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ORIGINAL ARTICLE

Endothelium and angiogenesis in white coat hypertension

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Hypertensive patients are at particular risk of cardiovascular complications, possibly related to endothelial damage or dysfunction, or to abnormal angiogenesis. The aim of this study was to compare the risk conferred by white coat hypertension (WCH) *vs* sustained hypertension in the development of the endothelial dysfunction and abnormal angiogenesis by evaluating nitric oxide ($NO = NO_2 + NO_3$), endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), and E-selectin levels in plasma. The study group included 102 subjects, 34 with WCH (17 male and 17 female patients) aged 49 ± 11 years, 34 sustained hypertensives (HT) (15 male and 19 female patients) aged 47 ± 11 years and 34 normotensive control subjects (NT) (16 male and 18 female patients) aged 48 ± 10 years. WCH was defined as clinical hypertension and daytime ambulatory blood pressure less than 135/85 mmHg. The subjects were matched for age, gender, body mass index and the patients with smoking habit, dyslipidaemia, and diabetes mellitus were excluded from the study. The NO, ET-1, VEGF and E-selectin levels were analysed by ELISA technique. The WCH subjects had significantly higher levels of NO than the HT (41.68 ± 2.23 *vs* 32.18 ± 2.68 μmol/l; $P < 0.001$) and significantly lower values than the NT (48.24 ± 4.29 μmol/l; $P < 0.001$). ET-1 levels of the WCH group were significantly higher than the NT (8.10 ± 0.92 *vs* 5.95 ± 0.26 ng/ml; $P < 0.001$) and significantly lower than the HT (11.46 ± 0.59 ng/ml; $P < 0.001$). Considering with VEGF, the WCH group had significantly higher levels than the NT (195.88 ± 11.84 *vs* 146.26 ± 18.67 pg/ml; $P < 0.001$), but the difference from the HT group was not significant (203.35 ± 7.48 pg/ml; $P = 0.062$). E-selectin in the WCH group was significantly lower than the HT (4.77 ± 0.52 *vs* 8.49 ± 2.85; $P < 0.001$), but the difference from the NT group was not significant (3.86 ± 0.67; $P = 0.077$). Our data demonstrate that WCH is associated with endothelial dysfunction and abnormal angiogenesis. The degree of these changes is not as severe as observed in hypertensive population.

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Introduction

It is well established that in hypertension, the process of endothelial damage and angiogenesis are abnormal.[1] Patients with hypertension exhibit loss of endothelial cell integrity by demonstrating impaired flow mediated dilatation.[2] Increased levels of plasma markers such as von Willebrand Factor (vWF) and soluble E-selectin are also reported.[2,3] Furthermore, epidemiologic studies show that increased levels of these plasma markers indicate an increased risk for a poor prognosis in hypertensives.[4] A beneficial change in indices of endothelial damage and angiogenesis by intensive cardiovascular risk factor management was also reported.[1] Little is known about the prognosis of the patients with white coat hypertension (WCH). It is said to be a harmless clinical situation by some authors,[5–7] whereas others have found an increased risk for cardiovascular disease.[8,9] Some authors reported that it might cause end-organ damage, some metabolic abnormalities such as hyperlipidaemia, impaired insulin sensitivity, elevated blood glucose and increased serum insulin levels and

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haemodynamic alterations.[10–12] Clinical surveys on endothelial dysfunction in WCH are controversial.[13–15] Some prognostic studies indicate that those disturbances might not be clinically significant at long-term outcome as WCH was not associated with high cardiovascular risk. Some others suggest increased cardiovascular risk for WCH *vs* NT.[16–18] The objective of this study is to assess the status of endothelial dysfunction (NO, ET-1, E-selectin) and presence of abnormal angiogenesis (VEGF) in patients with WCH.

