ABSTRACT

Title:	THE INFLUENCE OF ECE-1 GENE POLYMORPHISM AND EXERCISE TRAINING ON PLASMA AND URINARY ENDOTHELIN-1 LEVELS IN PRE- AND STAGE 1 HYPERTENSIVES
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Hypertension is an important risk factor for cardiovascular and renal diseases and has been identified as one of the main causes of mortality worldwide. Hence, factors contributing to this condition are of physiological and clinical importance. Endothelin-1 (ET-1) is a 21 amino acid peptide produced ubiquitously in the human body that mediates blood pressure. Vascular-derived ET-1 acts as a potent vasoconstrictor causing an increase in blood pressure. On the other hand, increases in ET-1 action in the nephron mediate diuresis and natriuresis, favoring a decrease in blood pressure. Moreover, a polymorphism, endothelin converting enzyme-1b-C-338A (ECE-1b-C-338A), in the 5′-regulatory region of the ECE-1 gene that alters ET-1 synthesis has been identified. Thus, the goal of this study was to investigate the association of the ECE-1b-C-338A polymorphism with plasma and urinary levels of ET-1 in pre- and stage 1 hypertensives. The effects of this polymorphism on aerobic exercise training-induced changes in these variables were also examined. Healthy, sedentary pre- and stage 1 hypertensive older adults underwent 24 weeks of supervised aerobic exercise training (AEX) after dietary stabilization. Plasma ET-1, urinary ET-1, and sodium excretion were assessed. Genotyping was carried out using standard PCR methods. Separate ANCOVAs were

performed using ECE-1 genotype as the independent variable and blood pressure, plasma ET-1, urinary ET-1, and sodium excretion as dependent variables. Possible confounding factors such as age and body mass index were used as covariates in the analyses when appropriate. ANCOVA was used to compare differences in exercise training-induced changes in all outcome variables between genotype groups after accounting for confounding variables. Baseline levels of blood pressure, plasma ET-1, urinary ET-1, and sodium excretion were not significantly different among genotype groups. When analyzed by carriers of the C allele and AA homozygotes, only plasma levels of ET-1 in men differed significantly with AA homozygotes exhibiting the greatest levels of plasma ET-1. After 24 weeks of AEX, there were no significant changes in the main outcome variables within genotype groups. Neither were these changes significantly different between genotype groups. These results suggest that the ECE-1b-C-338A gene polymorphism is associated with plasma levels of ET-1 in men at baseline but not with urinary levels of ET-1. In addition, this gene variant does not affect AEX-induced changes in blood pressure, plasma and urinary levels of ET-1, or sodium excretion.

THE INFLUENCE OF ECE-1 GENE POLYMORPHISM AND EXERCISE
TRAINING ON PLASMA AND URINARY ENDOTHELIN-1 LEVELS IN
PRE- AND STAGE 1 HYPERTENSIVES

by

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
AEX	Aerobic exercise training
AVP	Vasopressin
BMI	Body mass index
DBP	Diastolic blood pressure
ECE	Endothelin converting enzyme
EIA	Enzyme immunometric assay
ET	Endothelin(s)
ET-1	Endothelin-1
ET_A	Endothelin receptor type A
ET_B	Endothelin receptor type B
GFR	Glomerular filtration rate
HRT	Hormone replacement therapy
IMCD	Inner medulla collecting duct
ir-ET	Immunoreactive endothelin/ endothelin-1-like immunoreactivity
NEP	Neutral endopeptidase
RIA	Radioimmunoassay
SBP	Systolic blood pressure
TFA	Trifluroacetate
TPR	Total peripheral resistance
VSMC	Vascular smooth muscle cell(s)

INTRODUCTION

Hypertension is the chronic elevation of blood pressure that develops slowly over long periods of time.[1] The accompanying rise in arterial pressure causes baroreceptors to lose their sensitivity such that they reset at a new higher pressure, which effectively becomes their "normal setting".[1] In the United States, the number of deaths from hypertension rose 56 percent from 1993 to 2003.[2] Moreover, hypertension is an important risk factor for cardiovascular and renal diseases, such as coronary artery disease, atherosclerosis, stroke, and kidney failure. The prevalence of hypertension is unquestionable, yet, the cause of 90-95 percent of the cases of high blood pressure is not known.[2] Endothelin-1 and genetic variants may be among the many factors that contribute to the incidence of this "silent killer."

Endothelin-1 (ET-1) is a 21 amino acid peptide originally described as a highly potent vasoconstrictor produced by endothelial cells. It is now evident that ET-1 is produced ubiquitously in the human body. ET-1 produced in the vasculature and renal tubules influences blood pressure. ET-1 produced in these two distinct biological pools has complex and opposite effects on blood pressure. Vascular-derived ET-1 is synthesized and secreted by endothelial cells and acts as a potent vasoconstrictor, potentially causing an increase in blood pressure.[3] On the other hand, increases in ET-1 action in the nephron mediate diuresis and natriuresis,[4] favoring a decrease in blood pressure.[5]

The precursor of the mature peptide ET-1 is big endothelin. Big endothelin is converted into ET-1 by endothelin converting enzyme (ECE).[6] Evidence suggests that the enhanced expression of ECE increases ET-1 synthesis.[7] A polymorphism, ECE-1b-C-

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338A, in the 5′-regulatory region of the ECE-1 gene has been identified.[7] The A-allele has been associated with a higher promoter activity. A study by Funalot et al. found that older hypertensive women who were homozygous for the A allele had significantly higher blood pressure.[8] In contrast, AC heterozygotes had blood pressure levels similar to those of CC homozygotes. Thus, they suggested that the A allele may be recessive for this gene variant in the population.[8] Since the AA homozygotes are expected to have the highest levels of ECE-1 gene expression, the A-allele is associated with higher plasma levels of ET-1.[8]

Research suggests that plasma ET-1 is significantly elevated in individuals with severe hypertension, and cardiovascular and renal diseases.[4, 9-12] Moreover, plasma ET-1 levels are correlated with the degree of hypertension in patients with chronic renal failure.[13] Thus, elevated plasma ET-1 may be of pathophysiological significance.[12] Limited animal and human evidence suggests that aerobic exercise training (AEX) reduces the levels of circulating plasma ET-1.[14-16] This seems to result in blood pressure reduction especially in severe hypertensive subjects. Research on urinary ET-1 is limited when compared to that of plasma ET-1. One existing study suggest a positive correlation between urinary ET-1 excretion and urinary sodium excretion levels.[17] Renal sodium handling has a major effect on blood volume and blood pressure regulation. A decrease in ET-1 excretion may be indicative of sodium retention, leading to higher blood volume and a concomitant increase in blood pressure. Extremely low levels of circulating ET-1 appear in the urine because most (> 99%) of the ET-1 filtered from plasma is subject to degradation by neutral endopeptidase in the proximal tubule.[18] Therefore, urinary ET-1 excretion is indicative of intrarenal ET-1 generation and reduced production may be a

marker for hypertension.[19, 20] Unpublished data from our laboratory suggest that AEX increases urinary ET-1 and sodium excretion. Hence, AEX seems to favorably modify ET-1 levels in the renal tubules and the renal and peripheral vasculature.

While a number of studies have investigated the effect of exercise training on plasma and urinary ET-1, there are no data available on the effect of the ECE-1b-C-338A polymorphism on these variables at baseline or on their changes with AEX. Since ET-1 mediates its effects in the vasculature through modulation of vascular tone and in the renal system through modulation of sodium handling, it is important to understand the influence of this gene polymorphism because it regulates the synthesis and levels of ET-1. Thus, the goal of this study is to investigate the association of the ECE-1b-C-338A polymorphism with plasma and urinary levels of ET-1 in pre- and stage 1 hypertensives. This study will also examine if these associations are influenced by AEX.

HYPOTHESES

1. The ECE-1b-C-338A polymorphism will be associated with baseline levels of blood pressure, plasma and urinary ET-1, and sodium excretion.

    a. Compared to individuals who carry at least one C allele, individuals homozygous for the A allele will have significantly:

        i. higher blood pressure

        ii. greater plasma ET-1

        iii. lower urinary ET-1

        iv. lower sodium excretion

2. ECE-1b-C-338A polymorphism will be associated with exercise training-induced changes in blood pressure, plasma and urinary ET-1, and sodium excretion.

    a. Compared to individuals who carry at least one C allele, individuals homozygous for the A allele with exercise training will exhibit the:

        i. greatest reduction in blood pressure

        ii. greatest reduction in plasma ET-1

        iii. greatest increase in urinary ET-1

        iv. greatest increase in sodium excretion

METHODS

Subjects

Subjects between the ages of 50 and 75 years were recruited through print and media advertising and direct mailings from College Park, Maryland and surrounding areas. The age range was selected to permit the study of a homogenous group of subjects. Subjects responding to advertisements in newspapers and on the radio underwent a telephone interview to assess their initial eligibility. Subjects included in the study were sedentary (regular aerobic exercise ≤ 2 sessions/week and < 20 min/session and regular resistance exercise < 1 hour per week in the past year, sedentary occupation), nonsmokers, and non-diabetic (fasting glucose level < 126 mg/dl). They also had an average systolic blood pressure between 120-159 or a diastolic blood pressure between 80-99 mmHg (JNC VII Pre- and stage 1 hypertension) while not on medications; had a BMI < 37; had normal renal function (serum creatinine < 1.5 mg/dl); had a negative exercise test, and had no other medical conditions that would preclude vigorous exercise. Hypertensive subjects using antihypertensive medications were tapered off their medications under physician supervision before participating in the study. All women on hormone replacement therapy (HRT) agreed to maintain their regimen for the duration of the study. All subjects signed a written informed consent after the study and potential risks had been discussed and all subjects' questions had been answered. The written informed consent was in accordance with and was approved by the University of Maryland Institutional Review Board.

Screening

Subjects underwent two screening visits. Subjects' medical histories were reviewed on their first laboratory visit to ensure they met the study inclusion criteria. Subjects had blood samples drawn after a 12-hour overnight fast for blood chemistries, measurement of lipoproteins, and isolation of DNA. On the second visit, qualified subjects underwent a physical examination and a physician-supervised maximal treadmill exercise test (Bruce protocol) to screen for cardiovascular, pulmonary, or other diseases that would preclude exercise training. Blood pressure, electrocardiogram, and heart rate were measured before the treadmill test, at the end of each stage, and six minutes after the test. The test was terminated upon the onset of cardiovascular signs and/or symptoms, or when the participant could no longer continue. Subjects were included in the study if they did not exhibit any cardiovascular signs or symptoms.[21]

Dietary Stabilization

Qualified subjects were stabilized for six weeks on an American Heart Association (AHA) Step I diet (50-55% of calories from carbohydrates, 30-35 % from fat, 20-25% from protein, 350 mmol/day of cholesterol, and 3 g/day of sodium). This was necessary because diet has an effect on sodium excretion and blood pressure. Prior to beginning the baseline testing and the exercise intervention, a registered dietician instructed the subjects on the principles, application, and maintenance of the AHA Step I diet. Those who did not adhere to the prescribed diet for the last three weeks of the stabilization period were required to extend the dietary stabilization period until they complied and were weight stable for $\geq$ 3 weeks before undergoing baseline testing.

Compliance with the level of dietary sodium intake was checked by assessing 24-hour urinary sodium excretion. Subjects maintained this diet until they completed all testing and training.

Baseline Testing

At the completion of the dietary stabilization period and before exercise training began, all qualified subjects completed baseline testing to determine the main outcome variables.

Body mass index

A Detecto eye-level physician's scale was used to measure the height and weight of all subjects. Body mass index (BMI) was calculated as weight (kilograms) divided by height (meters) squared.

Casual blood pressure measurement

Casual blood pressure was measured on three separate days according to the JNC VII guidelines.[22] The average of the casual blood pressure measurements recorded on the three separate baseline testing days was the primary outcome variable used in analyses.

Body composition:

Percent body fat was measured using dual-energy X-ray absorptiometry (DPX-L software version 1.3z; Lunar Radiation, Madision, WI). Subjects were scanned at medium speed after a 12-hour overnight fast.

Maximal oxygen consumption (VO$_2$max):

Maximal oxygen consumption (VO$_2$max) was assessed using indirect calorimetry during a graded exercise test to exhaustion. The test began at 70% of the peak heart rate achieved on the subject's previous exercise test and treadmill grade was increased by 2% every two minutes. Blood pressure, heart rate, and electrocardiogram were monitored. Oxygen uptake was measured using a computerized VO$_2$ system (Mass Spectrometer MGA-1100, Marquette Electronics Inc., Milwaukee, WI) and a bi-directional turbine flow meter (Ventilation Measurement Module VMM-2, Interface Associates, Aliso Viejo, CA). The test was terminated in accordance with the ACSM termination criteria.[21] VO$_2$max was used to derive valid exercise prescriptions specific for the subject for the exercise training intervention.

Twenty-four hour urine collection:

The 24-hour urine collection period began immediately after the subject's first void in the morning. Subjects were asked to collect their urine in marked urine containers for 24 hours. On the following morning, the 24-hour urine collection period ended upon the first void. Urine samples were processed and stored at –80°C until analyzed for urinary ET-1 and sodium excretion.

