Original Article

Elevation of Serum Soluble E- and P-Selectin in Patients with Hypertension Is Reversed by Benidipine, a Long-Acting Calcium Channel Blocker

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Hypertension is a major risk factor for atherosclerotic cardiovascular disease. Selectins, cell-surface adhesion molecules involved in leukocyte rolling and attachment to the vascular endothelium, play a role in the initiation of atherosclerosis. We investigated whether or not serum levels of soluble adhesion molecules are elevated in patients with essential hypertension (EH) and examined whether antihypertensive therapy lowers such levels. Twenty-one patients who had untreated mild to moderate EH without diabetes mellitus, hyperlipidemia, or obesity were recruited at a clinic for hypertensive patients. Blood pressure was measured, and the serum levels of soluble E-selectin, P-selectin, L-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular-cell adhesion molecule 1 (VCAM-1) were determined by enzyme-linked immunosorbent assays before and after 12, 24, and 53 weeks of antihypertensive treatment with benidipine, a long-acting calcium channel blocker, given at a dose of 6 mg/day for 53 weeks. As a control, 21 age- and sex-matched patients without hypertension were studied. Serum E- and P-selectin levels were significantly higher in the subjects with EH than in the controls ($p<0.01$). There were no differences in serum levels of soluble L-selectin, VCAM-1, or ICAM-1 levels between the patients with EH and the controls. Treatment with benidipine decreased the elevated blood pressure over a 53-week study period (mean blood pressure: 119.8±6.5 mmHg at baseline, 101.0±5.9 mmHg at 12 weeks, 98.6±7.3 mmHg at 24 weeks, and 93.9±5.5 mmHg at 53 weeks). Serum levels of soluble E- and P-selectin decreased after the initiation of benidipine treatment and correlated with diastolic blood pressure. Serum levels of soluble L-selectin, VCAM-1, and ICAM-1 did not change significantly during the period of benidipine treatment. Benidipine treatment reduced the content of P-selectin in the platelets from patients with EH, as determined by Western blot analysis. In conclusion, decreased blood pressure may reduce the rate of progression of atherosclerosis by affecting the expression of E- and P-selectin in the endothelium, the platelets, or both. Benidipine may be protective against vascular damage in people with hypertension, not only by lowering blood pressure, but also by inhibiting the expression of selectins. (*Hypertens Res* 2005; 28: 871–878)

***Key Words*: hypertension, atherosclerosis, soluble E-selectin, soluble P-selectin, benidipine**

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Introduction

Hypertension is one of the most important risk factors for atherosclerotic diseases. Selectins are glycoproteins with three subtypes (E-selectin, P-selectin, and L-selectin). These molecules are expressed at sites of inflammatory or endothelial damage and they mediate leukocyte or platelet adhesion to the endothelial surface. This interaction between the endothelium and blood cells is thought to be an initial step in atherosclerosis (*1*).

The adherence of mononuclear cells to the endothelium is mediated by leukocyte adhesion molecules. E- and P-selectin are known to play important roles in the tethering of leukocytes to the endothelium, whereas intercellular adhesion molecule 1 (ICAM-1) and vascular-cell adhesion molecule 1 (VCAM-1) mediate the tight adhesion of leukocytes to the endothelium. Soluble forms of leukocyte adhesion molecules are released into the serum by a shedding mechanism, and these forms can be detected in the plasma (*2*).

Serum levels of soluble E- and P-selectin are higher in hypertensive patients than in healthy controls (*3*, *4*). The expression of E- and P-selectin is considered to be a marker of endothelial injury in patients with hypertension. However, it remains unknown whether or not serum levels of soluble E- and P-selectin can be reduced by the treatment of hypertension with a Ca^{2+} channel blocker. Ca^{2+} channel blockers are widely used to treat hypertension, as these drugs have been shown to reliably lower blood pressure, irrespective of age (*5*), and are also associated with reductions in dietary salt intake, salt-sensitivity status, plasma renin activity (*6*), or improvements in renal function (*7*). Recent clinical trials of Ca^{2+} channel blockers, such as INSIGHT (*8*) and PREVENT (*9*), have indicated that these drugs can retard the progression of atherosclerosis in patients with hypertension and coronary heart disease. The mechanisms underlying the anti-atherosclerotic effects of Ca^{2+} channel blockers have been reported, and they include various effects on leukocyte-endothelial cell adhesion (*10*), monocyte infiltration of the subendothelium (*11*), platelet aggregation (*12*), and the expression of vasoactive substances (*13*). To gain further insight into the anti-atherosclerotic effects of Ca^{2+} channel blockers, we investigated the effects of 53 weeks of antihypertensive therapy with benidipine, a long-acting Ca^{2+} channel blocker, on the levels of soluble adhesion molecules in the serum of hypertensive patients.

