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Ambulatory Blood Pressure Monitoring and Serum Nitric Oxide Concentration in Type 1 Diabetic Children

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Abstract. Blood pressure can be determined more precisely with the use of 24 hours ambulatory measurement in type 1 diabetics. Nitric oxide (NO) has been suggested to be responsible for the vascular changes described in early diabetic nephropathy. We aimed to investigate serum NO concentration along with ambulatory blood pressure monitoring (ABPM) parameters in type 1 diabetic patients and to find out whether there are correlation between serum NO level and ABPM parameters. Forty type 1 diabetic subjects and 35 controls were enrolled. Diabetic subjects were grouped as microalbuminuric (n=16) and normalbuminuric (n=24). Casual and ambulatory blood pressure parameters and serum NO concentrations were measured in all study population. Microalbuminuric subjects had higher nighttime systolic blood pressure (SBP), 24 hours diastolic blood pressure (DBP) and 24 hours mean arterial pressure (MAP) than controls. Both microalbuminuric and normalbuminuric subjects had also significantly higher nighttime DBP and nighttime MAP than controls. Serum NO concentrations were higher in normalbuminuric and microalbuminuric subjects than controls. Serum NO concentrations were positively correlated with daytime DBP and MAP, nighttime SBP, DBP and MAP, and 24 hours DBP and MAP in microalbuminuric subjects. Serum NO concentrations were also positively correlated with nighttime DBP in normalbuminuric subjects. Multiple linear regression analysis revealed that serum NO_2– + NO_3– concentrations and 24 hours DBP were independently associated with the development of microalbuminuria. Albuminuria seems to be closely associated with serum NO concentrations and ABPM parameters in type 1 DM patients. A prospective follow-up study on diabetic patients with normo- and micro- albuminuria is needed to confirm the predictive values of increased NO concentrations and ABPM parameters on the development of albuminuria.

Key words: Type 1 Diabetes, nitric oxide, blood pressure, ambulatory, nephropthy.

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DIABETES mellitus is the most common etiologic factor of chronic kidney disease worldwide. Approximately 30% of type 1 diabetic patients will develop diabetic nephropathy during the course of the disease [1]. Elevated blood pressure has important contributions for the development of diabetic nephropathy and progressive kidney damage in diabetic patients, and it is well known that lowering BP is accompanied by marked reduction in albuminuria and the rate of GFR decline [2]. Ambulatory blood pressure monitoring (ABPM) results have been shown to be better correlated with target organ damage than office blood pressure readings [3]. Besides clinically apparent hypertension, abnormal circadian variation of systemic blood pressure has been shown to be associated with diabetic nephropathy with the use of ABPM [4].

In previous reports, endothelial dysfunction has been suggested as an early event in diabetic vascular complication and seems to be relevant to the pathogenesis of diabetic microangiopathy [5, 6]. In fact,

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in several experimental and clinical studies [7, 8, 9, 10, 10], serum NO concentrations have been shown to increase in early course of diabetic nephropathy. Although the exact mechanism of increase in NO concentration in DM is still unclear, there are several suggestions. These include increased iNOS induction [11], increase in NO and superoxide anion production due to prolonged exposure of endothelial cells to high glucose level [12], and impaired action of NO accompanied by up-regulation of NO production [13].

In the present study, we aimed to investigate serum NO concentration along with ambulatory blood pressure monitoring (ABPM) parameters in type 1 diabetic patients with and without microalbuminuria and to find out whether there are correlation between serum NO level and ABPM parameters. In addition, we also aimed to investigate whether combining of serum NO concentration measurement and ABPM could be used for the early determination of type 1 diabetic subjects susceptible to the development of diabetic nephropathy.

Materials and methods

Subjects

Forty children with type 1 DM with 5 years or more of disease duration and 35 healthy children were enrolled. The study protocol was carried out in accordance with the Helsinki Declaration as revised in 1989 and approved by the local human institutional review committee. All subjects and their parents were informed about the study protocol and written consents were obtained from all participants.