Other plasma samples:

On a different day, additional blood samples were collected after another 12-hour overnight fast to assess plasma ET-1 concentration. Blood samples were drawn from each

subject in a seated position from an antecubital vein and collected in tubes containing

EDTA. Plasma from the blood sample was stored at –80 °C until assays were performed.

ET-1 immunoassay:

To measure either plasma or urinary ET-1, it was first extracted with C_{18} seppak

cartridges (Waters, Milford, MA). 500 μL of aprotinin was added to 1 mL of thawed

sample to inhibit any further reactions. 600 μL of the original 1 mL sample + aprotinin

was acidified with 600 μL of 0.1% trifluroacetate (TFA) and centrifuged at 3000 rpm for

15 minutes @ 4°C. A column chromatography setup with the C_{18} seppak cartridges was

equilibrated with 4 mL of 60% acetonitrile in 0.1% TFA and then washed with 10 mL of

0.1% TFA twice. 1 mL of sample supernatant was passed through the column. It was then

washed twice with 10 mL of 0.1% TFA. Liquid waste was thrown out before eluting with

3 mL of 60% acetonitrile in 0.1% TFA to collect desired sample. Samples were

evaporated to dryness. ET-1 assay was performed in duplicate with the Assay Designs'

Human Endothelin-1 TiterZyme Enzyme Immunometric Assay (EIA) kit. Briefly, the

assay involved extracted ET-1 binding to immobilized polyclonal antibody on a

microtiter plate. A labeled polyclonal antibody was added that binds to the ET-1 captured

on the plate. The concentration of ET-1 was measured by observing color generated when

the substrate reacted with the medium. According to the manufacturer, cross-reactivity

with other isoforms of ET-1 was < 0.1%. Intra-assay and inter-assay coefficients of

variance of low ET-1 levels (~4.76 pg/mL) were 8.5% and 8.1%, respectively. Detection

limit was 0.14 pg/mL. Endothelin-1 concentration was determined by using an Emax

Maxline Microplate Reader (Sunnyvale, CA). The plate was read at 450 nm.

Genotyping the ECE gene polymorphism:

Blood samples (10 mL) drawn into EDTA tubes were used to genotype for the ECE-1b-C-338A polymorphism. The anti-coagulated whole blood was centrifuged at 3,000 g for 20 minutes. Genomic DNA was isolated using the Genomic DNA Purification Kit (Gentra Systems, MN). Primers for the ECE-1b-C-338A polymorphism were: forward, 5'-TAGGGTTATAGGAGAGGGCTCAGG-3' and reverse, 5'-AAGTATCAGGAAGGTGCCCTCAAT-3'. PCR amplification was carried out in a reaction volume of 15 μL containing 1.5 μL of PCR Buffer (10X), 0.9 μL of $MgCl_2$ (25 mM), 2.4 μL dNTP (1.25 mM), 0.225 μL of each primer, 0.06 μL of Taq DNA polymerase, 8.49 μL of deionized water, and 1.2 μL of DNA. PCR was performed using an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealed at 56°C for 30 seconds (40 cycles), and extension at 72°C for 30 seconds. The PCR amplicon was digested overnight at 65ºC using Tsp509 I (New England BioLabs, MA) followed by electrophoresis for four hours in a gel composed of 3% agarose (FMC, ME). The A allele (presence of the Tsp I restriction site) yielded fragments of 243 bp, 178 bp, and 25 bp, and the -338C genotype (absence of the Tsp I restriction site) yielded fragments of 243 bp and 203 bp.

Exercise Training Protocol

The exercise training program consisted of three supervised sessions of aerobic exercise per week for 24 weeks. Exercise sessions started and concluded with appropriate warm-up and cool-down activities. Initial training sessions consisted of 20 minutes of exercise at 50% VO_2max. Training duration was increased by five minutes every week

until 40 minutes of exercise at 50% VO_2max were completed each session. Training

intensity was then increased by 5% VO_2max every week until an intensity of 70%

VO_2max was achieved. Increases in training duration or intensity occurred only if the

subjects completed their level of exercise for three consecutive sessions without

cardiovascular signs or symptoms or unwarranted fatigue. A fourth unsupervised exercise

session was added to the training program for the last 14 weeks of training. Thus, during

weeks 10 through 24, subjects performed aerobic training for 40 minutes at 70% VO_2

max, four times per week. Participants were required to maintain their weight within 5%

of their baseline body weight throughout the entire study.

Final Testing

At the completion of exercise training, subjects provided seven-day food records

to ensure dietary compliance. All final tests were performed 36-48 hours after each

subject's usual exercise session. This was necessary to avoid any acute effects of exercise

on the cardiovascular system and other outcome variables. Subjects continued their

exercise training until all final tests were complete.

Statistical Analyses

Statistical analysis was performed using SAS version 9.1 (Cary, NC). ANOVA

assumptions of homogeneity of variance and normality were examined. Variables that did

not meet these assumptions were log-transformed before ANCOVAs were performed.

Separate ANCOVAs were performed using ECE-1 genotype (AA, AC, and CC) as the

independent variable and blood pressure, plasma ET-1, urinary ET-1, and sodium

excretion as dependent variables. Possible confounding factors such as age and BMI were used as covariates in the analyses. ANCOVA was used to compare differences in AEX-induced changes in all outcome variables between genotype groups after accounting for confounding variables. Statistical significance in AEX-induced changes in outcome variables was tested using ANCOVA. Significance was established at $P < 0.05$. Values were expressed as means $\pm$ SEM.

RESULTS

Baseline Characteristics of Overall Group

This study included a total of 44 subjects (23 men and 21 women) with a mean age of 59 ± 1 years. Eight of the 21 women were on HRT. Fifty-five percent of the subjects were Caucasians (n = 24), 36% were African American (n = 16), and the remaining 9% were Pacific Islanders, Hispanic or Other (n = 4). Table 1 shows the subject characteristics of the overall group. The mean BMI, VO$_2$max, and HDL and LDL cholesterol of the overall group were 28.47 ± 0.53 kg/m^2, 25.14 ± 0.65 ml/kg/min, 49.03 ± 2.90 mg/dL, and 112.08 ± 5.11 mg/dL, respectively. The average blood pressure value was 133/88 mmHg. All subjects were pre- and stage 1 hypertensive, except two individuals whose diastolic blood pressure of 102 mmHg classified them as stage 2 hypertensives according to JNC VII guidelines. Data from these two subjects were kept in the analyses because they did not significantly influence the results. In addition, although the ranges reported in Table 1 may suggest that some subjects were normotensive, JNC VII guidelines require normotensive individuals to have both systolic and diastolic blood pressure below 120 mmHg and 80 mmHg, respectively. All subjects in the current study had systolic blood pressure between 109 and 159 mmHg and/or diastolic blood pressure between 77 and 102 mmHg. The average sodium excretion rate was 112.69 ± 9.23 mmol/d. Average plasma ET-1 level was 3.55 ± 0.52 pg/mL and average urinary ET-1 level was 2.21 ± 0.29 pg/mL. Normality test revealed an outlier in the urinary ET-1 data. This data point was excluded in analyses. The frequency of the CC, CA, and AA genotypes in this study group were 25%, 39%, and 36%, respectively with a

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minor allele frequency of 56% (Table 2). Also presented in Table 2 are the genotype distribution and allele frequency in men and women.

Table 1. Baseline characteristics of overall group

	N	Baseline	Range
Age, years	44	58.82 ± 0.94	$50.00 - 75.00$
Body mass index, kg/m²	43	28.47 ± 0.53	$21.37 - 35.06$
VO$_2$max, ml/kg/min	42	25.14 ± 0.65	$17.00 - 33.40$
Total body fat, %	39	37.16 ± 1.35	$20.50 - 55.50$
Triglycerides, mg/dL	39	116.92 ± 9.28	$42.00 - 316.00$
HDL cholesterol, mg/dL	39	49.03 ± 2.90	$27.00 - 112.00$
LDL cholesterol, mg/dL	39	112.08 ± 5.11	$48.00 - 177.00$
SBP, mmHg	36	132.56 ± 1.80	$109.40 - 159.20$
DBP, mmHg	36	87.84 ± 1.04	$77.00 - 102.00$
Na$^+$ excretion, mmol/d	42	112.69 ± 9.23	$43.00 - 300.00$
Plasma ET-1, pg/mL	35	3.55 ± 0.52	$0.00 - 11.60$
Urinary ET-1, pg/mL	31	2.21 ± 0.29	$0.00 - 6.30$

Values are expressed as means $\pm$ SEM.

Table 2. Distribution and allele frequency of the ECE-1b-C-338A polymorphism

	Men	Women	Total
Genotype, % (n)			
CC	30.5% (7)	19% (4)	25% (11)
CA	30.5% (7)	48% (10)	39% (17)
AA	39% (9)	33% (7)	36% (16)
Allele frequency			
A allele	54%	57%	56%

Baseline Differences

Residuals of HDL cholesterol, sodium excretion, plasma ET-1, and urinary ET-1 were not normally distributed. Therefore, these variables were log-transformed prior to analyses. However, means were presented using original data to allow for meaningful interpretation of results. Maximal oxygen consumption ($P < 0.001$), percent total body fat ($P < 0.001$), and HDL cholesterol ($P = 0.01$) differed significantly between men and women (Table 3). In addition, percent body fat significantly differed among the three ethnic groups with African-Americans manifesting the highest percent body fat ($P = 0.02$) (data not shown). Because gender distribution within ethnic groups could confound results, ethnic differences were analyzed in men and women. Since the "other" ethnic group was composed of only 4 subjects, it was not included in the sub-analyses. This sub-analysis revealed that African American women had a significantly higher BMI than Caucasian women (30.59 ± 1.09 vs. 25.73 ± 0.97, $P = 0.01$), while African American men and Caucasian men had similar BMI (27.78 ± 0.67 vs. 28.78 ± 0.79, $P = 0.67$) (Table 4). Also, there was a tendency towards a significant ethnic difference in urinary levels of ET-1 in men (3.73 ± 1.29 vs. 1.61 ± 0.39 in African American men and Caucasian men, respectively, $P = 0.05$) but not in women ($P = 0.13$). All other variables were similar between genders, among ethnic groups, and between African Americans and Caucasians in men and women.

None of the baseline measures, including the main outcome variables, were significantly different ($P > 0.05$) between carriers of the C allele and AA homozygotes (i.e. CC+CA vs. AA) except for plasma ET-1 (Table 5). Carriers of the C allele had significantly lower levels of plasma ET-1 compared to AA homozygotes (2.56 ± 0.57 vs.

4.93 ± 0.94 pg/mL, respectively, P = 0.02). However, when analyzed by the three

genotype groups, plasma levels of ET-1 were not significantly different (P = 0.12). No

covariates were used in the analysis because those considered did not differ between

groups and did not correlate with plasma levels of ET-1. However, AA homozygotes had

higher SBP, DBP, and sodium excretion values although these differences were not

statistically significant.

Similarly, there were no gender specific differences in all variables among

genotype groups except for plasma and urinary ET-1 levels. There was a gender specific

genotype difference in plasma levels of ET-1 in men (2.28 ± 0.73 pg/mL in CC + CA

genotype group vs. 5.80 ± 1.08 pg/mL in AA genotype group, P = 0.01) (Figure 1). While

the AA homozygote men had the highest levels of plasma ET-1, we observed similar

levels of plasma ET-1 in CC homozygotes and CA heterozygotes (1.96 ± 0.65 and 2.72 ±

1.58 pg/mL, respectively, P = 0.64). Plasma levels of ET-1 were similar among genotype

groups in females (Figure 1). There was no significant difference in urinary levels of ET-

1 between carriers of the C allele and AA homozygotes. Of interest, however, was the

finding that CA heterozygotes had the lowest levels of urinary ET-1 (0.98 ± 0.47 pg/mL)

compared to CC homozygotes (2.58 ± 0.96 pg/mL, P = 0.04) and AA homozygotes (2.27

± 0.66 pg/mL, P = 0.02) in men (Figure 2). Although urinary levels of ET-1 differed

significantly between CA heterozygotes and CC and AA homozygotes, this significance

was no longer evident when we accounted for baseline levels of LDL cholesterol. We

covaried for LDL cholesterol because this variable was correlated with urinary ET-1.

When the main outcome variables were analyzed by gender within genotype groups, no

significant differences were found. Sub-analyses by ethnicity within genotype groups could not be performed due to small sample size.

There was a significant correlation between sodium excretion and diastolic blood pressure (r = 0.37; P = 0.03) in the overall group (Table 6). There was also a significant correlation between plasma ET-1 levels and diastolic blood pressure in the overall group as well as in the CC+CA group (Table 6). Otherwise, there were no other significant correlations between any of the main outcome variables in the overall group, and when analyzed by genotype groups.