Methods

Twenty-one patients (age: 53.4±9.1 years; 13 males, 8 females) with mild to moderate essential hypertension (EH), defined as stage I according to the sixth report of the Joint National Committee on the Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC-VI), were enrolled in this study. Hypertension was defined as a systolic blood pressure of >140 mmHg, a diastolic blood pressure of >90 mmHg, or both. Blood pressure was measured with the use of a standard sphygmomanometer at the right arm of patients in a sitting position after 10 min rest. The blood pressure was measured at least twice on two different occasions and was subsequently confirmed on at least two more visits during the next 4 weeks. We excluded hypertensive patients who had severe complications (stage III according to the World Health Organization staging), as well as patients who had a history of diabetes mellitus, hyperlipidemia, obesity, or atherosclerotic diseases such as myocardial infarction, stable angina, stroke, or peripheral arterial disease. Each patient was given benidipine for 53 weeks at a dose of 6 mg/day, which has been shown to exert satisfactory and stable antihypertensive effects (*14–16*). As a control, 21 sex- and age-matched patients without hypertension (age: 54.3±9.9 years; range: 32–68 years; 13 males, 8 females) who visited an internal medicine outpatient clinic were included in the analysis. This study was performed in accordance with the 2nd Helsinki Declaration and was approved by the Ethical Committee of Fukushima Medical University. Informed consent was obtained from all subjects.

Table 1. Baseline Characteristics of Patients with Essential Hypertension and Age- and Sex-Matched Control Subjects

Variable	Essential hypertension (n=21)	Control subjects (n=21)
Age (years old)	53.4±9.1	54.3±9.9
Sex (M/F)	13/8	13/8
BMI (kg/mm^2)	22.1±2.2	22.4±2.4
MBP (mmHg)	119.8±6.5*	91.4±9.8
Creatinine (mg/dl)	0.8±0.2	0.7±0.2
Total cholesterol (mg/dl)	203.9±16.5	197.2±11.5
Platelet ($\times 10^4$ cells/μl)	24.5±4.3	23.5±5.2
Funduscopic grade ≥KWII (%)	14**	0
ECG LVH (%)	24**	0
Smoking (%)	33	33

Data are expressed as mean±SD. M, male; F, female; BMI, body mass index; MBP, mean blood pressure; KWII, grade II on the Keith-Wagener-Barker classification; ECG LVH, electrocardiographic evidence of left ventricular hypertrophy. *$p<0.01$ *vs*. control. **$p<0.001$ *vs*. control.

Measurement of Soluble Adhesion Molecules

Venous blood samples were obtained by standard phlebotomy. The samples were centrifuged at 3,000 × *g* for 15 min at 4°C, and the serum was removed and stored at −80°C until used for analysis (for the determination of routine chemical profiles according to standard methods). The serum levels of soluble adhesion molecules were determined with enzyme-linked immunosorbent assay (ELISA) kits purchased from

Table 2. Serum Levels of Soluble Adhesion Molecules in Patients with EH and Control Subjects

	E-selectin (ng/ml)	P-selectin (ng/ml)	L-selectin (ng/ml)	VCAM-1 (ng/ml)	ICAM-1 (ng/ml)
Controls (n=21)	44.7	122.2	996.4	530.1	268.1
	(30.5–51.6)	(103.5–146.6)	(756.0–1,429.3)	(465.3–738.8)	(206.8–308.5)
EH (n=21)	92.8*	288.7*	1,104.8	551.5	275.7
	(77.2–118.6)	(183.9–348.1)	(866.0–1,350.0)	(451.5–713.8)	(245.1–299.7)

Data are shown as medians and (25th to 75th percentiles). *$p<0.01$ *vs*. control. VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; EH, essential hypertension.