Exclusion Criteria

Exclusion criteria were as follows: casual systolic and diastolic BP on three consecutive measurements outside the 5^{th}-95^{th} percentile for age and height, use of drugs other than insulin, and the presence of systemic infection and other renal diseases evidenced by hematuria and abnormal urinary sediment or any chronic disease in addition to diabetes.

Determination of urinary albumin excretion rate

Determination of 24-h urinary albumin excretion (UAE) rate was performed in both diabetics and controls with the use of turbido-metric method (Integra-800 Roche). Diabetic subjects were divided into two groups as microalbuminuric (n=16) and normalbuminuric (n=24). Microalbuminuria was defined as urinary excretion of albumin ≥20 and <200μg/min in at least 2 out of 3 urine samples.

Casual and ambulatory blood pressure measurement

Blood pressure measurement was performed from non-dominant arm with the most appropriate cuff selected from three different sizes (12-20cm, 17-26cm, 24-32cm) available. Casual blood pressure of all participants was obtained by calculating the mean of 3 blood pressure measures recorded with a mercury sphygmomanometer after 5 minutes with the patient seated at rest. The Korotkoff phase I was used as the systolic blood pressure and phase V as the diastolic blood pressure. Ambulatory blood pressure measurements were performed with the use of oscillometric type of SpaceLabs 90207 recorder. The device was totally automatic and programmed to evaluate measures at an interval of 30 minutes between 08.00 and 22.00 hours (daytime period) and at every 60 minutes between 22.01 and 07.59 hours (nighttime period). The device was installed in the morning and the patients were instructed to perform their usual activities during monitoring and to document the time they went to sleep and woke up. The initial cuff inflation was 170 mmHg. Subsequent inflations were programmed to be 30 mmHg higher than the previous measured systolic blood pressure. Measurements that produced a pulse pressure of less than 20 mmHg or a heart rate of less than 40 beats/min were regarded as errors. The device was programmed to repeat the measurements that could not be recorded correctly within 2 minutes. The examinations had a minimum duration of 24 hours and they were considered valid when at least 85% of readings with valid measurements of 2 readings per hour [14]. Ambulatory blood pressure parameters were interpreted on the basis of guidelines constituted by Soergel et al [15]. Mean arterial pressure (MAP) value was obtained with the use of following formula; diastolic blood pressure (DBP) + [(systolic blood pressure (SBP) – DBP)/ 3].

Sample preparation and NO assay

All blood samples were collected in glass tubes and

allowed to clot spontaneously at room temperature, then the serum was prepared by centrifugation for 15 min at 3000 r.p.m. Aliquots were stored at –80°C until NO assay.

NO is rapidly converted to nitrite (NO_2–) and nitrate (NO_3–) in typical oxygenated aqueous solutions such as human serum. Because an excellent colorimetric reagent (the Griess reagent) exists for the determination of NO_2–, it is common practice to use enzymatic or chemical reduction to convert all NO_3– to NO_2– in samples and measure total NO_2– as an indicator of overall NO production [16].

In this study, serum NO_2–/NO_3– levels were measured in triplicate by conversion of NO_3– to NO_2 – by a commercially available kit (BIOXYTECH® NO-540™, Bio-Stat Research, Stockport, UK) based on the Griess reaction following the manufacturer's instructions. Briefly, serum samples (10–50µl) was adjusted to 190 µl with water, and then 10µl of 30% (wt/vol) ZnSO4 solution was added. Samples were then mixed by vortexing, incubated at room temperature for 15 min, and centrifuged (3,000rpm) for 5 min. One hundred microliters of the resulting supernatants were then transferred to microcentrifuge tubes containing 0.5 g granulated cadmium each as chemical reducent and incubated at room temperature overnight with agitation. Then, the samples were recentrifuged (3,000 rpm) at room temperature and the supernatants were applied to a 96-well microtiter plate, followed by 100 µl of Griess reagent (1g/l sulfanilamide, 25g/l phosphoric acid, and 0.1g/l N-1-naphthylethylenediamine dihydrochloride). After 10 min of color development at room temperature, the absorbance was measured on a microplate reader at a wavelength of 540 nm. Results are reported as NO_2– + NO_3– and expressed as micromoles per liter [9].

