contributes to the maintenance of coronary vascular tone in coronary artery disease and healthy controls [1]. In animal models of diabetes, reduced responsiveness to ET-1 is seen in both the large vessels and the microvasculature [2, 3]. In addition, both exogenous and endogenous ET-1 cause impaired vasoconstriction in forearm arteries of patients with type 2 diabetes mellitus [4, 5]. Greater compensatory vessel enlargement occurs in

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patients with unstable than in those with stable coronary syndromes, and is associated with increased coronary artery distensibility [6, 7]. In diabetic patients, coronary compliance is decreased [8]. Recently, we showed that ET_A receptor antagonists improve coronary artery compliance in patients with atherosclerotic vessels [9].

ET-1 binds to at least two receptors. The ET_A receptor appears to be the major receptor causing vasoconstriction in arteries; the ET_B receptor mediates release of endothelium-dependent vasodilator substances and is also present in some resistance and capacitance arteries, where it contributes to vasoconstriction [10]. ET-1 may play a part in the pathophysiology of several conditions associated with vasoconstriction, including chronic heart failure, essential hypertension, Reynaud's disease, and renal failure [10]. Furthermore, ET-1 influences salt and water homeostasis, increases central and peripheral sympathetic activity and stimulates the generation of renin, angiotensin II, aldosterone and adrenaline [11]. It has been shown recently that the effect of endogenous ET-1 on coronary artery stiffness is impaired in type 2 diabetes mellitus [12], and that ET_A antagonism causes decreased vasodilation, but does not have any differential effect on coronary artery compliance in hypertensive patients [13]. We also know that vascular smooth muscle and most other vascular tissues are less sensitive to the effects of ET in hypertensive animals [14].

We hypothesized that the mRNA expression of ET-1 and its receptors would be altered in patients with diabetes mellitus and systemic hypertension. The aim of the present study was to examine the mRNA expression of ET-1 and its receptors in the internal mammary artery in patients with coronary artery disease, with and without diabetes and systemic hypertension, undergoing coronary artery bypass grafting.

Methods

Selection of patients

Forty-nine consecutive patients, 18 of whom were type 2 diabetics (five having insulin-dependent diabetes), and 34 hypertensives undergoing coronary artery bypass graft for stable angina pectoris class II or more, were enrolled in the study.

Ten of the patients had both type 2 diabetes and hypertension. The patients' characteristics are presented in Table 1. Diabetes was defined as fasting plasma glucose ≥ 126 mg/dL or two hours post load plasma glucose ≥ 200 mg/dL or use of antidiabetic medications in order to maintain normal plasma glucose values. However, all patients with diabetes in our study were under antidiabetic medication treatment in order to control their glucose levels (only antidiabetic tablets [n = 13] and/or insulin [n = 5]). Hypertension was defined as systolic blood pressure more than 140 mm Hg or diastolic blood pressure more than 90 mm Hg or use of medication in order to maintain normal blood pressure values. Patients were excluded from the study in the presence of: acute myocardial infarction, unstable angina, heart failure, left ventricular ejection fraction < 45% (estimated by left ventricular angiography), systolic pulmonary artery pressure > 50 mm Hg (estimated by echocardiographic studies), plasma creatinine > 1.8 mg/dL, atrial fibrillation, additional cardiac disease or severe non-cardiac disease. Medical histories were collected from patients and their relatives and from medical files as well as from laboratory examinations performed in our institution. During the operation, a tiny part of the distal end of the left internal mammary artery was obtained from all patients, immediately frozen in liquid nitrogen and stored at –80°C until analyzed.

Table 1. Characteristics of the 49 patients studied.

Age	66 (59–73)
Male sex	42 (86%)
DM	18 (37%)
Non insulin-dependent DM	13 (27%)
Statin use	29 (59%)
Systemic hypertension	34 (69%)
History of myocardial infarction	23 (47%)
Beta-blocker use	12 (24%)
Nitrate use	39 (80%)
Calcium channel blocker use	14 (29%)
Diuretic use	8 (16%)
ACEI or AT-1 use	32 (65%)
Cholesterol [mg%]	202 (193–219)
Triglycerides [mg%]	155 (144–185)
LDL-cholesterol [mg%]	106 (63–124)
HDL-cholesterol [mg%]	38 (35–41)
LVEF (%)	55 (45–60)
Glycosylated hemoglobin	5.9 (5.5–6.8)

DM — diabetes mellitus; ACE — angiotensin converting enzyme inhibitor; AT1 — angiotensin 1 receptor blocker; LVEF — left ventricular ejection fraction

The Hospital Ethics Committee approved the study. All patients gave written informed consent.