Table 3. Effect of Benidipine on Adhesion Molecules and Blood Pressure in Hypertensive Patients

	Before	12 w	24 w	53 w
E-selectin (ng/ml)	92.8 (77.2–118.6)	69.2 (58.8–87.8)*	59.4 (48.5–77.3)*	58.4 (48.9–76.8)*
P-selectin (ng/ml)	288.7 (183.9–348.1)	145.8 (114.6–169.0)*	122.6 (87.5–152.8)*	101.2 (75.2–127.8)*
L-selectin (ng/ml)	1,104.8 (866.0–1,350.0)	1,152.5 (937.0–1,389.1)	1,144.0 (867.4–1,458.0)	1,184.3 (914.7–1,361.9)
VCAM-I (ng/ml)	551.5 (451.5–713.8)	578.3 (466.7–682.4)	591.2 (497.0–686.3)	582.4 (512.3–680.5)
ICAM-I (ng/ml)	275.7 (245.1–299.7)	249.8 (219.0–297.6)	247.2 (209.9–281.2)	244.8 (206.7–278.1)
SBP (mmHg)	162.3±10.2	135.7±9.4*	131.0±9.0*	125.0±7.6*
MBP (mmHg)	119.8±6.5	101.0±5.9*	98.6±7.3*	93.9±5.5*
DBP (mmHg)	98.6±8.5	83.8±7.3*	82.2±8.6*	78.4±7.5*

Data are shown as medians and (25th to 75th percentiles) or means±SD. *$p<0.01$ *vs*. before. w, weeks; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; SBP, systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure.

Bender MedSystems Diagnostics GmbH (Vienna, Austria) for the analyses of E-, P-, and L-selectins and from Genzyme Techne (Minneapolis, USA) for the analyses of VCAM-1 and ICAM-1.

Western Blot Analysis

Citrated (0.129 mol/l) fresh blood from patients (7 ml) was collected in vacutainer tubes (Becton Dickinson, Rutherford, USA). Platelet-rich plasma was obtained by centrifugation for 10 min at $350 \times g$, transferred to 15 ml polypropylene tubes, and centrifuged at $500 \times g$ for 10 min at room temperature in order to isolate the platelets. The pellets obtained in this manner were resuspended at 1×10^6 cells/100 µl in PBS and were added to a buffer containing 20 mmol/l Tris-HCl (pH 7.4), 2 mmol/l EDTA, 2 mmol/l phenylmethylsulfonyl fluoride (PMSF), 10 mmol/l Na_3VO_4, 100 mmol/l NaCl, 10% glycerol, 10 mg/ml leupeptin, and 10 mg/ml aprotinin at a density of 1×10^9 cells/ml. The mixture was stored −80°C until analysis.

The platelet proteins were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes, which were blocked with 5% normal goat sera in 10 mmol/l Tris-HCl, pH 7.5, containing saline and 0.1% Tween-20 (TBST buffer). Western blotting was performed with anti-P-selectin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, USA). The immunoblots were visualized with an ECL System and were quantified with an NIH image system.

Statistical Analysis

The data were statistically analyzed with the use of Mann-Whitney's *U* test, the Wilcoxon signed-rank rest, or analysis of variance (ANOVA), as appropriate. A *p*-value of less than 0.05 was considered to indicate statistical significance. The data are expressed as means±SD, or as medians (range 25th to 75th percentiles), unless otherwise indicated.

Results

The baseline clinical characteristics of the 21 patients with EH and the control subjects are summarized in Table 1. The clinical characteristics did not significantly differ between the two groups, except for with respect to blood pressure, the funduscopic grade of KW II or higher according to the Keith-Wagener-Barker classification (EH *vs*. control, 14% *vs*. 0%; $p<0.001$), and the electrocardiographic evidence of left ventricular hypertrophy (24% *vs*. control 0%; $p<0.001$).