Other parameters

Body weights of subjects with wearing standard clothes and no shoes were determined using a calibrated beam scale. A Harpender Statiometer was used in height measurement. Body mass index (BMI) was determined as the actual body weight divided by the square of height (kg/m^2). Serum and urinary creatinine levels were measured with an autoanalyzer (Aeroset®, Germany). Creatinine clearance was used for the estimation of glomerular filtration rate (GFR) and calculated using plasma creatinine and 24-hour urinary creatinine excretion.

Statistical analysis

Data were presented as mean ± SD. Qualitative variables were assessed by Chi-square test. Parametric variables were compared using one-way analysis of variance with post-hoc analysis using the Scheffe test. Pearson correlation analysis was used to find out the correlations between serum NO concentrations and ABPM parameters, eGFR and UAE rate. Multiple linear regression analysis was used to find out the factors associated with serum NO concentrations and UAE rate. Receiver operating characteristic (ROC) curves analysis was performed to select cut-off thresholds with the greatest discriminative power and to find out the sensitivity and specificity of serum NO concentrations, nighttime DBP and nighttime MAP in determining the presence of microalbuminuria. Differences were regarded as significant at $p<0.05$.

Results

The demographic and clinical characteristics of the study population are shown in Table 1. There was no statistically significant difference between microalbuminuric and normalbuminuric diabetic patients and controls in respect to age, gender, BMI and systolic and diastolic casual blood pressure levels (all $p>0.05$). Daily total insulin requirement was also similar in microalbuminuric and normalbuminuric diabetic patients ($p>0.05$). Microalbuminuric diabetic patients had higher estimated GFR (eGFR), HbA1c, AER and serum NO_2–+NO_3– concentration than both normalbuminuric diabetic patients and controls (all $p<0.05$). Normalbuminuric diabetic patients had also higher eGFR, HbA1c and serum NO_2– +NO_3– concentration than controls (all $p<0.05$).

Ambulatory blood pressure parameters of microalbuminuric and normalbuminuric diabetic subjects and controls are shown in Table 2. Microalbuminuric diabetic subjects had significantly higher nighttime SBP, 24 hours DBP and 24 hours MAP than controls (all $p<0.05$). Both microalbuminuric and normalbuminuric diabetic subjects had also significantly higher nighttime DBP (all $p<0.05$) and nighttime MAP than controls (all $p<0.05$). Other ambulatory blood pressure parameters, e.g. daytime SBP and DBP and MAP,

Table 1. Clinical and demographic characteristics of microalbuminuric (Group I) and normalbuminuric diabetics (Group II), and controls (Group III).

	Group I	Group II	Group III
n	16	24	26
Age (years)	13.8±2.6	12.7±2.4	12.8±2.5
Sex (F/M)	7/9	13/11	13/13
BMI	19.9±3.4	18.7±3.0	18.2±2.6
Diabetes duration (years)	7.81±2.33	6.62±2.12	-
HbAıc (%)	*11.6±1.8	9.1±2.5	4.9±0.4
Insulin requirement (U/kg/day)	1.0±0.28	0.92±0.28	-
Casual SBP (mm/Hg)	124.6±6.3	120.4±8.1	119.2±5.4
Casual DBP (mm/Hg)	78.4±5.6	76.2±5.1	74.2±6.4
AER (μg/min)	*36.7±20.9	7.2±4.4	7.6±3.2
GFR (ml/min/1.73m^2)	*170.1±8.2	**125.7±6.0	99.6±6.5
NO_2^-+NO_3^- (μmol/L)	*36.4±2.5	**26.6±2.9	22.6±3.2

*P<0.05 vs Group 2 and 3.

**P<0.05 vs Group 3.

BMI, body mass index; AER, albumin excretion rate; GFR, glomerular filtration rate; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table 2. Blood pressure parameters of microalbuminuric (Group I) and normalbuminuric diabetics (Group II), and controls (Group III).