Table 3. Baseline gender differences

	Gender		
Characteristic	Men	Women	P-value
Age, years	60.13 ± 1.46 (23)	57.38 ± 1.08 (21)	0.14
Body mass index, kg/m²	28.39 ± 0.64 (22)	28.55 ± 0.87 (21)	0.89
VO_2max, ml/kg/min	27.27 ± 0.80 (22)	22.79 ± 0.77 (20)	0.0002†
Total body fat, %	31.23 ± 1.03 (20)	43.40 ± 1.58 (19)	<0.0001†
Triglycerides, mg/dL	124.50 ±15.58 (20)	108.95 ± 9.82 (19)	0.41
[T]HDL cholesterol, mg/dL	42.15 ± 3.07 (20)	56.26 ± 4.50 (19)	0.01*
LDL cholesterol, mg/dL	106.75 ± 7.35 (20)	117.68 ± 7.04 (19)	0.29
SBP, mmHg	134.71 ± 2.34 (19)	130.15 ± 2.71 (17)	0.21
DBP, mmHg	89.12 ± 19 (19)	86.41 ±1.28 (17)	0.20
[T]Na^+ excretion, mmol/d	131.27 ± 13.83 (22)	92.25 ± 10.57 (20)	0.37
[T]Plasma ET-1, pg/mL	3.57 ± 0.71 (19)	3.52 ± 0.79 (16)	0.96
[T]Urinary ET-1, pg/mL	1.98 ± 0.44 (17)	2.51 ± 0.40 (13)	0.39

Values are expressed as means ± SEM.
Numbers in parentheses represent sample sizes.
* $P < 0.05$.
† $P < 0.001$.
[T] Represents log-transformed data. Although means ± SEM of original data are shown, the P-value of analyses with the log-transformed data was reported.

Table 4. Baseline ethnic differences

Characteristic	Men			Women		
	Caucasian	African-American	P-value	Caucasian	African-American	P-value
Age, years	60.35 ± 1.69 (17)	59.00 ± 4.76 (4)	0.75	58.29 ± 1.92 (7)	55.83 ± 1.31 (12)	0.29
BMI, kg/m²	28.78 ± 0.79 (17)	27.78 ± 0.67 (3)	0.61	25.73 ± 0.97 (7)	30.59 ± 1.09 (12)	0.01*
VO_2max, ml/kg/min	27.26 ± 1.02 (17)	27.43 ± 1.39 (3)	0.95	24.46 ± 1.12 (7)	21.70 ± 1.12 (11)	0.12
Total body fat, %	31.41 ± 1.39 (15)	30.67 ± 0.37 (3)	0.82	41.35 ± 2.51 (6)	45.09 ± 2.01 (12)	0.28
TG, mg/dL	129.88 ± 17.60 (16)	118.67 ± 45.94 (3)	0.81	99.43 ± 15.12 (7)	111.50 ± 15.41 (10)	0.60
[T]HDL-C, mg/dL	41.00 ± 3.40 (16)	41.33 ± 7.69 (3)	0.97	55.14 ± 5.94 (7)	58.20 ± 7.69 (10)	0.77
LDL-C, mg/dL	113.56 ± 7.45 (16)	88.67 ± 20.46 (3)	0.21	99.14 ± 11.49 (7)	123.50 ± 7.97 (10)	0.09
SBP, mmHg	134.24 ± 2.51 (14)	132.10 ± 7.22 (3)	0.55	129.32 ± 5.79 (5)	127.80 ± 3.03 (10)	0.80
DBP, mmHg	89.14 ± 2.04 (14)	90.57 ± 0.98 (3)	0.76	88.76 ± 2.54 (5)	85.59 ± 1.33 (10)	0.24
[T] Na^+ excretion, mmol/d	132.44 ± 18.42 (16)	135.00 ± 17.35 (3)	0.95	111.00 ± 29.31 (6)	88.25 ± 9.64 (12)	0.36
[T]Plasma ET-1, pg/mL	3.85 ± 0.86 (14)	2.73 ± 1.40 (4)	0.59	2.22 ± 0.56 (6)	4.76 ± 1.42 (8)	0.17
[T]Urinary ET-1, pg/mL	1.61 ± 0.39 (11)	3.73 ± 1.29 (3)	0.05	2.02 ± 0.26 (6)	3.27 ± 0.71 (6)	0.13

Values are expressed as means ± SEM

Numbers in parentheses represent sample sizes

* P < 0.05

† P < 0.001

[T] Represents log-transformed data. Although means ± SEM of original data are shown, the P-value of analyses with the log-transformed data was reported.

Table 5. Baseline genotype differences

Characteristic	Genotype		P-value
	CC + CA	AA	
Age, years	58.07 ± 1.07 (28)	60.13 ± 1.76 (16)	0.30
Body mass index, kg/m²	28.36 ± 0.61 (27)	28.64 ± 1.02 (16)	0.80
VO_2max, ml/kg/min	25.08 ± 0.70 (27)	25.23 ± 1.35 (15)	0.91
Total body fat, %	37.49 ± 1.51 (25)	36.57 ± 2.70 (14)	0.75
Triglycerides, mg/dL	111.54 ± 9.49 (24)	123.93 ± 19.11 (15)	0.56
[T]HDL cholesterol, mg/dL	50.08 ± 3.87 (24)	47.33 ± 4.43 (15)	0.61
LDL cholesterol, mg/dL	116.17 ± 6.44 (24)	105.53 ± 8.37 (15)	0.32
SBP, mmHg	131.01 ± 2.04 (26)	136.57 ± 3.55 (10)	0.17
DBP, mmHg	87.11 ± 1.19 (26)	89.71 ± 2.08 (10)	0.27
[T]Na^+ excretion, mmol/d	103.50 ± 9.11 (28)	131.07 ± 20.56 (14)	0.27
[T]Plasma ET-1, pg/mL	2.56 ± 0.57 (23)	4.93 ± 0.94 (13)	0.02*
[T]Urinary ET-1, pg/mL	2.28 ± 0.40 (19)	2.09 ± .0.42 (11)	0.42

Values are expressed as means ± SEM.
Numbers in parentheses represent sample sizes.
* $P < 0.05$.
† $P < 0.001$.
[T] Represents log-transformed data. Although means ± SEM of original data are shown, the P-value of analyses with the log-transformed data was reported.

Figure 1. Genotype difference in plasma levels of ET-1 by gender
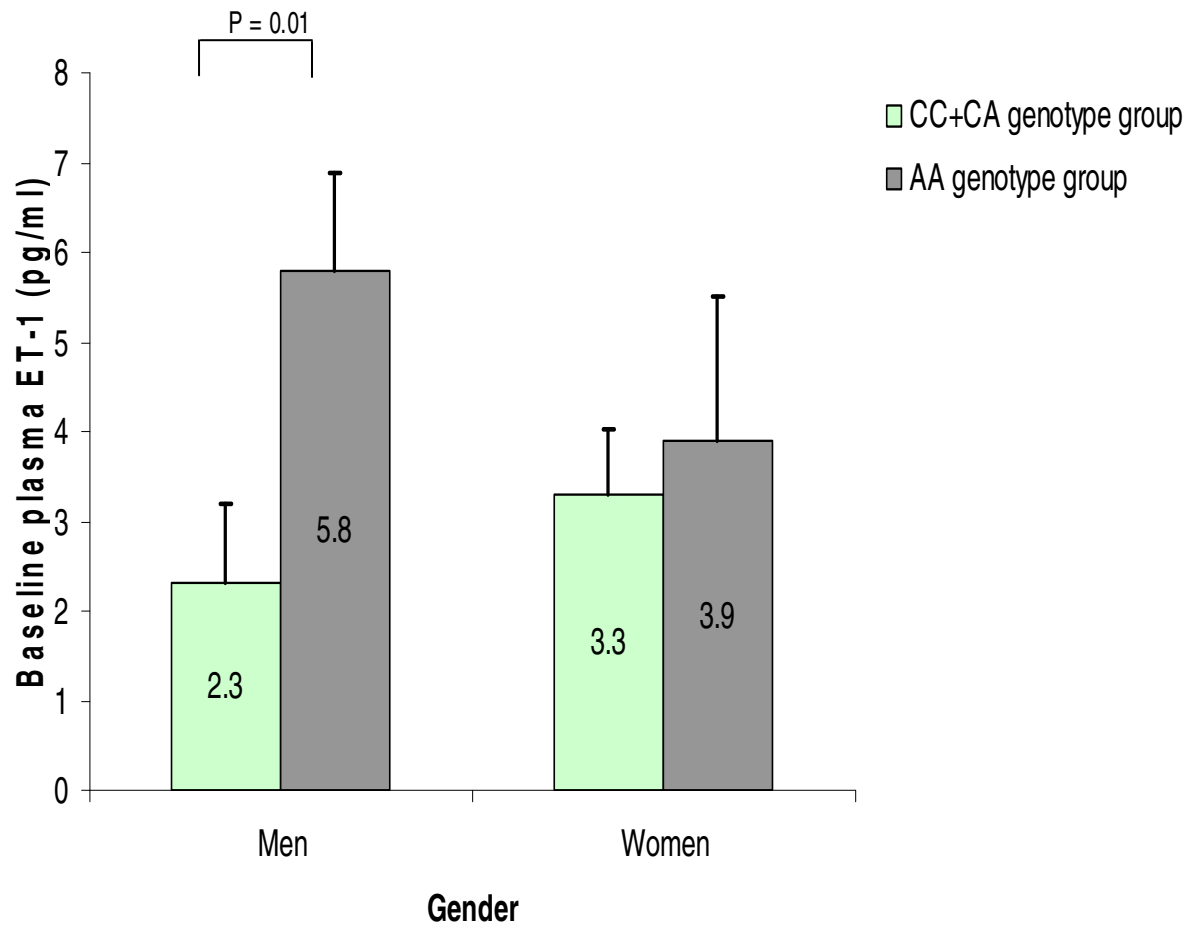

P = 0.01

Baseline plasma ET-1 (pg/ml)

8
7
6
5
4
3
2
1
0

☐ CC+CA genotype group
■ AA genotype group

2.3   5.8   3.3   3.9

Men   Women

Gender

Figure 2. Genotype difference in urinary levels of ET-1 by gender
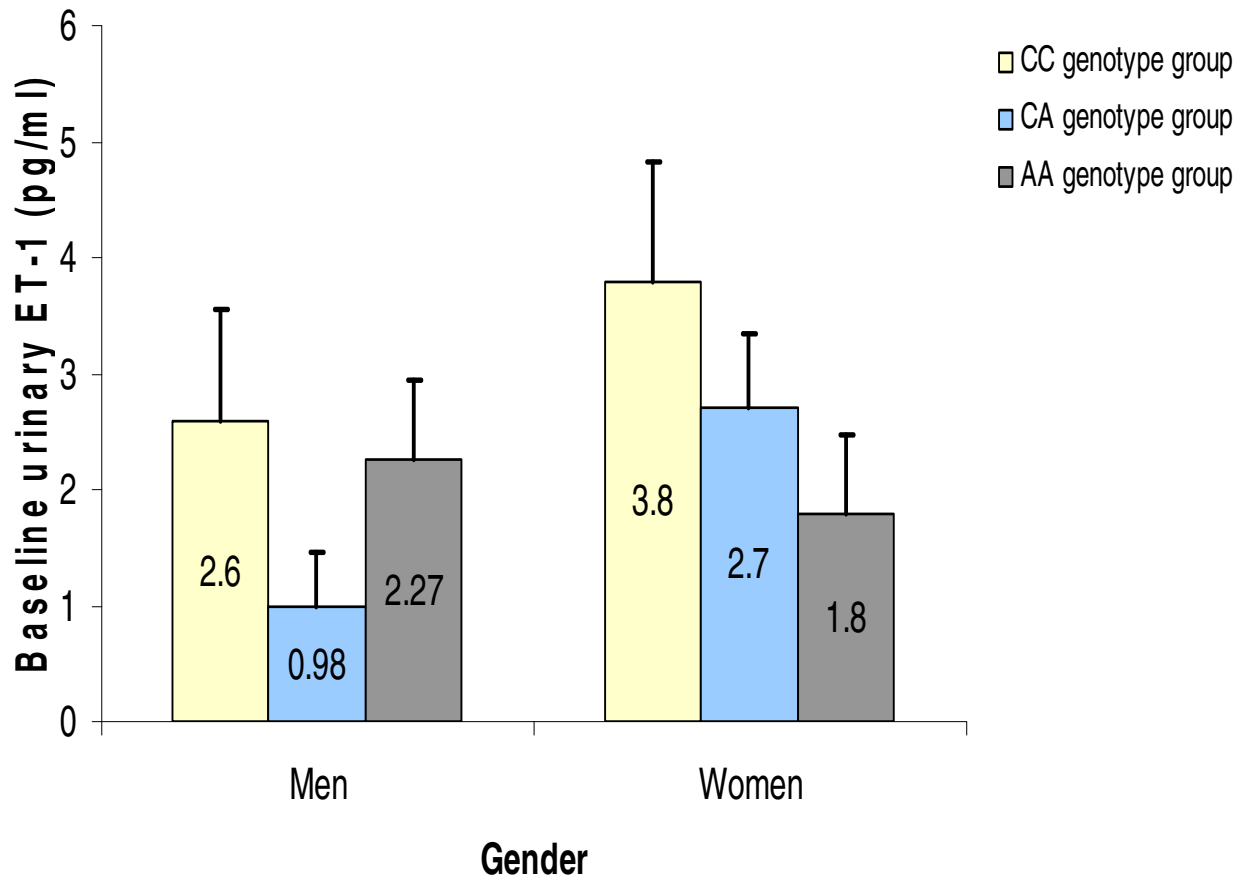
Table 6. Correlation coefficients of main outcome variables

		[T]Urinary ET-1	[T]Plasma ET-1	[T]Na$^+$ excretion	DBP
Overall Group					
SBP	r	0.07	-0.36	0.27	0.64
	P-value	0.76	0.13	0.11	<0.001*
	N	24	26	36	36
DBP	r	0.10	-0.42	0.37	
	P-value	0.65	0.03*	0.03*	
	N	24	26	36	
[T]Na$^+$ excretion	r	-0.05	0.14		
	P-value	0.80	0.45		
	N	29	32		
[T]Plasma ET-1	r	0.27			
	P-value	0.21			
	N	23			
CC+CA Group					
SBP	r	0.11	-0.36	0.10	0.65
	P-value	0.68	0.13	0.63	<0.001†
	N	17	19	26	26
DBP	r	0.13	-0.54	0.28	
	P-value	0.62	0.02*	0.16	
	N	17	19	26	
[T]Na$^+$ excretion	r	0.01	0.16		
	P-value	0.98	0.50		
	N	19	21		
[T]Plasma ET-1	r	0.32			
	P-value	0.22			
	N	16			
AA Group					
SBP	r	-0.33	0.01	0.51	0.58
	P-value	0.48	0.99	0.13	0.08
	N	7	7	10	10
DBP	r	-0.31	-0.32	0.47	
	P-value	0.50	0.48	0.17	
	N	7	7	10	
[T]Na$^+$ excretion	r	-0.42	0.04		
	P-value	0.22	0.91		
	N	10	11		
[T]Plasma ET-1	r	-0.17			
	P-value	0.72			
	n	7			

[T] Represents log-transformed data, Na$^+$ represents sodium.