RNA extraction and cDNA synthesis

Total cellular RNA was isolated using the Qiagen RNeasy Mini Reagent Set (Qiagen, Germany)

Table 2. Sequence of primers and probes used in this study.

Oligonucleotide	Sequence (5′-3′)	Base pair
ET-1 forward primer	CCAGAAACAGCAGTCTTAGGCG	22
ET-1 reverse primer	AACGTGCTCGGGAGTGTTGA	20
ET-1 probe	6FAM-CTCCTGCTCGTCCCTGATGGATAAAGAGTGTG-TMR	32
ET_A receptor forward primer	AACATCTTAAGCAGCGTCGAGAA	23
ET_A receptor reverse primer	GCAGAGGCATGACTGGAAACAAT	23
ET_A receptor probe	6FAM-ATTTTTGCTCTTTGCTGGTTCCCTGTTCATTTA-TMR	33
ET_B receptor forward primer	ACCTAAAGCAGAGACGGGAAGTG	23
ET_B receptor reverse primer	CCAATACCAACAGAAAGCTCAAAAG	25
ET_B receptor probe	6FAM-AACCGTCTTTTGCCTGGTCCTTGTCTTTGC-TMR	30

according to the manufacturer's recommendations. All preparation and handling steps of RNA took place in a laminar flow hood, under RNAse free conditions. The concentration and purity of the RNA were determined by spectrophotometric analysis at 260 and 280 nm and the isolated RNA was stored at –80°C until further manipulations. Reverse transcription of RNA was carried out with the SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen, California, USA) according to the manufacturer's instructions, using 1 μg of total RNA as template.

Real time PCR

For the quantification of each gene of interest, a real time PCR assay was developed. The primers and probes were designed using the Primer Premier software. The oligonucleotides designed were intron spanning in order to prevent amplification of genomic DNA; their sequences are presented in Table 2. For ET-1 the primers hybridize to exons 1/2 (F) and 2/3 (R) and therefore the mRNA that corresponds to the active peptide ET-1 is quantified.

Real time PCR was performed in the LightCycler Instrument (Roche Applied Science, Germany) in a total volume of 10 μL per glass capillary. For each reaction 1 μL of cDNA was placed in a 9 μL reaction mix containing 0.1 μL of a temperature-released *Taq* DNA polymerase (5 U/μL; Platinum DNA Polymerase; Invitrogen), 1 μL of the supplied 10 × PCR buffer, 0.7 μL (for ET-1) or 1.0 μL (for ET_AR and ET_BR) of the supplied $MgCl_2$ (50 mM), 0.2 μL of deoxynucleotide triphosphates (10 mM; Invitrogen), 0.15 μL of bovine serum albumin (10 μg/μL; Sigma), 0.5 μL of the primers (3 μM), 1 μL of the probe (3 μM), and diethylpyrocarbonate-treated H_2O. The cycling protocol was identical for the ET-1 and ET_B receptors and consisted of an initial five minute denaturation step at 95°C for activation of the DNA polymerase, followed by 45 cycles of denaturation at 95°C for ten seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 15 seconds. The cycling protocol for the ET_A receptor consisted of an initial five minute denaturation step at 95°C for activation of the DNA polymerase, followed by 45 cycles of denaturation at 95°C for ten seconds, annealing at 60°C for 15 seconds, and extension at 65°C for 20 seconds. For the normalization of our results, the glyceraldehyde-3--phosphate dehydrogenase (GAPDH) gene was used and the quantification was performed as previously described [15].

To establish a specific, sensitive, and reproducible real time PCR assay, we performed extensive optimization of primers, probes, and $MgCl_2$ concentrations, as well as reaction temperatures and times. The analytical evaluation of the assay and the quantification of the genes' expression levels were performed with calibrators prepared and quantified as previously described [14]. For each gene, a calibration curve was generated from serial dilutions ranging from 10^6 to 10^2 copies/μL of the target of interest. All calibration curves showed linearity over the entire quantification range with correlation coefficients > 0.99.