	Group I	Group II	Group III
	n=16	n=24	n=35
Day time SBP	116.3±6.7	115.7±7.1	113.8±6.9
Day time DBP	73.9±6.8	71.6±5.1	70±4.9
Day time MAP	88.4±6.2	86.6±4.4	84.8±4.8
Day time heart rate	92±13.4	91.1±9.2	89.3±11
Night time SBP	*108.7±6.8	108.4±7.4	104.3±6.1
Night time DBP	*65.4±6.7	*62.8±5.9	59±4.4
Night time MAP	*81±6.6	*79.9±5.5	75.2±4.1
Night time heart rate	80±14.8	79.3±9.1	74.1±8.3
24 hour SBP	113.1±6.3	112.5±6.7	109.8±5.9
24 hour DBP	*70.1±6.6	67.8±4.8	65.5±4
24 hour MAP	*85.1±6.1	83.8±4.3	80.8±4.1
24 hour heart rate	86.8±13.2	86.3±8.4	82.8±9.1

*P<0.05 vs. controls.

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

and 24 hours SBP were not significantly different in 3 groups (all p>0.05).

Serum NO_2– + NO_3– concentrations were significantly higher in microalbuminuric diabetic subjects than normalbuminuric diabetic subjects and controls (both p<0.05). Normalbuminuric diabetic subjects had also higher serum NO_2– + NO_3– concentrations than controls (p<0.05). In both microalbuminuric and normalbuminuric diabetic subjects, linear regression analysis revealed that serum NO_2– + NO_3– concentrations were significantly associated with diabetes duration (β=0.443, p<0.05 and β=0.347, p<0.05), BMI (β=0.523, p<0.05 and β=0.406, p<0.05) and HbA1C (β=0.338, p<0.05 and β=0.431, p<0.05). No association was observed between serum NO_2– + NO_3– concentrations and age or gender (p>0.05).

Pearson correlation analysis revealed that serum NO_2– + NO_3– concentrations were significantly cor-