Training-induced Changes in Overall Group

Of the 44 subjects, thirty-six (20 men and 16 women) completed the 24 week

aerobic exercise training (AEX) intervention. Fifty-six percent of those completing the

study were Caucasians (n = 20), 33% were African Americans (n = 12), and the

remaining 11% were Pacific Islanders, Hispanic or Other (n = 4). The CC, CA, and AA

genotype frequency in this group was 19%, 39%, and 42%, respectively (Table 7). Also

reported in Table 7 are the genotype distribution and allele frequency in men and women

who completed the AEX intervention.

Presented in Table 8 are the AEX-induced changes in all outcome variables in the

entire group. After AEX, VO_2max was significantly elevated (3.80 ± 0.45 ml/kg/min, P <

0.0001), confirming the efficacy of the exercise training protocol. Although there was a

significant reduction in BMI (-0.34 ± 0.11 kg/m², P = 0.004), all changes were within 5%

of the initial body weight as mentioned in the methods section. Concomitantly, there was

a significant reduction in percent body fat (-1.87 ± 0.37 %, P < 0.0001). Though there

was a significant increase in HDL cholesterol (3.00 ± 0.97 mg/dL, P = 0.01) in response

to AEX, there was a non-significant decrease in LDL cholesterol (-0.25 ± 3.95 mg/dL, P

= 0.95). Also, there was a non-significant decrease in TG levels (-10.71 ± 10.28 mg/dL, P

= 0.34), SBP (-1.14 ± 1.08 mmHg, P = 0.30), and DBP (-1.40 ± 0.80 mmHg, P = 0.09).

Conversely, there was a non-significant increase in sodium excretion (3.38 ± 8.22

mmol/d, P = 0.68). Surprisingly, the entire group manifested a significant increase in

plasma ET-1 level instead of the anticipated decrease. Urinary levels of ET-1 increased,

as expected, by 0.79 pg/mL. However, this exercise training-induced change was not

significant.

Table 7. Distribution and allele frequency of the ECE-1 polymorphism after AEX

	Men	Women	Total
Genotype, % (n)			
CC	25% (5)	6% (1)	19% (7)
CA	30% (6)	56% (9)	39% (14)
AA	45% (9)	38% (6)	42% (15)
Allele frequency			
A allele	60%	66%	69%

Table 8. Training-induced changes in overall group

	N	Change	P-value
Body mass index, kg/m²	36	-0.34 ± 0.11	0.004*
VO_2max, ml/kg/min	36	3.80 ± 0.45	<0.0001†
Total body fat, %	30	-1.87 ± 0.37	<0.0001†
Triglycerides, mg/dL	28	-10.71 ± 10.28	0.34
HDL cholesterol, mg/dL	28	3.00 ± 0.97	0.01*
LDL cholesterol, mg/dL	28	-0.25 ± 3.95	0.95
SBP, mmHg	28	-1.14 ± 1.08	0.30
DBP, mmHg	28	-1.40 ± 0.80	0.09
Sodium excretion, mmol/d	29	3.38 ± 8.22	0.68
Plasma ET-1, pg/mL	26	1.06 ± 0.44	0.02*
Urinary ET-1, pg/mL	23	0.79 ± 0.49	0.15

Values are expressed as means ± SEM.
* Indicates significant ($P < 0.05$) exercise training-induced changes.
† Indicates significant ($P < 0.001$) exercise training-induced changes.

Differences in Training–induced Changes

Residuals of plasma and urinary ET-1 were not normally distributed. Therefore, these variables were log-transformed prior to analyses. As with baseline results, means were presented using original data to allow for meaningful interpretation of results. As shown in Table 9, all training-induced changes in all variables were not significantly different ($P > 0.05$) between carriers of the C allele versus the AA homozygotes. However, AA homozygotes experienced the greatest reductions in BMI, percent body fat, and diastolic and systolic blood pressure, but again, these differences were not statistically significant. They also experienced the least decrease in TG levels but the most increase in sodium excretion. There was an unexpected increase in plasma levels of ET-1 in the CA and AA genotype groups (Figure 3). As mentioned earlier, all these changes were not statistically different among genotype groups. However, as with the baseline results, there were significant gender differences in VO_2max ($P = 0.0013$), percent body fat ($P = 0.01$), and plasma ET-1 level ($P = 0.04$) changes with exercise training (Data not shown). While men manifested no significant change in plasma levels of ET-1 with AEX, women demonstrated a significant increase. There were no ethnic differences in the main outcome variables. All analyses were covaried for baseline levels, as well as variables that correlated with the variable of interest.

Table 9. Genotype differences in training-induced changes

| Characteristic	Genotype		P-value
	CC + CA	AA	
Body mass index, kg/m²	-0.18 ± 0.14 (21)	-0.57 ± 0.17* (15)	0.09
VO$_2$max, ml/kg/min	3.67 ± 0.61† (21)	3.99 ± 0.68† (15)	0.74
Total body fat, %	-1.33 ± 0.43* (18)	-2.68 ± 0.60† (12)	0.07
Triglycerides, mg/dL	-11.93 ± 8.25 (15)	-7.92 ± 20.48 (13)	0.85
HDL cholesterol, mg/dL	3.13 ± 1.62 (15)	2 ± 1.02* (13)	0.89
LDL cholesterol, mg/dL	-2.13 ± 5.87 (15)	1.92 ± 5.33 (13)	0.62
SBP, mmHg	-0.09 ± 1.35 (19)	-3.36 ± 1.63 (9)	0.16
DBP, mmHg	-0.95 ± 0.93 (19)	-2.33 ± 1.58 (9)	0.43
Sodium excretion, mmol/d	-1.95 ± 10.45 (19)	13.5 ± 13.21 (10)	0.38
[T]Plasma ET-1, pg/mL	0.75 ± 0.49 (15)	1.48 ± 0.81 (11)	0.79
[T]Urinary ET-1, pg/mL	1.22 ± 0.84 (13)	0.12 ± 0.22 (10)	0.22

Values are expressed as means ± SEM.
Numbers in parentheses represent sample sizes.
* Indicates significant (P < 0.05) exercise training-induced change.
† Indicates significant (P < 0.001) exercise training-induced change.
[T] Represents log-transformed data. Although means ± SEM of original data are shown, the P-value of analyses with the log-transformed data was reported.

Figure 3. Change in plasma levels of ET-1 with AEX in genotype groups
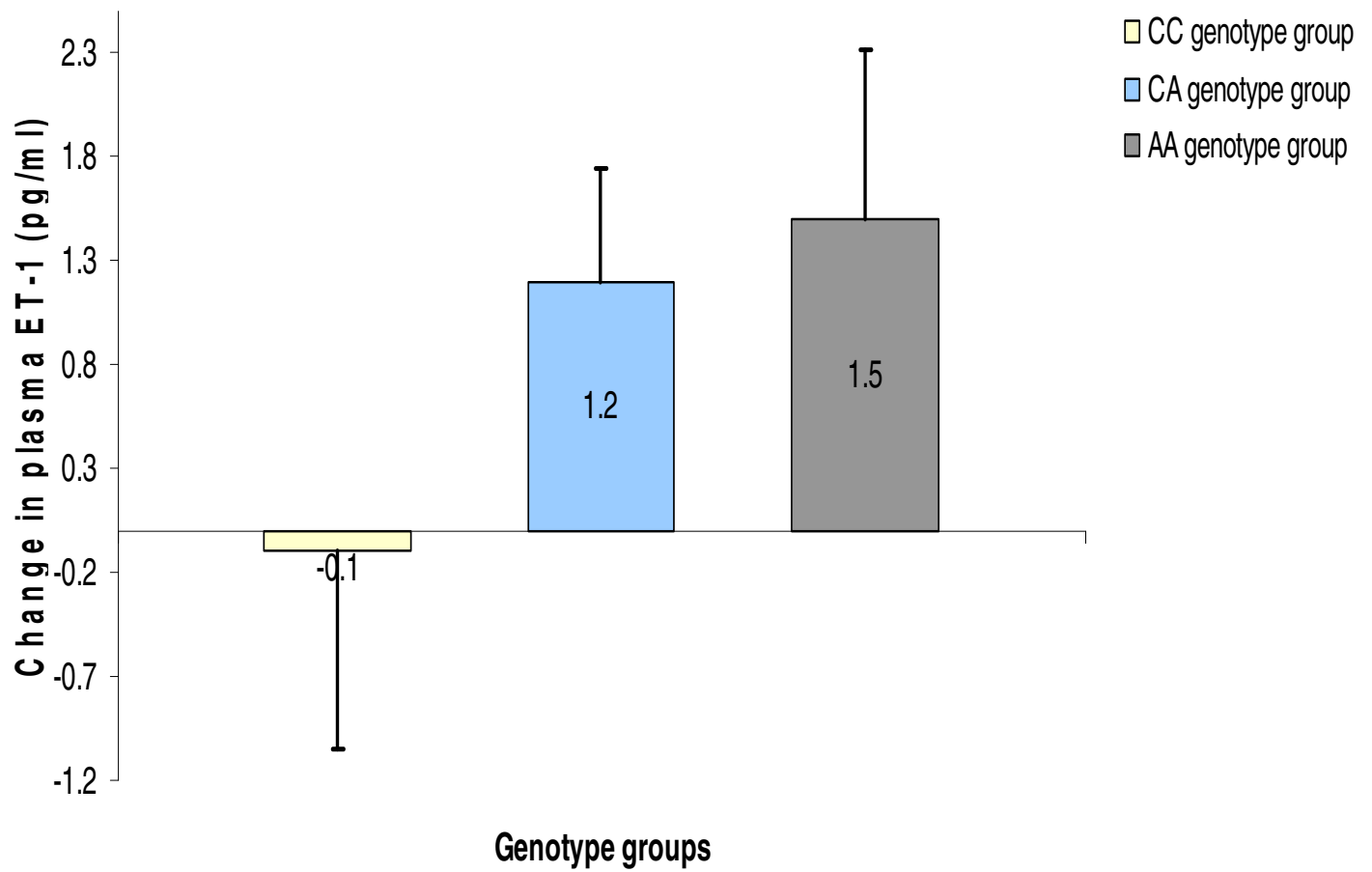
DISCUSSION

Baseline Measures

Blood Pressure

Endothelin-1 (ET-1) is a potent vasoconstrictor and many lines of evidence support its involvement in the regulation of blood pressure.[23-28] Plasma levels of ET-1 have been found to be elevated in subjects with severe hypertension.[29-34] On the other hand, urinary levels of ET-1 have been found to be reduced in individuals with hypertension.[35, 36] Moreover, a study by Jackson et al.[17] reported an association between urinary levels of ET-1 and sodium excretion. This finding was particularly significant in light of the fact that evidence clearly shows a strong association between abnormal renal handling of sodium and blood pressure.[37] Therefore, the main protease, endothelin converting enzyme (ECE), responsible for the genesis of ET-1 by cleavage of its functionally inactive precursor big ET-1 has been reported as a candidate gene for essential hypertension.[38] A polymorphism in this gene, ECE-1b-C-338A, has been suggested to influence blood pressure. Only two studies thus far have investigated the influence of this polymorphism on blood pressure.[7, 8] Both studies found an association of this polymorphism with blood pressure in females only.[7, 8] In the study by Funke-Kaiser et al.,[7] ambulatory blood pressure measurement, which has been suggested to give more valid blood pressure values for research purposes, was used. These investigators found an association of AA homozygosity with higher blood pressure levels in 354 German women. Funalot et al., using casual blood pressure measurements, confirmed the results of Funke-Kaiser et al. in 698 French women. Nonetheless, the current study found

no association between this gene variant and casual systolic and diastolic blood pressure in males and/or females even after accounting for age and body mass index (BMI). Age and BMI were used as covariates because although these variables did not differ between genotype groups, there was a tendency towards a significant correlation with systolic and diastolic blood pressure. However, individuals homozygous for the A allele did have the highest systolic and diastolic blood pressure values in the overall group, as well as in males and females. However, these differences were not statistically significant. Since the subject characteristics of all three studies were similar, the reason for the inconsistency in the present study may be the small sample size, which may have rendered the study with insufficient statistical power to detect differences between genotype groups.