Statistical analysis

Data for each continuous variable was examined with the Shapiro-Wilk's W test to determine whether assumptions of normality were valid. Continuous variables are summarized as median (25^{th}, 75^{th} centiles) unless stated otherwise. Since the data was non-normally distributed, non-parametric tests were used. Comparisons between continuous variables were done using the Mann-Whitney U test. Unadjusted associations between the genes investigated and independent variables were tested using Spearman's rank R. Adjusted associations were tested using multiple linear regression analysis with

log ET-1 expression, log ET_A receptor and log ET_B receptor as independent variables. Variables that reached levels of significance ≤ 0.20 during univariate analysis were included in the multivariate analysis. Descriptive data for continuous variables are summarized as median (25^{th}, 75^{th} centiles) unless stated otherwise. For hypothesis testing, two-sided p values below 0.05 were considered to be statistically significant. Data was analyzed with the Statistica software (version 7.0, StatSoft Inc, USA).

Results

Endothelin-1 mRNA expression

Diabetic patients tended to have lower ET-1 mRNA expression than non-diabetic patients (8235 [4707, 15635] *vs*. 18055 [5724, 31621], p = 0.09). Hypertensive patients had higher ET-1 mRNA expression (16438 [8417, 23917]) than normotensive patients (2974 [2283, 18055], p = 0.008) (Fig. 1).

The results of the univariate analysis are shown in Table 3. Six variables were associated with ET-1 expression with a p value < 0.20 and were entered into the multivariate analysis. The presence of arterial hypertension was the only independent predictor of log ET-1 expression levels. After adjustment for other variables, the presence of arterial hypertension accounted for a mean increase of 0.88 in log ET-1 expression.

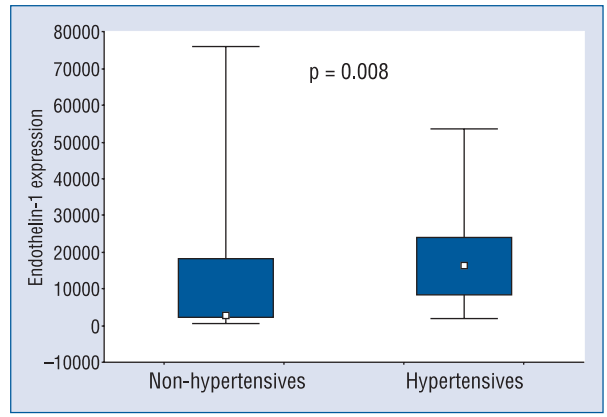

Figure 1. Endothelin-1 mRNA expression in non-hypertensive (n = 15) and hypertensive (n = 34) patients.

Endothelin A receptor mRNA expression

Diabetic patients had significantly lower ET_A receptor levels than non-diabetic patients (455 [167,

Table 3. Univariate and multivariate predictors for endothelin 1 (ET1) expression.

Univariate predictors	Spearman R	t	p
Age	0.07	0.47	0.64
Female sex	0.18	1.24	0.22
Diabetes mellitus	0.25	1.76	0.09
Non insulin-dependent diabetes mellitus	–0.20	–1.37	0.18
Insulin-dependent diabetes mellitus	–0.11	–0.76	0.45
Glycosylated hemoglobin	–0.16	–0.64	0.52
Cholesterol	0.21	1.49	0.14
High density lipoprotein-cholesterol	–0.30	–2.17	0.04
Statin use	–0.22	–1.55	0.13
Systemic hypertension	0.38	2.83	0.008
Left ventricular ejection fraction	0.13	0.92	0.36
Old myocardial infarction	–0.08	–0.54	0.59
Beta-blocker use	0.14	1.00	0.32
Clopidogrel use	–0.02	–0.12	0.91
Nitrate use	0.06	0.42	0.68
Calcium channel blocker use	0.01	0.04	0.97
ACEI or angiotensin 1 receptor blocker use	0.07	0.48	0.63
Multivariate predictors for Log ET-1 expression	**Beta**	**SE beta**	**p**
Diabetes mellitus	0.30	0.53	0.58
Non insulin-dependent diabetes	–0.23	0.56	0.90
Cholesterol	0.01	0.01	0.20
High density lipoprotein-cholesterol	–0.02	0.03	0.49
Statin use	0.03	0.36	0.94
Systemic hypertension	0.98	0.38	0.01

ACEI — angiotensin converting enzyme inhibitor

1496] *vs*. 1660 [700, 3190], p = 0.003). Patients with insulin-dependent diabetes mellitus had lower values than non-diabetic patients, whereas the difference between non-diabetic patients and those with non insulin-dependent diabetes was of borderline significance (Fig. 2). Univariate predictors for ET_A receptor levels are shown in Table 4. After adjustment for other variables, both systemic hypertension and insulin-dependent diabetes were independent predictors for log ET_A receptor levels. Log ET_A receptor levels were associated with ET-1 expression (R = 0.39, p = 0.008) (Fig. 3).