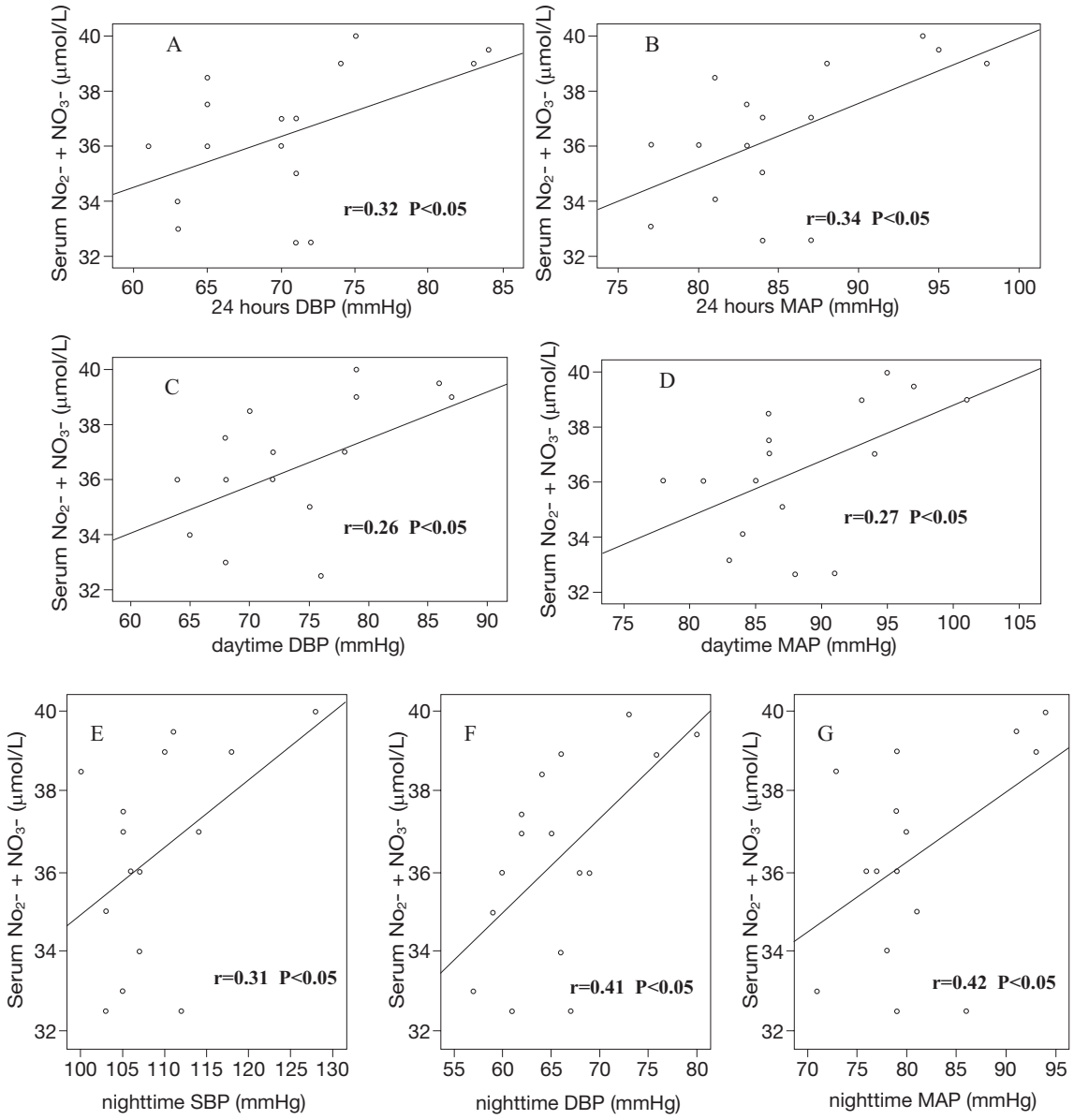

Fig. 1. The correlations of serum nitric oxide concentration with 24 hours DBP (A) and MAP (B), daytime DBP (C) and MAP (D), and nighttime SBP (E), DBP (F) and MAP (G) in type 1 diabetic patients with microalbuminuria.
DBP, diastolic blood pressure; MAP, mean arterial pressure; SBP, systolic blood pressure.

related with 24 hours DBP (r=0.32, p<0.05) and MAP (r=0.34, p<0.05), daytime DBP (r=0.26, p<0.05) and MAP (r=0.27, p<0.05), and nighttime SBP (r=0.31, p<0.05), DBP (r=0.41, p<0.05) and MAP (r=0.42, p<0.05) in microalbuminuric diabetic subjects (Figure 1), while no association was observed between serum NO_2– + NO_3– concentrations and daytime or 24 hours SBP (both p>0.05). In normalbuminuric diabetic subjects, serum NO_2– + NO_3– concentrations were correlated with nighttime DBP (r=0.40, p<0.05) (Figure 2). Serum NO_2– + NO_3– concentrations were also correlated with both eGFR (r=0.560, p<0.05) and UAE rate (r=0.708, p<0.05) in microalbuminuric diabetic subjects, while only correlated with eGFR (r=0.659, p<0.05) in normalbuminuric diabetic subjects. In controls, serum NO_2– + NO_3– concentrations were not correlated with any of those ambulatory blood pressure parameters and eGFR or UAE rate (all p>0.05).

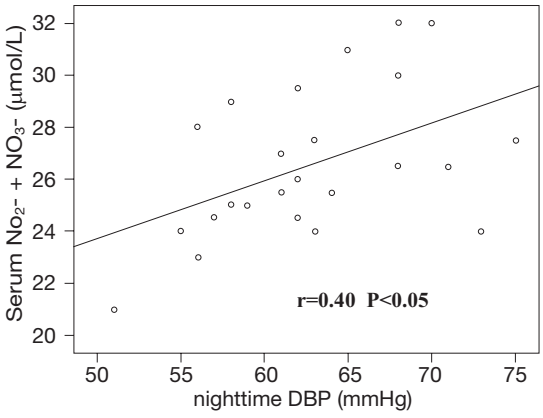

Fig. 2. The correlation of serum nitric oxide concentration with nighttime DBP in type 1 diabetic patients with microalbuminuria.
DBP, diastolic blood pressure.