Since blood pressure is a multi-factorial complex trait, intermediate phenotypes (plasma and urinary levels of ET-1 and sodium excretion) were analyzed to determine how the ECE-1 gene variant might perhaps influence blood pressure. It was hypothesized that individuals homozygous for the A allele would have the greatest levels of plasma ET-1 and lowest levels of urinary ET-1 and sodium excretion due to the association of the A allele with higher promoter activity.

Plasma Levels of ET-1

The levels of plasma ET-1 found in the current study are in agreement with several other reports, falling in the range of 0.8 – 10 pg/mL.[36, 39-41] However, the current study found an association between the ECE-1b-C-338A polymorphism and plasma levels of ET-1 in men only with AA homozygotes having the highest levels of plasma ET-1 compared to carriers of the C allele (Figure 1). Because Funke-Kaiser et al.[7] and

Funalot et al.[8] found an association between this polymorphism and blood pressure in females rather than males, we anticipated an association of the polymorphism with plasma ET-1 levels in females. The results of the present study contradict what was expected. However, Funalot et al.[8] reported similar blood pressure levels in CC homozygotes and CA heterozygotes in women. Somewhat consistent with this result, we found similar plasma levels of ET-1 in CC homozygote and CA heterozygote males, signifying that the C allele might have a dominant effect. Ultimately, the contradictory gender-specific genotype observations are difficult to reconcile and remain conjectural.

Evidence suggests a strong influence of gender on plasma levels of ET-1. Estradiol inhibits ECE-1 gene expression and ET-1 synthesis.[42-44] Consequently, Polderman et al. showed that women have lower plasma levels of ET-1 in comparison to men.[45] Our data seems to be in disagreement with the previous result reported by Polderman et al. because we did not find a significant gender difference in the levels of plasma ET-1 in the overall group, nor within genotype groups. Instead, our results were in accordance with a recent large epidemiological study by Hirai et al.[46] who found no gender difference in plasma ET-1 in 584 men and 866 women. However, the apparent inconsistency in the present study and the study by Polderman et al. may be due to the different subject characteristics (i.e. healthy young male-to-female and female-to-male transsexual patients in the study by Polderman et al. vs. older hypertensive men and women in the present study). Most importantly, while the subjects in Polderman's study were young healthy patients, the women in the present study were postmenopausal for > 2 years. Having lower estrogen levels, postmenopausal women tend to lose the beneficial effects of this hormone, thus possibly increasing their plasma ET-1 to levels comparable

to men. Nonetheless, in the present study, when women on hormone replacement therapy (HRT) were compared with women not on HRT, there was no significant difference between the two groups. This finding is not in accordance with a report by Ylikorkala et al.[47] who found that postmenopausal women on HRT had reduced plasma levels of ET-1. Since the type, dosage, mode of delivery, and duration of HRT use was not standardized in women on HRT in the present study, specific conclusions cannot be drawn. With regards to experimental methods, most of the studies reported, (i.e. studies by Polderman et al.,[45] Hirai et al.,[46] and Ylikorkala et al.[47]) measured plasma levels of ET-1 by radioimmunoassay (RIA) and had cross-reactivity with big ET, ET-2, and/or ET-3 ranging from < 0.1% to 96%. In contrast, the current study utilized enzyme immunometric assay (EIA) and had practically < 0.1% cross-reactivity with all isoforms of ET but a 100% cross-reactivity with ET-1. These variations in methods, subject characteristics, and even sample size, might explain the discrepancies in results.

Systemic administration of ET-1 above physiological levels induces a prolonged increase in blood pressure, suggesting a role in hypertension.[48] In fact, some older studies found a positive correlation between plasma levels of ET-1 and blood pressure.[33, 34, 49] Although a few other studies did not find a significant correlation between these two variables,[40, 46, 50, 51] we found a slight negative correlation of plasma levels of ET-1 with diastolic blood pressure in the overall group (r = -0.42, P = 0.03), as well as in the "CC+CA" group (r = -0.54, P = 0.02) after log transformation. This result suggests a possible inverse relationship between plasma levels of ET-1 and diastolic blood pressure, a finding that is counterintuitive and in disagreement with published results. Although

there is great variability, studies suggest normal or only slightly increased levels of

plasma ET-1 in hypertensive animals and humans.[39, 52, 53]

Physiological levels of ET-1 are so low that they do not produce any vascular

effects. Under "physiological" conditions in the vessel wall, ~80% of ET-1 is released

abluminally by endothelial cell predominantly on the underlying VSMC.[7, 54] Only 20% of

ET-1 produced by endothelial cells is secreted luminally and, thus, ends up in the

circulatory system.[16] Furthermore, circulating ET-1 may be eliminated through the

kidneys, lungs, and/or liver.[55-58] This suggests that ET-1 acts as an autocrine/paracrine

system rather than a systematic peptide.[7] Also, worthy of attention is the fact that plasma

levels of ET-1 reflect partly ET-1 produced in other organs. Since plasma levels of ET-1

are a result of "spillover into the blood stream"[59] and do not necessarily reflect ET-1

synthesis and/or secretion occurring in tissues,[59] assessment of plasma ET-1 seems not to

be an appropriate parameter[7, 60] for ECE activity and blood pressure levels (in

physiological states and pathological conditions of moderate intensity). Instead, new

techniques must be designed to enable the measurement of ET-1 at the vascular smooth

muscle interface and in tissues. Until then, reports from studies utilizing plasma ET-1 as a

marker should be interpreted with caution.

Urinary Levels of ET-1 and Sodium Excretion

As we discuss urinary and sodium excretion findings from the present study, bear

in mind that unlike plasma levels of ET-1, urinary levels of ET-1 represent ET-1

synthesis in the kidneys. Thus, urinary ET-1 excretion is a marker of nephron ET-1

metabolism.[11] In the nephron, ET-1 acts in an autocrine manner to directly inhibit sodium

and water reabsorption.[11] Hence elevated levels of renal ET-1 will promote natriuresis and favor hypotension, while a reduction would favor sodium and water retention, and contribute to a hypertensive state.

There is great variability in reported "units" of urinary ET-1. In order to compare mean urinary levels of ET-1 in hypertensives subjects across studies, units in this study and other studies were converted to ng/day. Studies have reported levels of urinary ET-1 in hypertensives ranging from 0.03 ng/day[61] to 29 ng/day[36]. The mean concentration of 4.96 ng/day (2.21 pg/mL) urinary ET-1 in the present study falls within the range reported above. Furthermore, this value is similar to values (4.38 ng/day) found in the lab of Ferri et al.[62] Similarly, the average 24-hour sodium excretion rate in the overall group (112.69 mmol/d) in the present study was within normal range.

No previous study has investigated the influence of the ECE-1b-C-338A polymorphism on urinary excretion and/or sodium excretion, although one earlier study has investigated the influence of salt intake on the expression of ECE in the renal medulla.[63] In this study, Fattal et al.[63] found elevated expression of ECE-1 mRNA and ECE-1 immunoreactive protein in the renal medulla of Wister rats exposed to high salt diet than in rats on a normal salt diet.[63] This suggests that increased salt intake up-regulates ECE-1 expresssion.[63] Thus, polymorphisms in ECE most likely result in reduced ET-1 production leading to an inadequate natriuretic response to salt loading.[63] Impaired renal ET-1 generation has been reported in patients with essential hypertension, especially in the salt sensitive compared to salt resistant types.[63, 64] Specifically, urinary ET-1 level has been reported to be lower in hypertensive humans and rats.[35, 36, 65, 66] Based on these findings, it seems that salt loading, rather than hypertension, upregulates

ECE-1 expression in the medulla.[63] Accordingly, studies have documented a positive

association between urinary ET-1 and sodium intake.[67, 68] Thus, we expected sodium

excretion to be correlated with urinary excretion and be associated with this gene variant.

Such correlations and associations were not found in the current study.

In addition, the current study found no significant association between urinary

levels of ET-1 and this gene variant. Neither was there a significant gender specific

genotype association between these variables. Furthermore, while we found no gender

difference in the levels of urinary ET-1, there was a tendency towards a significant ethnic

difference in urinary levels of ET-1. African Americans had higher urinary levels of ET-1

compared to Caucasian men; however, this difference was of borderline statistical

significance (P = 0.05). Since urinary levels of ET-1 tend to be lower in hypertensives in

comparison to normotensives[36] and hypertension is more prevalent in African American

men, we expected African American men to have lower levels of urinary ET-1 even if

these differences were not statistically significant. However, the initial blood pressure

values of African Americans in the present study may explain this discrepancy in results.

As reported previously, blood pressure values did not differ significantly among ethnic

groups; however, African Americans (both men and women) in our study had the lowest

systolic blood pressure. Although these differences were not statistically significant,

based on the blood pressure values, we would expect to see greater urinary ET-1 levels in

African Americans. Interestingly, we found no statistically significant gender differences

in sodium excretion, although men had much greater sodium excretion levels (131.27 ±

13.83 vs. 92.25 ± 10.57, P = 0.37 after log transformation). In addition, we found no

ethnic differences in sodium excretion in the overall group and in men and women.

Studies suggest a strong association between altered renal sodium handling and high blood pressure.[37] Particularly, reduced sodium excretion may increase blood pressure by increasing blood volume. We found sodium excretion to be significantly correlated with diastolic blood pressure (r = 3.7, P = 0.03) but not with systolic blood pressure (r = 0.27, P = 0.11). However, we did not find a significant association between urinary ET-1 and blood pressure. This latter finding is in accordance with data from the labs of Lemne et al.[33] and Hoffman et al.[36] Hoffman and colleagues found a weak negative correlation between mean arterial pressure and urinary ir-ET-1 levels only when his controls and hypertensive subjects were grouped together.[36] Otherwise, Hoffman found no correlation between urinary levels of ET-1 and blood pressure in his hypertensive subjects.

Additionally, we found no relationship between urinary levels of ET-1 and sodium excretion. This finding is somewhat in disagreement with reports from the labs of Hwang et al.[69] and Saito et al.[32] Both investigators found a positive correlation between urinary ir-ET-1 and sodium excretion rate in patients with essential hypertension. In addition, using 24-hour ambulatory blood pressure monitoring, Hwang et al.[69] found that urinary sodium excretion showed a rhythm similar to blood pressure, and urinary ET-1 excretion rate paralleled the sodium excretion rate in both normotensives and hypertensives. They also showed increased urinary ir-ET-1 excretion rate in response to saline loading in normotensive and hypertensive subjects. One reason that may explain the discrepancies in results is the small sample size in the present study. Furthermore, most studies discussed in this section measured ir-ET-1. Immunoreactive ET-1 is a

combination of ET-1, big ET-1, and ET-2.[70] Hence, assessments of ET-1 in the currents study versus the older studies are different.

Aerobic Exercise Training-induced Changes

Blood Pressure

In the present study, we observed non-significant reductions in systolic and diastolic blood pressure (SBP and DBP) in the overall group (-1.14 ± 1.08 mmHg and -1.40 ± 0.80 mmHg, respectively) with AEX. The reductions observed in the present study are small in comparison to those reported in reviews by Hagberg et al.[71] and Brown et al.[72] (i.e. reductions of ~10 mmHg in SBP and ~8 mmHg in DBP). The remarkable differences in the amount of reduction observed can be attributed to initial blood pressure values of subjects in the studies. The average baseline SBP and DBP of the subjects in the studies used in the review by Hagberg et al. and Brown et al. were > 140 mmHg and > 90 mmHg, respectively.[71, 72] Conversely, the average baseline blood pressure of the subjects in the present study was 133/88 mmHg. Since reductions in blood pressure are most pronounced in individuals with severe hypertension,[73] we expected to see smaller reductions. High intensity exercise training is less effective in reducing blood pressure. However, lower training intensities have been consistently shown to effectively reduce blood pressure.[71, 74] Thus, the intensity of 70% of VO_2max, used in the current study, may have blunted the magnitude of changes experienced in this study group.

Because evidence suggests that exercise training reduces blood pressure in humans, and even more so, in individuals with severe hypertension, we hypothesized those individuals homozygous for the A allele, having the highest initial blood pressure

values, would experience the greatest reductions in blood pressure. However, we observed similar non-significant reductions in systolic and diastolic blood pressure in both genotype groups (P = 0.28 and 0.65, respectively). Although the absence of a significant genotype difference in the reductions experienced with AEX may indicate that this gene polymorphism does not influence AEX-induced changes in blood pressure levels in pre- and stage 1 hypertensives, this premise may not be valid due to the exercise intensity utilized and the small sample size.