Endothelin B receptor mRNA expression

Univariate and multivariate predictors for ET_B receptors are shown in Table 5. In multivariate analysis there was only a borderline association between the presence of systemic hypertension and ET_B receptors.

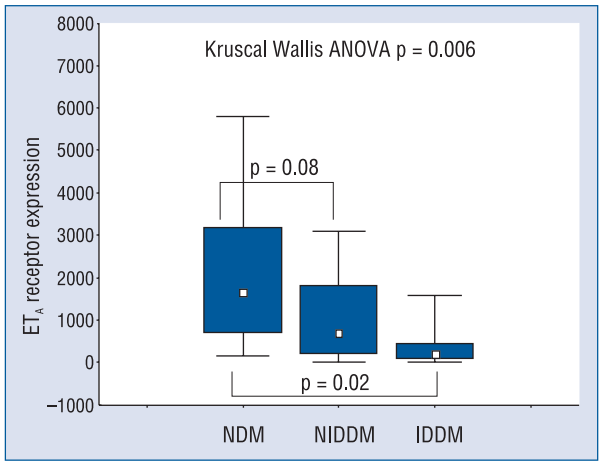

Figure 2. Endothelin A (ET_A) receptor mRNA expression in non-diabetics (NDM) (n = 31), insulin-dependent diabetics (IDDM) (n = 5) and non insulin-dependent diabetics (NIDDM) (n = 13).

Discussion

Our study demonstrates, for the first time, that systemic hypertension increases ET-1 and ET_A receptor mRNA expression, whereas insulin-dependent diabetes down-regulates ET_A receptor mRNA expression in the internal mammary artery in patients with coronary artery disease undergoing bypass grafting.

Table 4. Univariate and multivariate predictors for endothelin-1 (ET-1) receptors.

Univariate predictors	Spearman R	t	p
Age	0.04	0.25	0.80
Female sex	0.08	0.50	0.62
Diabetes mellitus	0.45	3.31	0.002
Non insulin-dependent diabetes	–0.25	–1.68	0.10
Insulin-dependent diabetes	–0.34	–2.43	0.02
Glycosylated hemoglobin	–0.12	–0.70	0.49
Cholesterol	0.18	1.22	0.23
High density lipoprotein-cholesterol	–0.23	–1.55	0.12
Statin use	–0.10	–0.68	0.50
Systemic hypertension	0.35	2.44	0.02
Left ventricular ejection fraction	–0.12	–0.79	0.43
Old myocardial infarction	0.00	0.03	0.97
Beta-blocker use	–0.04	–0.25	0.81
Clopidogrel use	–0.07	–0.48	0.64
Nitrate use	0.03	0.21	0.84
Calcium channel blocker use	0.00	0.01	0.99
ACEI or angiotensin 1 receptor blocker use	–0.10	–0.69	0.50
Multivariate predictors	**Beta**	**SE beta**	**p**
Non insulin-dependent diabetes	–1.0	0.67	0.14
Insulin-dependent diabetes	–2.8	0.8	0.0006
Glycosylated hemoglobin	0.1	0.27	0.71
High density lipoprotein-cholesterol	0.02	0.04	0.54
Systemic hypertension	1.06	0.43	0.03

ACEI — angiotensin converting enzyme inhibitor

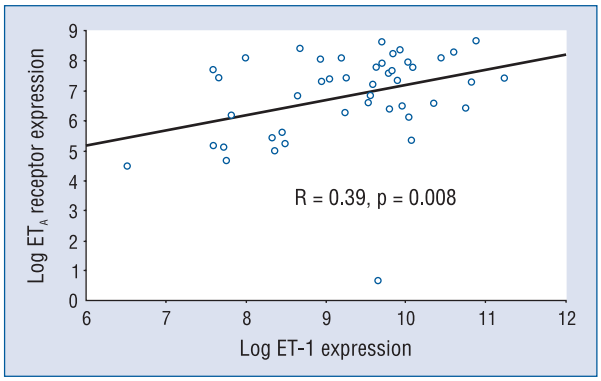

Figure 3. Relation between log endothelin-1 (ET-1) and log endothelin A (ET_A) receptor mRNA expression.