Materials and methods

The study group consisted of a total of 102 subjects: 34 with WCH (17 male and 17 female patients) aged 49 ± 11 years, 34 sustained hypertensives (HT) (15 male and 19 female patients) aged 47 ± 11 years and 34 normotensive control subjects (NT) (16 male and 18 female patients) aged 48 ± 10 years. The groups were matched for age and gender (Table 1). Subjects with other risk factors for atherosclerosis (hyperlipidaemia LDL < 130 mg/dl, diabetes mellitus, obesity BMI > 27 kg/m^2, smoking), subjects having signs or symptoms of atherosclerotic vascular disease and other endocrine diseases or alcoholism are not included. Patients using drugs that may affect blood pressure and lipid metabolism were also excluded. All patients were free of concomitant vascular and renal diseases, malignancy and connective tissue diseases. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975.

Blood pressure measurements

Measurements of brachial arterial pressures in the patients referred to our clinic due to high blood pressure (diastolic pressure > 90 mmHg) were obtained in our outpatient department by a nurse with a mercury sphygmomanometer, which was standardized in accordance with the approval of American and British Hypertension Society and World Health Organization. Measurements were obtained with the subject in the sitting position after resting for 20–30 mins.[19] Korotkoff phase I was used to determine the systolic pressure and phase V for diastolic pressure. Measurements were performed on three different days within 5 days. The average of three measurements were taken as the mean systolic and diastolic pressures.

An ambulatory 24-h arterial blood pressure recording was performed in patients whose diastolic pressure was found to be higher than 90 mmHg in the outpatient department, with an instrument (A and D Engineering, TM- 2421) that was approved and suggested by European Society of Hypertension.[20] Measurements were performed as British Society of Hypertension suggested on the left arm.[21] According to the results of the ambulatory measurement patients were classified as WCH *vs* HT groups. WCH was defined as clinical hypertension and daytime ambulatory blood pressure less than 135/85 mmHg. Patients with the daytime ambulatory diastolic blood pressure more than 85 mmHg were considered as the hypertensive group (Table 1).

Table 1 Characteristics of the groups and ambulatory blood pressure measurements of the groups

	NT, n = *34*	*WCH*, n = *34*	*HT*, n = *34*
Age (years)	48 ± 10	49 ± 11	47 ± 11
Gender: M/F	16/18	17/17	15/19
BMI (kg/m^2)	24.3 ± 5	25.3 ± 4	25.7 ± 6
SBP (mmHg)	127 ± 4	129 ± 2	159 ± 7
DBP (mmHg)	80 ± 3	79 ± 2	94 ± 2

NT: Normotensive group; HT: hypertensive group; WCH: white coat hypertensive group; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure.
SBP of WCH is significantly different from HT ($P < 0.001$); DBP of WCH is significantly different from HT ($P < 0.001$). SBP of NT is significantly different from HT($P < 0.001$); DBP of NT is significantly different from HT ($P < 0.001$).

Laboratory methods

Fasting peripheral venous blood samples for vascular endothelial growth factor (VEGF), s-E selectin and NO were collected before any medication into heparinized vacutainer tubes. Plasma samples were isolated by 2500 *g* centrifugation at 4°C for 10 minutes. All plasma samples were kept at −70°C until the experiments were performed. Subjects were administered low-nitrate diet for 2 days before measurements.

VEGF

For the measurement in plasma, a commercially available ELISA technique was used (Oncogene Human VEGF ELISA). Briefly, samples were incubated in duplicates in microtitre plates precoated with a monoclonal antibody specific for VEGF at room temperature for 2 h. Unbound material was washed away and a polyclonal horseradish peroxidase (HRP)-conjugated anti-VEGF antibody was added to the wells. The HRP catalyses the conversion of the chromogenic substrate tetramethylbenzidine (TMB) from a colourless solution to a blue solution the intensity of which was proportional to the amount of human VEGF protein in the test sample. The coloured reaction product was quantified using a spectrophotometer. Results were calculated from a standard curve (recombinant human VEGF; range 31.2–1000 pg/ml) generated by a four-parameter logistic curve-fit and expressed in pg/ml of plasma. The assay has been reported to recognize both natural human VEGF and recombinant VEGF and not to exhibit crossreactivity with

a series of cytokines and growth factors. The manufacturer claims a sensitivity of less than 9.0 pg/ml and an intra- and interassay variation of less than 10%.