Plasma Levels of ET-1

The current study found an unexpected significant increase in plasma levels of ET-1 after 24 weeks of AEX (1.06 ± 0.44, P = 0.02). The finding is not in accordance with the reports of Callaerts-Vegh et al.,[75] and Maeda et al.[15, 16] While Callaerts-Vegh et al.[75] reported no change in plasma levels of ET-1 after eight weeks of AEX in patients with congestive heart failure (n = 9), Maeda et al. reported reduced plasma levels of ET-1 with AEX in healthy normotensive young men (n = 7),[15] and older healthy women (n = 8).[16] Differences in their observations may be due to the difference in subject characteristics i.e. patients with a cardiovascular disease vs. healthy subjects. Also, in comparison to the current study (n = 26), the studies by Callaerts-Vegh et al.[75] and Maeda et al.[15, 16] had small sample sizes where a single subject could have dramatically influenced results. In addition, Otusuki et al. found plasma ET-1 levels to be lower in endurance trained athletes compared to strength trained athletes (1.1 pg/mL vs. 1.6 pg/mL).[76] Evidence suggest that nitric oxide (NO) production and bioavailability[12, 77-80] and plasma prostaglandin E (PGE_2)[81] is elevated with AEX. Since NO and PGE_2 are

inhibitory factors of ET-1, one could postulate that exercise training would reduce ET-1 production by the endothelium. However, a case could be made for increases in plasma levels of ET-1 observed with AEX.

Evidence suggests that oxidative stress increases ET-1 production.[82, 83] Moreover, high-intensity AEX ($\geq$ 75% of VO_2max) has been found to possibly increase oxidative stress.[84, 85] Thus, high-intensity AEX possibly increases ET-1 synthesis. However, since plasma levels of ET-1 most probably does not reflect the synthesis of ET-1 in the vasculature, interpretation of previous and current data is compromised and the reason for the discrepancies in results is hard to reconcile. Further studies in larger populations are required to verify these results and to shed more light on the complex interactions among ET-1, oxidative stress, shear stress and AEX.

While there was a tendency towards a significant increase in plasma ET-1 levels in CA heterozygotes (P = 0.06), there was a non-significant increase in AA homozygotes (P = 0.10) and a non-significant reduction in CC homozygotes (P = 0.92) (Figure 3). However, statistical analysis revealed that these differences were not statistically significant. Thus, this gene variant, most likely, does not influence training-induced changes in plasma levels of ET-1.

Urinary Levels of ET-1 and Sodium Excretion

We found no significant increases in urinary ET-1 and sodium excretion with AEX in the overall group. Both findings confirm preliminary results attained from this lab. No previous study has reported the influence of AEX on urinary levels of ET-1, although published data from our lab reported a non-significant increase in sodium

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excretion with AEX.[86] Exercise training has been documented to reduce blood pressure in hypertensives.[71, 87, 88] Although research has shed some light on mechanisms responsible for AEX-induced reductions in blood pressure in hypertensives, there is still more to be elucidated. One of the mechanisms through which AEX may work to reduce blood pressure may be its influence on intrarenal ET-1 production and/or sodium excretion and the complex interactions between these two systems. Given that ET-1 affects renal sodium handling, and consequently blood pressure, the present study investigated the influence of this gene polymorphism on sodium excretion. We found no genotype differences in the AEX-induced changes. Neither did we observe gender-specific genotype differences. This finding suggests that this gene variant may not be associated with urinary levels of ET-1 and sodium excretion.

Limitations

Although we utilized a highly specific EIA (100% cross-reactivity with ET-1 and < 0.1% cross reactivity with other endothelin isoforms), the current study is limited by small sample size, inability to measure ET-1 synthesis in the vasculature, influence of ECE-independent pathways on ET-1 levels, different technicians conducting extractions and assays, compliance of study subjects to the exercise training protocol outside the lab, and inclusion of different ethnic groups when assessing genotype differences.

Many steps were taken to minimize confounding variables. For instance, subjects were asked to maintain their body weight within 5% of their baseline body weight. This precaution was taken so that only the effect of exercise training was examined. Subjects who were not weight stable were excluded from analyses. This elimination further

reduced our already small sample size. Thus, one of the main limitations of this study was the small sample size, limiting the statistical power of this study to detect significant differences between genotype groups. Future studies are required to duplicate this study in larger populations.

Secondly, we were unable to effectively assess ET-1 synthesis in the vasculature. As discussed previously, plasma level of ET-1 is a result of "spillover into the blood stream"[59] and does not reflect vascular synthesis of this peptide.

Thirdly, although urinary ET-1 level is a good marker of ET-1 production in the nephron, there are many ECE-independent pathways that contribute to the genesis of ET-1[89] both in the nephron and in the vasculature. As discussed by Reiterova et al.,[90] other enzymes besides ECE, for instance, chymase A, a major angiotensin II-forming enzyme,[91] and non-ECE metalloproteases, can synthesize ET-1.[89] These pathways are practically impossible to control in human studies. Currently, no available technique exists that can eliminate their influence.

In addition, two different technicians performed ET-1 extractions, purifications, and assays. Although results obtained were identical, personal differences in procedural implementation may affect results.

Results obtained in this study can only be generalized to similar populations i.e. non-smoking and non-diabetic hypertensive middle-aged to older adults who are free of cardiovascular disease.

Furthermore, although subjects in this study were required to strictly follow the prescribed exercise protocol, compliance outside of the lab could not be controlled.

Last but not the least, genotype differences could not be performed in the various ethnic groups. Thus, ethnic differences may confound results.

Conclusion

It is not known how the ECE-1b-C-338A polymorphism affects plasma and urinary levels of ET-1 and AEX-induced changes in these variables. The present study showed for the first time that this gene variant may be associated with plasma levels of ET-1 in men but not with urinary levels of ET-1, signifying that the expression of this gene variant differs in these two biological systems. Additionally, this polymorphism, most likely, does not influence AEX-induced changes in these variables, suggesting no relationship with AEX-induced changes in blood pressure. This finding is of empirical importance, given that accumulation of studies on single nucleotide polymorphisms would facilitate personalized exercise training prescription and/or programs in the near future. However, this study needs to be replicated in larger populations. Additionally, new techniques must be designed to measure the synthesis of ET-1 in humans.

LITERATURE REVIEW

<u>Hypertension</u>

Hypertension, chronically elevated arterial blood pressure, affects one in three adults in the United States.[2] In the 2002 World Health Report, it was estimated that approximately 7.1 million deaths per year may be attributable to hypertension.[92] The high prevalence of hypertension is unquestionable; however, the cause of 90-95 percent of the cases of high blood pressure is not known (primary or essential hypertension).[2] Secondary hypertension, which occurs when elevated blood pressure is a result of another disease, such as renal hypertension, makes up the remaining 5-10 percent. Regardless of the cause of hypertension, baroreceptor reflexes or any of the normal mechanisms that regulate blood pressure cannot compensate for hypertension because under conditions of chronically elevated pressure, the baroreceptors reset and the regulatory mechanisms work to maintain the new, high pressure.[1]

Hypertension has several adverse effects on the cardiovascular system. Elevated arterial blood pressure increases the workload on the heart, hence, it can increase the likelihood of a myocardial infarction. It can also lead to heart failure because it increases afterload (the pressure that the ventricles have to work against as they pump blood) and can chronically elevate end-diastolic volume (the volume of blood contained within each ventricle at the end of diastole). Hypertension-induced damage to blood vessels can also lead to kidney failure and loss of vision.[1] Consequently, hypertension is a major risk factor for cardiovascular and renal diseases, such as coronary artery disease, atherosclerosis, stroke, and kidney failure.

Specific factors that elevate blood pressure include increased cardiac output, total peripheral resistance, and blood volume. Elevated stroke volume and/or heart rate cause cardiac output to increase, while vasoconstriction causes total peripheral resistance (TPR) to increase. Failure of the kidneys to excrete adequate amounts of salt and water results in the retention of excess fluid in the body. This excess fluid causes blood volume to increase, which raises mean arterial pressure. Thus, any condition, genetic variation, hormone, or peptide that inhibits or stimulates any of these factors will influence blood pressure. One such peptide is endothlein-1, a potent vasoconstrictor. Consequently, a genetic variant in the enzyme responsible for the production of endothelin-1 from its precursor influences blood pressure.

Treatments for hypertension include pharmacologic interventions such as diuretics, beta blockers, calcium channel blockers, and angiotensin converting enzyme (ACE) inhibitors, and nonpharmacologic interventions such as exercise, dietary sodium reduction, weight loss, and psychological stress management. Diuretics promote increased excretion of salt and water by the kidneys, thus reducing blood volume and blood pressure. Beta blockers reduce cardiac output while calcium channel blockers reduce the flow of calcium into vascular smooth muscle cells (VSMC), which prevents vasoconstriction and lowers TPR. ACE inhibitors lower TPR by reducing plasma levels of angiotensin II, a potent vasoconstrictor. Aerobic exercise training (AEX) is a major nonpharmacological method for treating hypertension. Specific mechanisms underlying the reduction in blood pressure with AEX in individuals with hypertension are unknown. However, AEX seems to modify many of the factors that influence blood pressure. For instance, AEX reduces resting heart rate and heart rate at any given submaximal exercise

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intensity. Although blood volume is increased with AEX, TPR is reduced.[93] All of these

factors ultimately aid to reduce blood pressure.

As mentioned earlier, ET-1 is a potent vasoconstrictor that influences blood

pressure. Its potency is 10 times that of angiotensin II.[94] Thus, elevated levels of ET-1 in

the vasculature increase TPR by its vasoconstriction action, ultimately having a

hypertensive effect. Interestingly, ET-1 action in the renal tubule has a hypotensive effect

by decreasing total blood volume.[4] The discovery, general biology, and function of ET-1

will be discussed in the next section. Then a general review of the actions of ET-1 in the

vasculature and renal tubule will be presented after the discussion of a genetic variant in

the endothelin converting enzyme that may be associated with hypertension. Lastly, the

influence of acute exercise and aerobic exercise training will be presented.

Endothelin-1

Hickey et al. showed for the first time in 1985 the presence of a vasoconstrictor

peptide secreted by porcine aortic endothelial cells.[95] This observation was confirmed by

Gillespie et al.[96] and O'Brian et al.[97] in 1986 and 1987 respectively. In 1988, Yanagisawa

and his colleagues isolated, sequenced, and cloned the endothelin (ET) peptide from the

supernatant of cultured porcine aortic endothelial cells.[3] The structure of ET is very

unique and does not belong to any previously known mammalian peptide family, but is

remarkably similar to sarafotoxin S6b, the venom of the Israeli burrowing snake

Attractaspis Endaddensis.[98, 99]

Structure of Endothelins

The endothelin family consists of three ET isoforms, ET-1, ET-2, and ET-3. All isoforms are made up of 21 amino acid residues and show complete similarity at ten positions, including all four cysteine (Cys) residues, which are located at positions 1, 3, 11, and 15.[100] These four Cys residues form two intrachain disulfide bonds (Cys1-Cys15 and Cys3-Cys11).[3] Both the disulphide bonds and the C-terminal tryptophan (Trp) residue are essential for its high affinity binding to its receptor(s) and biological activity.[101] All three ET isoforms are synthesized as larger preproforms, which are the primary translation product.[100] Processing of the 212 (human) amino acid prepro-ET peptides occur in three stages. First, neutral endopeptidase (NEP) cleaves prepro-ET at Arg^{52}-Cys^{53} and at Arg^{92}-Ala^{93}. Carboxypeptidase then sequentially trims the Arg^{92} and Lys^{91} residues from the COOH terminus to produce propeptides called big ET. Finally, big ET-1, a 38 (human) or 39 (porcine) amino acid residue, is cleaved at the Trp^{73}-Val^{74} bond to form the final 21-amino acid ET peptide by endothelin converting enzyme (ECE) located in the plasma membrane.[6, 94] Apparently, cleavage of propeptides by ECE is insufficient in vivo and in vitro because big ETs are present in plasma and in the media of cultured cells.[100, 102] Secreted ET stimulates the secretory cell itself (autocrine) and/or neighboring cells (paracrine).[4]

Endothelin-1 Gene

The three isoforms of ET, ET-1, ET-2, and ET-3, are encoded by three distinct genes on different chromosomes.[103-108] ET-1, the predominant isoform and the focus of

this paper, is the most powerful natural vasoconstrictor substance known.[6] It appears to be the ET isoform responsible for most of the pathophysiology associated with alterations in ET production. In humans, the ET-1 gene has been mapped to chromosome 6p23-24 and the complete nucleotide sequence is known. The gene spans 6836 bp of DNA, and contains five exons, four introns, and 5′- and 3′-flanking regions.[109-112]

While the coding region of ET-1 gene is 6.8 kb in length, the primary transcript, ET-1 mRNA, which directs translation of the precursor preproET-1 peptide, is 2.3 kb in length.[100] The human ET-1 mRNA has a half-life of approximately 15-20 minutes, possibly due to three AUUUA motifs in the 3′ non-translated region that may mediate mRNA destabilization.[6, 112, 113] The five exons of the ET-1 gene encodes a portion of preproET-1. For instance, exon 1 encodes the 5′-untranslated region and the first 22 amino acids of the precursor including the entire signal sequence.[100, 114] Exon 2 encodes the sequence for the mature ET-1 peptide, the Trp-Val cleavage site at which ECE processes big ET-1, and the first four residues of the COOH-terminal portion of big ET-1.[100] Exon 3 contains the coding region of the remainder of big ET-1, while exon 5 specifies the COOH-terminal portion of preproET-1 and the 3′ untranslated region of the mRNA.[100] The ET-1 promoter has two functional transcription start sites[115] and several important regulating sequences, including TATAA and CAAT boxes, and binding sites for nuclear factor-1 (NF-1), acute phase reaction regulatory elements (APRE), GATA motif, and AP1 sequence.[100] The GATA motif recognizes zinc finger DNA-binding proteins, while octanucleotide AP-1 sequence binds proto-oncogene products such as c-fos and c-jun complexes.[113]