Serum levels of soluble adhesion molecule at baseline are summarized in Table 2. Only the E- and P-selectin levels were significantly higher in the EH group than in the control group (both $p<0.01$); the levels of L-selectin, VCAM-1, and ICAM-1 did not significantly differ between the EH and control groups.

The time courses of the serum adhesion molecules after the

Table 4. Serum Chemical Variables in Hypertensive Patients before and after Benidipine Treatment

	Before	12 w	24 w	53 w
TP (g/dl)	7.3±0.4	7.5±0.4	7.4±0.3	7.5±0.3
GOT (IU/l)	20.1±6.7	19.9±4.4	23.5±6.3	22.4±8.2
GPT (IU/l)	21.0±5.6	23.8±10.4	24.9±7.3	23.3±8.8
γGTP (IU/l)	36.8±25.1	28.4±13.1	28.3±9.8	24.7±7.2
TB (mg/dl)	0.7±0.2	0.6±0.1	0.7±0.1	0.8±0.1
Cr (mg/dl)	0.8±0.2	0.8±0.2	0.8±0.1	0.8±0.1
BUN (mg/dl)	12.9±2.6	14.5±2.5	13.1±2.8	13.6±2.7
TC (mg/dl)	203.9±16.5	202.7±18.6	192.8±22.3	203.5±14.4
TG (mg/dl)	108±12	103±15	107±12	105±10
Plt ($\times 10^4/\mu l$)	24.5±4.3	25.4±4.1	25.5±4.1	26.0±5.0

w, weeks; TP, total protein; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; γGTP, gamma glutamyl transpeptidase; TB, total bilirubin; Cr, creatinin; BUN, blood urea nitrogen; TC, total cholesterol; TG, triglyceride; Plt, platelets.

initiation of benidipine treatment are shown in Table 3. The serum levels of soluble E- and P-selectin significantly decreased in a time-dependent manner (Table 3), whereas the serum L-selectin level did not exhibit such a decrease. Systolic, mean, and diastolic blood pressures were reduced by benidipine throughout the treatment period (Table 3). Benidipine treatment did not alter any of the other clinical variables (Table 4). The level of expression of P-selectin in the platelets from the patients with EH was higher than that of the platelets from the controls. P-selectin expression in the platelets was reduced by benidipine treatment for 53 weeks (Fig. 1).

Discussion

This study demonstrated that the serum levels of E-selectin and P-selectin were significantly elevated in patients with EH. These levels were reduced in a time-dependent manner by treatment with benidipine, a Ca^{2+} channel blocker. These findings suggest that the levels of expression of both E- and P-selectin are regulated by the exposure of endothelial cells, platelets, or both to blood pressure-induced mechanical stress. Our results also revealed that the P-selectin content of platelets from patients with EH was higher than that of the platelets from the control subjects. To the best of our knowledge, this is the first study to demonstrate that antihypertensive treatment can reduce to normal the abnormally high levels of soluble selectins in the serum and platelets of patients with EH.

Our findings are consistent with those of previous studies demonstrating elevated levels of soluble E- and P-selectin in EH (*4*, *17*–*20*). In patients with EH, plasma soluble E-selectin levels have been shown to correlate with minimal vascular resistance, an index of hypertension-related vascular damage (*21*). Furthermore, E-selectin levels are known to positively correlate with left ventricular relative wall thickness (*17*). Progressive vascular damage in hypertension has also been associated with increased levels of soluble P-selectin (*4*).

Although several studies have demonstrated a lack of significant elevations in E-selectin levels in EH (*22*–*24*), the patients in those studies had uncomplicated or mild hypertension, without indices of end-organ damage such as cardiac hypertrophy or urinary albumin excretion. In contrast, our patients presented with cardiac hypertrophy. The inconsistency in the reported levels of soluble selectins in EH patients may be related to the magnitude of hypertensive vascular damage among the study subjects. It is also possible that the stage of hypertension is associated with the increased secretion of E- and/or P-selectin.