s-E selectin
s-E selectin levels were assayed by ELISA technique with commercially available assay kit (Hycult Biotechnology, The Netherlands). The procedural details recommended by the manufacturer were strictly followed. The colour formation after the complex formation between s-E selectin and its respective antibody was quantified spectrophotometrically at 450 nm. All determinations were carried out in duplicate. Concentrations in unknown samples were calculated from the standard curve. The minimal detectable level was 50 pg/ml. No significant crossreactivity and interference with factors related s-E selectin have been noted with this assay. The coefficients of intra- and interassay variations for s-E selectin levels were 9.1% ($n=15$) and 9.7% ($n=13$), respectively.

NO
Plasma concentration of NO (NO_2+NO_3) was estimated using commercially available colorimetric assay (Roche, Cat. No. 1 756 281).[22] Nitrogen monoxide was detected in plasma via nitrite. The nitrate present in the plasma is reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of the enzyme nitrate reductase. The nitrite formed reacts with sulphanilamide and *N*-(1-naphthyl)-ethylenediamine dihydrochloride to give a red-violet diazo dye. The diazo dye was measured on the basis of its absorbance in the visible range at 550 nm. The coefficients of intra- and interassay variations for NO levels were 4.9% ($n=15$) and 5.6% ($n=13$).

ET-1
The blood samples for *ET-1* were also collected in the vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). The tubes were rocked gently several times for anticoagulation. The samples then were transferred to the centrifuge tubes containing aprotinin (0.6 TIU/ml of blood) and were rocked gently for several times to inhibit the activity of proteinases. The blood samples were centrifuged at 1600 g for 15 min at 4°C and the plasma was obtained. All samples were kept at −70°C until the experiments were performed. Immunoreactive ET-1 was quantified in extracts of 1.0 ml aliquots of frozen plasma. Thawed samples were acidified with an equal volume of 1% trifluoroacetic acid (TFA), extracted over C18 Sep-pak column (Millipore, Bedford, MA, USA) and eluted with 60% acetonitrile in 1% TFA. The extracts were evaporated to dryness and dissolved with assay buffer. Plasma ET-1 was evaluated by a commercial kit of competitive enzyme immunoassay (Phoenix Pharmaceuticals, Inc., CA, USA). All determinations were carried out in duplicate. The detection range of plasma ET-1 was 0–25 ng/ml. The inter- and intra-assay variations were <14 and <5%, respectively. According to the manufacturer, the crossreactivities to ET-2 and ET-3 were 7% and the crossreactivity to higher ET-1 and sarafotoxin were 17 and 3%, respectively. No significant crossreactivity to other substances has been reported. The coefficients of intra- and interassay variations for ET-1 levels were 8.9% ($n=15$) and 9.3% ($n=13$) respectively.

Statistical method
The values of selected variables were defined as mean ± SD. The ET-1, NO, E-selectin and VEGF values of the groups (NT, WCH and SH) were compared with one-way ANOVA (Tukey HSD). '*P*' value <0.05 was considered statistically significant.

Results

Basic characteristics and ambulatory blood pressure values of the groups are given in Table 1. Patients with WCH have similar ambulatory blood pressure values with NT. Plasma NO, ET-1, E-selectin and VEGF levels of the three groups are given in Table 2. The WCH subjects had significantly higher levels of NO than the HT (41.68 ± 2.23 *vs* 32.18 ± 2.68 μmol/l; $P<0.001$) and significantly lower values than the NT (48.24 ± 4.29 μmol/l; $P<0.001$). ET-1 levels of the WCH group were significantly higher than the NT (8.10 ± 0.92 *vs* 5.95 ± 0.26 ng/ml; $P<0.01$) and significantly lower than the HT (11.46 ± 0.59 ng/ml; $P<0.001$).