So to reiterate the process of ET-1 production, transcription of the ET-1 gene by ET-1 mRNA leads to the production of the 212 amino acid PreproET-1.[113] PreproET-1 is hydrolytically cleaved by NEP to form big ET-1, which is then cleaved by ECE to form ET-1.[6, 94]

Endothlein-1 Gene Expression and Regulation

ET-1 is produced in almost all cell and tissue types. For instance, endothelial cells appear to express ET-1 mRNA.[100] ET-1 gene expression also occurs in other cells types such as epithelial cells,[116, 117] keratinocytes,[118, 119] macrophages,[120] bone marrow mast cells,[121] astrocytes,[122] cardiomyocytes,[123] and mesangial cells.[124] Additionally, ET-1 expression has been observed in tissues such as the lungs, brain, uterus, stomach, heart, adrenal gland, and kidney.[125, 126]

The regulation of the transcription of ET-1 mRNA seems to play an important and probably predominant role in the regulation of gene expression.[100] Thus, it has been suggested that ET-1 secretion is determined largely by the levels of transcription and translation.[113] Agents that stimulate the release of ET-1 by enhancing ET-1 mRNA expression fall into three categories: vasoconstrictors/thrombogenic agents, inflammatory cytokines, and physical factors.[113] Vasoconstrictors/thrombogenic agents such as thrombin,[127] angiotensin II,[128-130] vasopressin (AVP),[130] and ET-1 itself[130] increase endothelial cell ET-1 release via sequential activation of phosphorylase C, protein kinase C (PKC), c-Jun/c-Fos, and the AP-1 site in the ET-1 promoter.[113] Examples of inflammatory agents that stimulate ET-1 release are cytokines such as interleukin-1 (IL-1),[127] transforming growth factor β (TGF-β),[130-133] and tumor necrosis factor (TNF).[127,]

[134-136] Finally, some physical factors that elevate endothelial cell ET-1 production are mechanical strain and low shear stress (5 dynes/cm^2).[113, 137] Conversely, ET-1 release is inhibited by anticoagulants and vasodilators such as heparin, bradykinin, prostaglandin E$_2$ (PGE$_2$), prostacyclin (PGI$_2$), atrial natriuretic peptide (ANP), and nitric oxide (NO).[5, 113, 138] High shear stress (25 dynes/cm^2) also decreases ET-1 mRNA expression.[139, 140]

Endothelin-1 Receptors

Both endothelin A (ET$_A$) and endothelin B (ET$_B$) receptors are G protein-coupled receptors. Thus, they contain seven hydrophobic membrane spanning helices joined together by three extracellular and three intracellular loops, an extracellular NH$_2$-terminal region and an intracellular COOH-terminal region.[100] ET$_A$ receptors consist of 427 amino acids and are found on vascular smooth muscle. ET$_B$ receptors contain 442 amino acids and are found on both vascular smooth muscle and endothelium.[113] Although ET$_A$ and ET$_B$ receptors are identical in structure, they are encoded by two distinct genes spanning 40 and 24 kb of DNA, respectively.[141-144] The human ET$_A$ gene is found on chromosome 4 and contains eight exons and seven introns, while the human ET$_B$ gene is located on chromosome 13 and contains seven exons and six introns.[100]

Signal Transduction Mechanisms

When ET-1 binds to ET$_A$ or ET$_B$ receptors found on the vascular smooth muscle, it leads to the formation of inositol triphosphate (IP$_3$). The formation of IP$_3$ leads to calcium mobilization and smooth muscle vasoconstriction.[6, 145] However, when ET-1 binds to ET$_B$ receptors located on endothelial cells, it produces vasodilation (or

vasorelaxation) through endothelial nitric oxide synthase (eNOS).[6] Thus, ET_B receptors localized on endothelial cells participate in vasodilation, whereas ET_A and ET_B receptors localized on vascular smooth muscles mediate vasoconstriction.

Endothelin-1 Receptor Agonists and Antagonists

The development of several ET_A and ET_B receptor antagonists and agonists has helped confirm the operation of ET-1 receptors and holds promise for the treatment of disorders associated with ET-1.[5] Thus, the following is a list of agonists and antagonists of ET receptors, some of which were utilized in various studies that will be discussed throughout this document. Antagonists of the ET_A receptor include BQ-123 and [Dpr[1]-Asp[15]]ET-1. An antagonist of the ET_A receptor is IRL 1038ET-1. An antagonist of the ET_B receptor is BQ-788.[9] Cochinmicins and PD 145065 are antagonists of both ET_A and ET_B receptors. Finally, some agonists of the ET_B receptor are IRL 1620 and sarafotoxin 6c.[113]

As stated previously, the binding of ET-1 to ET_A receptors produces vasoconstriction. Accordingly, ET_A receptor antagonists cause vasodilation. However, ET_A receptor antagonists produce a greater degree of vasodilation in forearm vessels of essential hypertensive patients compared with normotensive subjects.[146] On the other hand, the binding of ET-1 to ET_B receptors produce vasodilation through eNOS or vasoconstriction through calcium mobilization in endothelial cells and VSMC, respectively. Thus, although the ET_B receptor antagonist BQ-788 induces vasoconstriction and increases forearm resistance in normotensive subjects,[147] it stimulates a vasodilator action in the forearm circulation of hypertensive subjects.[146]

Findings on the distribution of ET receptors in African Americans shed some light on this seemingly contradictory finding. Apparently, in African Americans, who have an increased prevalence of hypertension, there is a modification of the ratio between endothelial and smooth muscle ET_B receptors that may lead to an overall ET_B-mediated vasoconstrictor effect.[148, 149] Specifically, Ergul et al.[148] found that the total number of ET_B receptors was lower in black patients in comparison to white patients. Additionally, black patients possessed both ET_A and ET_B receptor subtypes on vascular smooth muscle cells, whereas white patients possessed only the ET_A receptors on vascular smooth muscle cells. Thus, it seems that a modification in the tissue distribution of ET receptors, between vasodilatory and vasoconstrictor receptors, may partly explain the increased prevalence of hypertension in African Americans.[149] Likewise, this may explain for the seemingly opposing effects of BQ-788 on blood pressure in normotensives and hypertensives.

Functions of Endothelin-1

Although ET-1 was originally reported to be produced by endothelial cells, it is now evident that it is produced by many tissues and organs and exerts diverse biological effects on every organ system.[150] Some biologic actions of ET-1 include stimulation of smooth muscle contraction as discussed above, cell proliferation, extracellular matrix accumulation, diuresis, and natriuresis. ET-1 also causes bronchoconstriction[3] and serve as a neurotransmitter.[151] Nonetheless, the vasoconstrictor property of ET-1 is the most widely studied and best understood. For instance, it is now known that ET-1-induced contractions of isolated blood vessels are more slowly developing and are maintained for

a longer time in comparison to other vasoconstrictors such as angiotensin II.[100] Functions of endothelin-1 relevant to this paper are smooth muscle contraction, diuresis, and natriuresis.

Endothelin Converting Enzyme

Endothelin converting enzyme (ECE) is a phosphoramidon-sensitive membrane bound metalloprotease found on endothelial cell membrane. The ECE gene is located on chromosome 1p36, spanning over 120 kbp and consisting of 20 exons.[152-154] It cleaves big ET-1 to ET-1 via proteolytic cleavage between a Trp and valine (Val) residue. There are two different ECE isoforms, ECE-1 and ECE-2.[7] ECE-1 is mainly expressed in the vascular endothelium, while ECE-2 is expressed in neural tissues.[7] ECE-1 exists in four different isoforms (ECE-1a,[155] ECE-1b,[155] ECE-1c,[156] and ECE-1d[153]) which differ by their N-terminal amino acid tails, and result from the existence of four isoform-specific alternative promoters in the gene encoding ECE-1. The N-terminal amino acid is responsible for their cellular location.[8]

ECE-1b, the enzyme of interest in the present study, is an intracellular enzyme, while the other isoforms have their catalytic domain toward the outside of the cell. The ECE-1b isoform is expressed in endothelial and vascular smooth muscle cells and may, therefore, contribute to vascular ET-1 generation. A genetic variation in the ECE-1 gene (ECE-1b-C-338A polymorphism) that may influence blood pressure will be discussed next.

ECE-1b-C-338A Polymorphism

A polymorphism of the ECE-1 gene, ECE-1b-C-338A, in the 5′-regulatory region of the ECE-1 gene (338bp upstream from the translation start site), has been identified that results in a binding site for the transcription factor E2F-2 and GATA proteins.[7, 9] ECE-1b-C-338A is associated with increased promoter activity, with the A-allele showing an increase in promoter activity compared with the wild type promoter.[9] Two studies, one by Funke-Kaiser et al.[7] and the other by Funalot et al.,[8] studied this polymorphism in two cohorts of hypertensive patients. In the study by Funke-Kaiser et al.,[7] the A-allele had a codominant effect on blood pressure in untreated hypertensive German women. Specifically, these German women had a significantly higher daytime and nighttime ambulatory systolic and diastolic blood pressure.[7] In the study by Funalot et al.,[8] performed in a cohort of 1198 subjects (491 men and 698 women) from the French epidemiological study Étude du Vieillissement Artériel (EVA), a similar association was found in women. Since the AC heterozygotes had BP levels similar to those of CC homozygotes, Funalot and his colleagues concluded that it suggests a recessive effect of this gene variant in the population.[8] If this is true, then AA homozygotes are expected to have the highest levels of ECE-1 gene transcription and ECE-1 enzymatic activity. So, while this gene variant seems to have a codominant effect in German women, it seems to have a recessive effect in French women.

As shown by the experiments discussed above, the A-allele is associated with higher promoter activity.[8] Since the ECE-1 gene appears to be the rate-limiting enzyme in the biosynthesis of mature ET-1 in vivo, it has been suggested that the enhanced

expression of the enzyme (i.e. in AA homozygotes) could increase ET-1 synthesis in the vessel wall.[90] However, this is yet to be proven. This present study will seek to confirm this association. Also, ET-1 has been suggested to be a disease promoting factor in the kidney.[90] In a study by Reiterova, Merta, and Stekrova, there was a tendency toward faster decline of renal function in AA homozygous individuals.[90] However, the effect of this polymorphism on renal-derived ET-1 is still yet to be elucidated. Moreover, the reason why an association between this gene variant and blood pressure was observed only in females is yet to be elucidated, but could be related to interactions between sex hormones (estrogen) and the ET system. Stimulation of the ET system by androgens could be an explanation for the lack of effect in males.[9]

Endothelin-1 in the Vasculature (Plasma ET-1)

In the vasculature, elevated ET-1 action predominantly causes vasoconstriction. However, very low levels of ET-1 are found in the plasma due to a number of reasons. First, circulating endothelin may bind to its receptors to produce its biological actions. Additionally, it may be eliminated by pulmonary uptake.[55, 56] Remaining portions may be eliminated through the kidneys by NEP cleavage and a very small amount through the liver.[57, 58, 157] Hence, while the half-life ET-1 mRNA is 15-20 minutes, the half-life of mature ET-1 in the plasma only range from 1-7 minutes.[6, 70, 158-160] In a study by Anggard et al., intravenously injected labeled ET-1 was quickly eliminated from the blood stream showing that ET that is released is quickly eliminated and/or taken up and bound to one of its two possible cell surface receptors.[160, 161] Due to these low levels of ET-1 in the plasma, extraction and purification are usually performed before assay to obtain more

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valid results.[70] In humans and animals, levels of ET-1 and big ET-1 in the blood range

from 0.3 to 3 pg/ml, but there is a great deal of variablility.[57, 102, 162, 163]

Some investigators argue that plasma levels of ET-1 may not accurately reflect

changes in endothelial synthesis or the concentration of the peptide in the vascular wall

because other organs in the body may contribute to the circulating levels of ET-1, and the

majority of ET-1 derived from endothelial cells is released towards the vascular tunica

media[70] and not the lumen. Evidence has confirmed that ET concentration at the

endothelium/smooth muscle interface where it is released is higher than in the

bloodstream.[94] Nonetheless, levels of ET-1 in the plasma reflect a balance between its

production and clearance.[70] So although ET-1 causes vasoconstriction, which increases

total peripheral resistance and hence blood pressure, studies measuring plasma levels of

ET-1 as a maker of elevated blood pressure produce conflicting results.

Consequently, some studies have reported elevated levels of plasma ET-1 in

essential hypertension,[29-34, 164] while others have not.[39, 53] Kohno et al.[29] found that

plasma immunoreactive ET (ir-ET) is elevated in hypertensive individuals with severe

hypertension or renal involvement. In another study by Ergul et al.,[30] both male and

female hypertensive blacks had significantly elevated levels of plasma ir-ET-1 compared

with normotensive control blacks. The difference in plasma ir-ET-1 between black

hypertensives and normotensives was approximately 10 pmol/L. Similarly, male and

female hypertensive whites had significantly elevated levels of plasma ir-ET-1 compared

to normotensive whites. However, the difference in plasma ir-ET-1 between the white

hypertensives and white normotensives was smaller (~ 1.5 pmol/L). In a case-control

study by Lemne et al.,[33] they also found a significantly elevated plasma ir-ET levels in

pre- and stage 1 hypertensives compared to age-matched normotensive men. However, this difference was even smaller (~0.5 pmol/L).