The pre-treatment levels of E- and P-selectin of our hypertensive patients relative to those of our normotensive controls were higher than those reported previously (*4*, *16*, *17*). Our hypertensive patients had mild to moderate, but not severe hypertension, and we excluded from the present analysis patients with histories of events involving severe vascular damage such as myocardial infarction, cerebrovascular disease, or obstructive peripheral vascular disease. Differences among studies in the baseline values of selectins may also be related to the kits used to measure selectin levels: the kits used in the present study had steeper standard curves than those of other commercially available kits (data not shown).

In contrast to the results of several previous studies (*15*, *16*, *20*, *22*), we found no evidence of increased serum levels of either VCAM-1 or ICAM-1, both of which are immunoglobulin superfamily cell adhesion molecules, among our hypertensive subjects. Verhaar and co-workers (*4*) reported observing that the plasma levels of VCAM-1 and ICAM-1 in EH patients were not higher than those of normotensive subjects. They also showed that the plasma levels of VCAM-1, but not those of ICAM-1, were increased, but only concomitant with progressive vascular damage, which is associated with conditions such as malignant hypertension or septicemia. Other groups have observed that cell adhesion molecules are induced by obesity (*25*, *26*), impaired glucose tolerance/hyperglycemia (*27*, *28*), and diabetes (*29*), thus suggesting that these molecules are induced by metabolic abnormalities in a manner independent of hypertension. These latter results

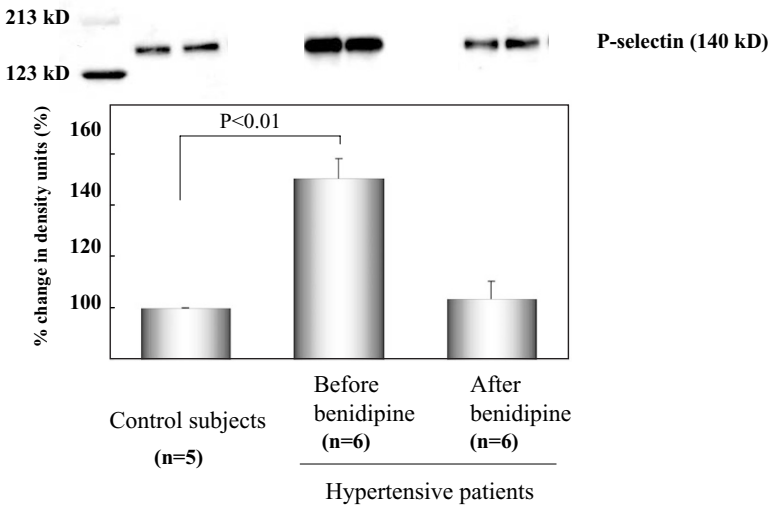

Fig. 1. *Western blot analysis of P-selectin in human platelets before and after benidipine treatment.*

support the present findings. The available evidence thus suggests that the levels of expression of E- and P-selectin can serve as markers of endothelial and platelet activation in hypertensive patients.