Considering VEGF, the WCH group had significantly higher levels than the NT (195.88 ± 11.84 *vs* 146.26 ± 18.67 pg/ml; $P<0.001$), but the difference from the HT group was not significant (203.35 ± 7.48 pg/ml; $P=0.062$). E-selectin in the WCH group was significantly lower than the HT

Table 2 Plasma NO, ET-1, E-selectin and VEGF levels in groups

	NT, n = *34*	*WCH*, n = *34*	*HT*, n = *34*
NO (μmol/l)	48.24 ± 4.29	41.68 ± 2.23	32.18 ± 2.68
ET-1 (ng/ml)	5.95 ± 0.26	8.10 ± 0.92	11.46 ± 0.59
E-selectin (ng/ml)	3.86 ± 0.67	4.77 ± 0.52	8.49 ± 2.85
VEGF (pg/ml)	146.26 ± 18.67	195.88 ± 11.84	203.35 ± 7.48

NO: Nitric oxide; ET-1: endothelin-1; VEGF: vascular endothelial growth factor; WCH: white coat hypertensive group; NT: Normotensive group; HT:Hypertensive group.
The NO level of WCH, NT and HT are different from each other ($P<0.001$).
The ET-1 level of NT is different from WCH ($P<0.01$) and HT ($P<0.001$).
The E-selectin level of HT is different from WCH and NT ($P<0.001$).
There is no difference between the E-selectin levels of NT and WCH ($P=0.077$).
The VEGF level of NT is different from WCH and HT ($P<0.001$).
There is no difference between VEGF levels of WCH and HT ($P=0.062$).

(4.77 ± 0.52 *vs* 8.49 ± 2.85; $P < 0.001$), but the difference from the NT group was not significant (3.86 ± 0.67; $P = 0.077$).

Discussion

Endothelial cells produce both vasodilatating compounds as nitric oxide, prostacycline, endothelial derived hyperpolarizing factor and counteracting substances known as endothelial derived contracting factors: endothelin, thromboxane A_2, prostaglandin H_2, free oxygen radicals. [23,24] Natural balance between both groups affects blood perfusion and constitutes an important element in blood pressure control. Endothelial dysfunction is thought to be a marker of future cardiovascular events in patients with hypertension. In a number of studies, endothelial dysfunction in hypertensive patients was found out as decreased release of nitric oxide or increased production of endothelin.[23,25] There are very few studies concerning endothelial dysfunction in WCH despite many in sustained hypertension.[13,23–25] Gomez *et al*,[26] using a noninvasive method, showed the presence of endothelial dysfunction by means of endothelium-dependent flow-mediated dilatation in WCH as in essential hypertension.[26] But this method (ultrasound) is not favourable for application to large epidemiologic studies, which require specialized equipment and technical expertise. Therefore to analyse the molecules produced by endothelial cells by biochemical methods may be a more appropriate marker for the assessment of endothelial dysfunction.[13,27] Clinical surveys on endothelial dysfunction in WCH are controversial.[11,18,27]

Nitric oxide is a free radical considered to be the major endothelium-derived relaxing factor. It is released in response to shear stress or to the stimulation of several receptors on the endothelial cell surface.[28] Compared with NT, patients with HT have a blunted endothelium-dependent relaxing response.[29] In this study the NO levels of WCH were significantly lower than NT but higher than HT. Pierdomenico found that WCH subjects had significantly higher levels of NO than HT patients; but they found no significant difference between WCH and NT groups. They concluded that middle-aged WCH subjects without other cardiovascular risk factors do not show endothelial dysfunction.[13] When we compare our study with Pierdomenico's, the exclusion criteria and methodologies were nearly the same. Our study group was slightly older than Pierdomenico's. This small age difference and the duration of WCH, a factor not assessed in the study, may have played a role in the discrepancies.