With reference to the negative studies, Predel et al.[53] found no significant increase in plasma ET-1 in hypertensives compared to normotensives although the difference between groups was ~1.1 pmol/L. Likewise, Haynes et al.[39] found similar circulating ET levels in hypertensives and normotensives. However, they found that patients with essential hypertension had enhanced vasoconstriction to ET-1 that is positively correlated with blood pressure. Nevertheless, Forgari et al.[165] found that although plasma levels of ir-ET-1 were not correlated with casual SBP and DBP in pre- and stage 1 hypertensives, they where significantly correlated with 24-hour ambulatory blood pressure (both nighttime and daytime SBP and DBP values), suggesting that the method of acquiring blood pressure measurements may affect the results.

Elevated levels of plasma ET-1 have been observed in other cardiovascular and renal conditions.[113] However, there seems to be little or no correlation between plasma ET-1 and blood pressure in pre- and stage 1 hypertensives except in the presence of other diseases (such as renal diseases).[166] It has been suggested that the high concentrations of plasma ET-1 found in hypertension secondary to renal disease might be due to impaired renal clearance of ET-1.[39] Thus, most studies conducted in pre- and stage 1 hypertensive patients with normal renal function tend to find concentrations of ET-1 similar to normotensives.[51, 58, 167] In the absence of other diseases, there seems to be an association between plasma ET-1 and blood pressure in stage 2 hypertensives.

<u>**Endothelin-1 in the Kidney (Urinary ET-1)**</u>

Research thus far has focused on two major areas of renal ET-1 involvement in essential hypertension: the renal vasculature and renal tubules. Most cells in the renal vasculature, such as endothelial, mesangial, and vascular smooth muscle cells, are capable of producing ET-1.[168-170] ET-1 stimulates mesangial cell and vascular smooth muscle cell vasoconstriction through activation of ET_A and ET_B receptors.[171] However, it stimulates vasodilation in endothelial cells through ET_B receptors.[172] Infusion of ET-1 causes a reduction in glomerular filtration rate (GFR), in addition to its renal vasoconstrictive effect.[172] Specifically, increasing plasma levels of ET-1 from basal levels to approximately 10 pmol/L causes a significant reduction in renal blood flow, GFR, urinary flow rate, and sodium excretion.[13] However, a study by Warren et al. found no such correlation between plasma ET-1 and GFR.[172] Although elevated circulating levels of ET-1 have been reported by Anand et al. to reduce sodium excretion, which would suggest an increase in blood volume resulting in hypertension,[5] ET-1 action in the nephron seems to decrease blood volume by enhancing sodium and water excretion, favoring hypotension.[19] In order to appreciate the function of ET-1 in the kidney, this section will briefly discuss the production and major functions of ET-1 in the nephron.

Production of Endothelin-1 in the Kidney

A year after the discovery of ET-1 in porcine aortic endothelium, Berbinschi and Ketelslegers reported finding ir-ET in human urine at concentrations six times higher than those found in the blood.[173] Since then, many studies have confirmed that the human kidney is rich in ET-1[174] and urinary ET-1 is of renal origin.[175] Although every region of

the kidney has been found to be potentially regulated by and produce ET-1, the renal

medulla emerges as the predominant ET-1 production site. ET-1 synthesis is high in the

inner medulla, and less within the outer medulla (inner stripe > outer stripe), and

markedly decreased in the cortex. The production of ET-1 in the medulla exceeds that of

anywhere else in the human body.[5] Within the inner medulla, the inner medulla

collecting duct (IMCD) is clearly the major source of ET-1,[176] producing 10-fold more

ET-1 than any other nephron segment.[150] Renal ET receptor expression parallels that of

ET-1 synthesis, with the greatest receptor expression in the inner medulla. The medulla

expresses the greatest density of ET_B receptors within the kidney.[113] Because the medulla

is the predominant site of ET-1 production and receptor expression in the kidney, this

section largely discusses the synthesis and action of ET-1 in the medulla. The net effect

of ET-1 in the renal medulla is diuretic and natriuretic.[177, 178]

Again, very little circulating ET-1 appears in the urine because the kidney has

several degrading enzymes in the proximal tubule that contribute to renal clearance of

ET-1 from the blood.[177] Abassi et al. found that the clearance of 125I-labeled ET-1 from

the blood into the urine is very low.[19] Specifically, only 0.2 – 0.3% of the total

radioactivity injected as 125I-ET-1 into normal rats was recovered in the urine within 30

minutes.[19] However, when rats were treated with neutral endopeotidases (NEP) inhibitors,

a significantly elevated amount of 125I-ET was recovered in the urine suggesting that

filtered ET-1 is subject to proteolytic degradation by NEP.[19] As explained in the review

by Abassi et al., NEP is predominantly located along the brush border of the proximal

tubules and has a very high affinity for all isoforms of ET.[19] Consequently, it is very

unlikely that urinary ET-1 is derived from the blood.[19, 113]

Since urinary ET-1 is most likely of renal origin,[179] it been proposed that urinary ET may be a possible marker of renal disease/damage. Many studies support this claim. For instance, urinary ET-1 is elevated in nephritic patients such as those with glomerular disease,[179] proteinuria,[180] mesangial proliferative glomerulonephritis,[181] focal segmental glomerulosclerosis (prerequisite for renal failure),[20] and progressive kidney disease due to reduced kidney mass.[19, 182]

Function of Endothelin-1 in the Kidney

Endothelin-1 produced in the kidney is multifunctional and regulates a variety of renal functions such as cell proliferation, extracellular matrix accumulation, renin release, blood flow, and electrolyte and water transport.[183, 184] Of interest in this review is ET-1's regulation of electrolyte and water transport. Definitive evidence from gene targeting studies[183] show that ET-1 inhibits its Na^+/K^+ ATPase activity and AVP-stimulated cAMP generation that results in decreased AVP-dependent water absorption. In a study by Ge et al.,[184] collecting duct ET-1 knockout mice could not eliminate an acute volume load as well as wild-type mice. However, there was no difference in the ability of the two strains to eliminate a chronic water load. This study and another similar study by Ahn et al.[19] show unequivocally that under physiological circumstances, ET-1 produced in the collecting duct promotes diuresis. Data on similar experiments in pathological conditions such as hypertension are not available.

ET-1 also modulates sodium transport by the collecting duct. A high sodium diet increases medullary ET-1 mRNA, medullary ECE-1 mRNA, and protein.[185] Thus, these in vivo studies suggest that medullary ET-1 reduces blood pressure by inhibiting sodium

and water reabsorption. Moreover, it appears that it is the ET_B receptor that mediates these known actions of ET-1 in IMCD. Generally, it is believed that in the kidney, ET_A receptors mediate vasoconstriction while ET_B receptors mediate other functions.[63]

Reduced urinary ET-1 has been observed in rats and individuals with hypertension. Hughes et al. found significantly reduced levels of urinary ET-1 in SHR compared with values obtained in age-matched WKYs.[35] Before the development of hypertension, levels of urinary ET-1 in both rats were similar.[35] It has been suggested that there is a reduction in the synthesis of ET-1 in the kidney after the onset of hypertension because cultured IMCD cells from SHR released less ET than those from WKY.[186-188] Zoccali et al.[34] found that ET-1 excretion in essential hypertensives and in normal subjects were significantly lower than in renal hypertensives. They concluded that synthesis of ET-1 in the nephron is elevated in individuals with hypertension secondary to renal disease[34] but not in individuals with essential hypertension.

As proposed by Serniri et al.[189] elevated levels of ET-1 in the plasma above physiological values may influence renal regulation of blood pressure by reducing urinary volume and sodium excretion via the vasoconstrictor activity of ET-1 at ET_A receptors (especially in the vasa recta, arcuate arteries, and in the peritubular capillaries). On the other hand, elevated levels of ET-1 in the nephron may counteract the effect of AVP and of renal vasoconstriction via ET_B receptor mediated operations.[189] and contribute to the maintenance of urine flow, causing an increase in free water clearance.[189] These seemly conflicting operations of ET-1 may work together to maintain blood pressure in physiological conditions.

Effect of Acute Exercise and Exercise Training

Acute Exercise, Plasma and Urinary Endothelin-1

Studies on the effect of acute exercise on plasma levels of ET-1 have been fairly consistent. Circulating ET-1 is slightly elevated immediately after acute exercise of moderate intensity in humans and animals with and without cardiovascular and renal disease.

Most studies found an increase in plasma levels of ET-1 immediately after an acute bout of exercise[59, 186, 187, 189-194] while some others have not.[195, 196] Maeda et al.[186] found that venous plasma levels of ET-1 were increased after ergometer cycling at 90 and 130% of the ventilatory threshold in intercollegiate athletes with the greatest increase at 30 minutes after exercise at both intensities. They concluded that the greater the intensity of exercise, the greater the increase in plasma ET-1 concentration. Also, Predel et al.[190] found elevated levels of plasma ET-1 in patients with coronary artery disease five minutes after ergometer cycling. In another study by McKeever et al,[191] plasma ET-1 levels were significantly elevated immediately and two minutes after steady state exercise at 60% of VO_2max in horses; however, these levels were unchanged during a graded exercise test. These and other studies by Maeda et al.,[192, 193] Mangieri et al.,[194] Serneri et al.,[189] and Miyauchi et al.[187] have confirmed that plasma levels of ET-1 are elevated immediately after acute exercise of moderate intensity. Nonetheless, Cosenzi et al.[195] found that plasma levels of ET-1 were not affected by 15 minutes of moderate intensity ergometer cycling in young healthy individuals. Additionally, Cruden et al.[196] found no change in circulating levels of ET-1 24 hours after low altitude mountaineering in healthy

males. These differences in results are not difficult to reconcile considering that the duration of the exercise protocol in the study by Cosenzi was only 15 minutes and the length of time that elapsed prior to plasma ET-1 testing in the study by Cruden et al. must have been enough to allow circulating ET-1 to return to baseline levels. A few inconsistencies that may not be easily reconciled are the observations of Matsakas et al.[197] and Lewczuk et al.[198] The former observed opposite effects of acute exercise on plasma levels of ET-1 in the trained and untrained groups (reduced vs. elevated, respectively), while the latter found a significant increase and decrease in plasma levels of ET-1 after jogging and cycling, respectively. Serneri et al. suggested and showed that the reduction in blood volume that accompanies exercise is responsible for the increase in ET-1 concentration in plasma. Thus, their study,[189] and all other studies[186, 187] that measured and found a reduction blood volume with their exercise protocol, observed an increase in plasma levels of ET-1.

While many studies have confirmed that plasma levels of ET-1 are elevated immediately after acute exercise, there is conflicting data on changes in plasma levels of ET-1 during exercise. Some studies found an increase in plasma ET-1 during exercise,[188, 199] while one did not.[200] For instance, Letizia et al.[188] found an increase in plasma ET-1 in patients with coronary artery disease who showed no ECG signs of myocardial ischemia performing moderate intensity exercise. However, these values returned to baseline levels during a six minute recovery period. Moreover, they found no change in plasma levels of ET-1 at peak exercise and during the recovery period in normal subjects. All the same, Ahlborg et al.[199] found an increase in plasma ET-1 in healthy subjects performing moderate intensity exercise. Conversely, Richter et al.[200] found that forearm venous

plasma levels of ET-1 declined in healthy men during the first 30 minutes of ergometer

cycling at 65% of VO$_2$max; however, they increased back to resting values after 60

minutes of exercise.

Although there are studies on the effect of acute exercise on plasma ET-1, there

seems to be little data available on the effect of acute exercise on urinary ET-1. To the

best of the author's knowledge, the only study available was conducted by Serneri et

al.[189] In this study, the investigators found increased renal ET-1 with acute exercise.

Aerobic Exercise Training, Plasma and Urinary Endothelin-1

The effect of aerobic exercise training (AEX) on plasma levels of ET-1 has also

been fairly consistent. The trend has been a reduction in the levels of plasma ET-1 with

AEX.[15, 16, 59] However, there is one study[75] that found no change in circulating levels of

ET-1 after AEX. Maeda et al., through a succession of studies, found that plasma ET-1 is

higher in middle-aged than in young men and it is significantly decreased by AEX in

healthy young men,[15] and older women.[16] Also, AEX prevented the increase in plasma

levels of ET-1 induced by acute exercise in normotensive offspring of hypertensive

patitents.[59] The only study that found no change in plasma levels of ET-1 after AEX was

conducted in patients with congestive heart failure after acute myocardial infarction.[75]

Thus, there is available data on the effect of AEX on plasma ET-1 that show that AEX

reduces circulating levels of plasma ET-1. However, other investigators need to verify

this finding.

To the best of the author's knowledge, there are currently no published data on the effect of AEX on urinary ET-1. Nevertheless, preliminary data from our laboratory suggest that AEX causes a non-significant reduction in urinary levels of ET-1.

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