To our knowledge, no studies have examined the mRNA expression of ET-1 and its receptors in the diabetic and hypertensive human internal mammary arteries in coronary artery disease patients.

We chose to study the internal mammary artery because this special artery is resistant to atherosclerosis [16].

It has been demonstrated that ET-1 exerts a tonic stiffening effect on the in vitro common carotid artery and that this effect is mediated via the ET_A receptor [17]. In animal models of diabetes and in patients with type 2 diabetes mellitus, a reduced responsiveness to ET-1 is seen in both the large peripheral vessels and the microvasculature [2–5]. Recently, a human study demonstrated that the effect of endogenous ET-1 on ET_A receptors in the periphery is enhanced in the resistance vessels of patients with diabetes, whereas their sensitivity to exogenous ET-1 is blunted [18]. These discrepancies between the results of the different studies could be ascribed to the following:

— the patient's quality of glucose control. It is possible that a worse metabolic milieu may have affected the vasodilatory mechanisms secondary to ET_A receptor blockade;
— the medications the patients were taking, and whether those medications had been stopped before the study. Also, the average duration of diabetes in the population may have contributed to the difference between the groups' responses;

Table 5. Univariate and multivariate predictors for endothelin B receptors.

Univariate predictors	Spearman	t	p
Age	0.06	0.39	0.70
Female sex	–0.10	–0.65	0.52
Diabetes mellitus	0.22	1.54	0.13
Non insulin-dependent diabetes	–0.07	–0.50	0.62
Insulin-dependent diabetes	–0.24	–1.70	0.10
Glycosylated hemoglobin	–0.16	–1.15	0.26
Cholesterol	0.01	0.08	0.94
Triglycerides	0.14	0.95	0.35
High density lipoprotein-cholesterol	–0.24	–1.70	0.10
Statin use	–0.18	–1.22	0.23
Systemic hypertension	0.33	2.34	0.02
Left ventricular ejection fraction	–0.07	–0.46	0.65
Old myocardial infarction	–0.08	–0.58	0.57
Blood glucose levels	–0.18	–1.26	0.21
Beta-blocker use	0.07	0.47	0.64
Clopidogrel use	0.00	0.00	1.00
Nitrate use	0.00	0.03	0.98
Calcium channel blocker use	0.11	0.72	0.47
ACEI or angiotensin 1 receptor blocker use	0.11	0.78	0.44
Multivariate predictors	**Beta**	**SE beta**	**p**
Diabetes mellitus	0.41	0.39	0.29
Insulin-dependent diabetes	–0.40	0.62	0.52
High density lipoprotein-cholesterol	–0.01	0.03	0.68
Systemic hypertension	0.68	0.39	0.09

ACEI — angiotensin converting enzyme inhibitor

— inter-individual variability, or other unrecognized factors;
— gender issues;
— small study sample.

The down-regulation of the ET_A receptor mRNA expression we found in diabetics could explain the reduced responsiveness to ET-1 that is demonstrated in large vessels and the impaired response to ET_A receptor antagonists as regards coronary artery compliance in diabetic patients. This is in accordance with a previous study of ours [12].

The present study, showing that systemic hypertension increases ET-1 and ET_A receptor mRNA expression in the left internal artery in patients undergoing coronary artery bypass grafting, could explain our previous findings showing that ET_A antagonism causes decreased vasodilation in coronary arteries [13] and that the epicardial coronary vasculature in hypertensive patients is less responsive to baseline ET during coronary angioplasty [19]. Schneider et al. demonstrated that plasma ET is increased in early essential hypertension [20]. The higher ET-1 and ET_A receptor mRNA expression found in the arteries of hypertensive patients might result in a condition where higher levels of ET_A receptor blockers are needed in order to reverse these patients' increased vasomotor tone.

Conclusions

Systemic hypertension is associated with increased ET-1 and ET_A receptor mRNA expression, while insulin-dependent diabetes down-regulates ET_A receptor mRNA expression in the internal mammary artery in patients with coronary artery disease undergoing bypass grafting. This could help explain the differential response of hypertensive and diabetic animals and humans to external and internal stimulation and blockade of ET-1 and its receptors.

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