Several studies have examined the effects of antihypertensive drugs on cell adhesion molecules (*19, 30–32*), but such investigations have yielded inconsistent findings. The mechanisms related to these effects remain poorly investigated. Benidipine has been reported to inhibit the formation of E- and P-selectin in cultured endothelial cells from human umbilical cord veins (*33*). A Ca^{2+} channel blockade is a very unlikely mechanism to account for the benidipine-induced inhibition of E- and P-selectin secretion, since endothelial cells have no voltage-sensitive (L-type) calcium channels. A more likely explanation for the inhibition of the secretion of selectin by benidipine is a reduction in mechanical stress. In a recent study by Nomura and co-workers (*19*), decreases in the levels of both E- and P-selectin were observed after 8 weeks' treatment with the dihydropyridine derivative efonidipine in hypertensive patients with diabetes; furthermore, the reduction in E-selectin was greater in the former group than in patients who exhibited a decrease in systolic blood pressure of less than 10%. Studies of angiotensin converting enzyme inhibitors (ACE-I) have demonstrated a reduction in E-selectin levels in association with a decrease in blood pressure (*30*), as well as no change in E-selectin levels in the absence of a decrease in blood pressure (*31*). Reports of angiotensin receptor blockers (ARBs) (*30*, *32*) have noted a lack of reduction in E-selectin levels in non-diabetic hypertensive patients, in spite of an association with decreases in blood pressure. However, in those reports, the post-treatment blood pressure levels remained higher than the levels used in the criteria for hypertension (*34–37*). On the other hand, the treatment protocol used in the present study was able to achieve a low mean blood pressure value of 125/78.4 mmHg, which is below 140/90 mmHg, *i.e.*, the recommended value determined by several therapeutic guidelines (*34–37*); furthermore, to the best of our knowledge, the post-treatment blood pressure values of the patients considered here were lower than those of patients analyzed in previous reports (*19*, *30–32*). In non-diabetic hypertensive patients, a reduction of blood pressure values may be related to a reduction of serum selectin levels. Hypertension increases mechanical stretching, tension, or both, which may in turn enhance the expression of adhesion molecules *via* the nuclear factor-κB (NF-κB) system; in addition, hypertension may enhance oxidative stress and superoxide production in endothelial cells (*38*, *39*) and vascular smooth muscle cells (*40*, *41*). Oxidative stress is known to promote the inflammatory response *via* the NF-κB system. The human E-selectin promoter has been shown to possess three NF-κB binding sites (*42*). Mechanical stress can induce E-selectin expression, and reduced mechanical stress accompanying decreased blood pressure during treatment with a Ca^{2+} channel blocker may reduce E-selectin levels in the serum.

A second possible mechanism for the benidipine-induced reductions in E- and P-selectin levels may be related to the antioxidant activity associated with several dihydropyridine Ca^{2+} channel blockers, the antioxidant activity of which has been reported clinically (*43*, *44*), *in vivo* (*45*, *46*) and *in vitro* (*47*, *48*). The antioxidant effects of benidipine have been demonstrated in a clinical study showing that benidipine reduces increased levels of serum lipid peroxidation products and it increases serum superoxide dismutase activity (*43*); in addition, benidipine has been shown to inhibit oxidative stress in polymorphonuclear cells (*44*) in patients with EH. Recent findings have indicated that certain radical-scavenging antioxidants inhibit the expression of adhesion molecules (*49*) and inhibit monocyte adhesion to endothelial cells by the

suppression of NF-κB mobilization (*50*). Given that antioxidant activity inhibits the expression of adhesion molecules by the suppression of NF-κB mobilization, dihydropyridine Ca^{2+} channel blockers such as benidipine that exhibit antioxidant activity are likely to inhibit the expression of E-selectin.

Although the effects of dihydropyridine Ca^{2+} channel blockers on nitric oxide (NO) synthesis remain controversial, benidipine has been reported to augment NO production clinically (*51*) and *in vivo* (*52*, *53*). The pre-treatment of platelets with a NO-donor has been shown to prevent the induction of adhesion molecules (*54*). Benidipine may thus reduce serum levels of endothelial selectins by enhancing NO production.

Finally, the inhibition of platelet function might also contribute to the beneficial effects of dihydropyridine Ca^{2+} channel blockers such as benidipine. Hypertensive patients tend to have elevated levels of both plasma fibrinogen and adhesion molecules such as P-selectin, both of which promote platelet activation and atherogenesis (*55*). Previous clinical studies have reported that dihydropyridine Ca^{2+} channel blockers such as benidipine (*55*) and amlodipine (*3*) inhibit platelet activation in patients with EH by blocking calcium channels, which increases intracellular calcium levels after platelet activation (*17*). Thus, benidipine might inhibit the formation of E- and P-selectin *via* several mechanisms and may also reduce *in vivo* platelet activation while improving endothelial cell function. However, the confirmation of these mechanisms will require further investigation. The present results also suggested that soluble E- and P-selectin levels or the P-selectin content of platelets, or both, may be clinical markers of vascular damage in EH patients.

In conclusion, treatment with the Ca^{2+} channel blocker benidipine led to reductions in the serum levels of soluble E- and P-selectin, and it also reduced the P-selectin content of platelets in patients with EH. These effects may be clinically relevant markers for the hemodynamic effects of antihypertensive treatment.

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