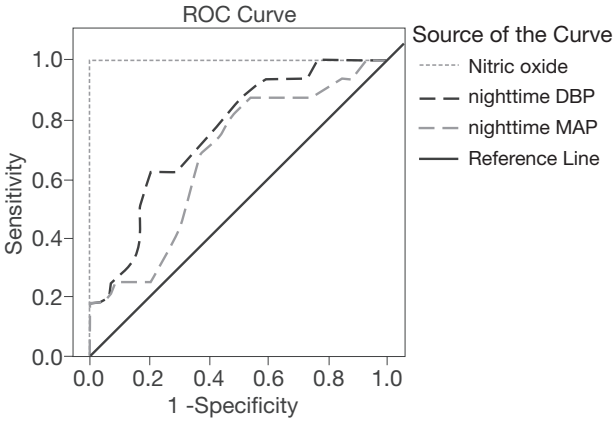

Fig. 3. Receiver operating characteristic (ROC) curves of the serum nitric oxide concentration, nighttime DBP and nighttime MAP.

Pearson correlation analysis applied to find out the correlations between AER and ABPM parameters revealed significant correlations between AER and daytime SBP ($p<0.05$, $r=0.574$), daytime DBP ($p<0.05$, $r=0.672$), daytime MAP ($p<0.05$, $r=0.757$), nighttime SBP ($p<0.05$, $r=0.598$), nighttime DBP ($p<0.05$, $r=814$), nighttime MAP ($p<0.05$, $r=0.692$), 24 hours SBP ($p<0.05$, $r=0.657$), 24 hours DBP ($p<0.05$, $r=0.762$) or 24 hours MAP ($p<0.05$, $r=810$). In order to find out the predictive factors for the development of microalbuminuria, we applied a multiple linear regression analysis method using AER as a dependent variable and other clinical parameters, e.g. serum $NO_2– + NO_3–$ concentrations, ABPM parameters, diabetes duration, age, and BMI as independent variables. The variables are selected based on their generalization ability using stepwise variable selection method. After applying multiple linear regression model, we found that only serum $NO_2– + NO_3–$ concentrations ($p<0.05$, $\beta=0.645$) was independently associated with the presence of microalbuminuria. No association was observed between AER and ABPM parameters, diabetes duration, age or BMI in multiple linear regression analysis (all $p>0.05$).

ROC curve analysis revealed that among the variables were tested, serum $NO_2– + NO_3–$ concentrations showed highest sensitivity and specificity with a cut-off of >32.3 µmol/L (area under curve [AUC]: 1.00; sensitivity: 100%, specificity: 100%, $p<0.05$). Sensitivity and specificity of nighttime DBP and MAP for microalbuminuria were lower than from serum $NO_2– + NO_3–$ concentrations with a cut-off of > 64.5 mmHg (AUC: 0.752; sensitivity: 56%, specificity: 81%, $p<0.05$) and >78.5 (AUC: 0.671; sensitivity: 69%, specificity:67%, $p<0.05$), respectively (Figure 3).

Discussion

Microalbuminuria is considered as a risk factor for diabetic nephropathy, and closely related to the progression of the kidney disease and to both fatal and nonfatal cardiovascular events [17]. Among patients with type 1 DM, microalbuminuria has been reported to revert to normoalbuminuria in 35 to 60 percent of patients over follow-up periods of approximately 5 to 18 years [18, 19]. However, in a recent study of Perkins et al [20], they reported that early decline in renal function has occurred in 68, 32, 16, and 9 percent of patients with progression, stabilization, regression of microalbuminuria, and normoalbuminuria, respectively. Thus, together with testing for microalbuminuria, developing novel strategies for identifying diabetic patients at high risk for the development of diabetic nephropathy will lead to a better follow-up and early application of interventional strategies to reduce renal morbidity and mortality.