Endothelin-1 (ET-1) is a peptide secreted mostly by vascular endothelial cells and is the most potent vasoconstrictor currently known. ET-1 also has inotrophic, chemotactic and mitogenic properties. In addition, it influences salt and water homeostasis through its effects on the renin–angiotensin–aldosterone system, vasopressin, and atrial natriuretic peptide and stimulates the sympathetic nervous system. The overall action of endothelin is to increase blood pressure and vascular tone. ET increases vascular resistance, decreases renal flow and glomerular filtration and also stimulates pulmonary fibroblasts to produce collagen, increases mucus secretion, contracts bronchial smooth muscle. It also affects the conductivity of parasympathetic ganglia and nerves. ET-1 levels are elevated in patients with essential hypertension with no other risk factors for atherosclerosis, and this may serve as markers of endothelial dysfunction in uncomplicated hypertension.[30] A study by Vaindirlis *et al*[18] supported the existence of endothelial dysfunction in uncomplicated hypertension by showing increased amount of ET-1 in the urine analysis of adult patients with WCH.[18] Hlubocka *et al*[14] showed not only increased ET-1 levels in uncomplicated hypertension but also a decrease in ET-1 levels after treatment with ACE inhibitors. Similar to these results, we found that hypertensives have significantly higher plasma ET-1 levels. WCH showed to be an intermediate group with ET-1 levels significantly higher than NT ($P < 0.01$) but significantly lower than hypertensives ($P < 0.001$).

In addition to NO and ET- 1, soluble endothelial leucocyte adhesion molecules have been recently proposed as new markers of endothelial function. In vascular diseases, E-selectin, an endothelium-specific molecule has an important role in tethering of leucocytes to the endothelium.[31,32] In essential hypertension, increased levels of E-selectin in a small number of patients compared to NT controls have been reported.[33,34] This has been subsequently confirmed in some, but not in all similar investigations.[35–38] Results of a restricted number of studies on E-selectin levels in essential hypertension are controversial. The mechanism by which hypertension causes an increase in soluble adhesion molecules are not yet fully understood. With increased blood pressure, shear stress may cause an inflammatory condition and endothelial cells may be activated by inflammatory cytokines.[39]

In our study, E-selectin levels were found to be increased significantly in the HT group compared with the NT and WCH groups. The difference between the NT and WCH groups are not significant.

Our early findings about the endothelial dysfunction in WCH were published as a letter.[40] At that time, we observed that E-selectin levels increased significantly in the WCH group compared with the NT, but this increase was not as high as in the HT subjects. As we went on gathering data and increased the numbers of the groups, we noticed that the levels of E-selectin in WCH were similar with NT. It seems like a contradiction because in endothelial dysfunction, all endothelial molecules may be thought to be affected by cell injury. Blann showed that E-selectin and vWF levels raised

together in hypertension, but there was no correlation between them. He suggested that raised levels of soluble E-selectin in hypertension do not indicate endothelial cell injury but may be related to endothelial cell activation.[33] Therefore, the stimulus for release of soluble E-selectin might be different from the others and the stimulus in WCH might not be as strong as in HT. There was no other previous published work about E-selectin in WCH to compare with our study and the restricted studies about E-selectin in sustained hypertension were controversial due to small numbers.

There also exists impaired vascular development in essential hypertension and this is based on structural alterations of microvascular beds such as capillary rarefaction.[1,41–43] As the angiogenic growth factors are major regulators of blood vessel formation, abnormal angiogenesis may affect these markers. Evidence of angiogenesis in full-blown atherosclerosis includes increased numbers of vasa vasorum in arteries burdened with atheroma with increased expression of angiogenic growth factor.[1,41,44–46] Felmeden *et al*[1] reported that one of such growth factors, VEGF increased in plasma in hypertensive patients. Our data indicate an increased level of VEGF in WCH compared to NT group and this increase was similar with the HT. The increasing level of VEGF emphasizes the presence of abnormal angiogenesis in WCH as in HT group.

In summary, our data demonstrate that WCH, with increased levels of ET-1 , VEGF and decreased NO levels is associated with endothelial dysfunction and abnormal angiogenesis although the degree of these changes is not as severe as observed in hypertensive population. The presence of endothelial dysfunction and abnormal angiogenesis suggests that WCH has common features with sustained hypertension and should not be considered as a harmless trait.

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