Among normalbuminuric type 1 diabetic subjects, the prevalence of hypertension determined on the basis of blood-pressure readings at office visits is similar to that in the general population [21]. It has been re-

ported that in contrast to type 2 diabetic subjects, hypertension is usually absent when microalbuminuria, an indicator of incipient nephropathy, is first detected in type 1 diabetic subjects [22. However, in studies, in which blood pressure parameters were determined with the use of ambulatory blood-pressure monitoring, microalbuminuric type 1 diabetic subjects have been shown to have higher nocturnal blood pressure than either normalbuminuric type 1 diabetic subjects or age-matched controls [23, 24]. Theochari et al [25] reported that hemodynamic changes associated with hypertension may appear early in children with diabetes and the increase in blood pressure early in type 1 diabetes mellitus can be determined more precisely with the use of 24 hours ambulatory measurement. In the present study, casual BP parameters were comparable between type 1 diabetic subjects with or without microalbuminuria and controls. However, in accordance with previous reports [25, 26], night-time SBP, DBP and MAP, and 24 hours DBP and MAP were found significantly higher in microalbuminuric diabetic subjects than controls. More strikingly, normalbuminuric diabetic subjects had also higher night-time DBP and MAP than controls.

Nitric oxide, an endothelium-derived relaxing factor, has been identified as a pleiotropic intercellular messenger that regulates a variety of cellular functions [27]. As NO may increase both blood flow and vascular permeability, it has been suggested as an important mediator responsible for the vascular changes that have been described in early diabetic nephropathy [28]. However, in several studies [29, 30, 31, 32], inhibition of NO action and genetic variation of NO synthetase exacerbate, not attenuate, diabetic nephropathy. These findings suggest that increased production of NO in early diabetic nephropathy is compensative for blood pressure elevation or vascular endothelial damage. In fact, in a recent study of Brand et al [33], they suggested that NO may actually be playing an important role in keeping blood pressure under control at the onset of diabetes. In addition, the positive correlation observed between serum NO concentrations and ABPM parameters in the present study further support this suggestion. Furthermore, despite the high serum NO concentration observed in our microalbuminuric type 1 diabetic subjects, elevation in ABPM parameters and positive correlation between serum NO concentrations and these ABPM parameters may also reflect the NO resistance, which may be due to vascular endothelial damage.

In the present study, we also observed that HbA1c and diabetes duration were the factor associated with increased serum NO concentration. Increase in NO generation has been suggested to be due to impaired action of NO accompanied by up-regulation of NO production [13]. Indeed, glucose-dependent increase in NO production and action has been suggested for the pathogenesis of early diabetic nephropathy by Choi et al [34]. In addition, Cosentino et al demonstrated that prolonged exposure of endothelial cells to high glucose increases both NO and superoxide anion production [12]. More recently, the association between chronic hyperglycemia and serum NO concentration has been reported by Apakkan Aksun et al [10]. Thus, on the basis of the present study and literature data, we can suggest that chronic hyperglycemia may be one of the factors responsible from the increased NO levels in our type 1 diabetic patients with and without microalbuminuria.

In conclusion, ABPM is an important tool in the detection of early increase in systolic and diastolic blood pressure which may be overlooked by the casual measurement. Serum NO concentration is increased in early stages of diabetic nephropathy, and seems to be relevant to glycemic control, glomerular hyperfiltration, ABPM parameters and UAE rate. In addition, albuminuria seems to be closely associated with serum NO concentrations and ABPM parameters in type 1 DM patients. A prospective follow-up study on diabetic patients with normo- and micro- albuminuria is needed to confirm the predictive values of increased NO concentrations and ABPM parameters on the development of albuminuria.

Conflict of interest statement

The author(s) declare that they have no competing interests.

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