Original Article

Benidipine, a Long-Acting Calcium Channel Blocker, Inhibits Oxidative Stress in Polymorphonuclear Cells in Patients with Essential Hypertension

Kenichi YASUNARI, Kensaku MAEDA, Munehiro NAKAMURA,

Takanori WATANABE, and Junichi YOSHIKAWA

To study the relationship between blood pressure and oxidative stress in leukocytes, the effect of benidipine on these variables was compared with that of a placebo. Hypertensive patients were randomly assigned benidipine 4 mg ($n=40$) or placebo ($n=40$), and treated for 6 months. Oxidative stress in polymorphonuclear cells (PMNs) was measured by gated flow cytometry. There was a significant relationship between systolic or diastolic arterial pressure and reactive oxygen species (ROS) formation by PMNs in the benidipine group ($r=0.61$, $p<0.01$) and in the placebo group ($r=0.58$, $p<0.01$). After administration of 4 mg benidipine, ROS formation by PMNs fell by 32 arbitrary units ($n=40$, $p<0.01$). After administration of placebo, ROS formation by PMNs decreased by 0.6 arbitrary units ($n=40$, $p=0.31$) ($p<0.01$ for differing treatment effects). There was a significant relationship between the decrease in systolic arterial pressure and the decrease in ROS formation by PMNs in the benidipine group ($r=0.52$, $p<0.01$), but not in the placebo group ($r=-0.08$, $p=0.61$). There was also a significant relationship between the decrease in diastolic arterial pressure and decrease in ROS formation by PMNs in the benidipine group ($r=0.65$, $p<0.01$) but not in the placebo group ($r=-0.09$, $p=0.59$). In hypertensive patients, we observed a significant relationship between systolic or diastolic blood pressure and ROS formation by PMNs, and found that benidipine decreased oxidative stress in PMNs of hypertensive patients, at least in part by decreasing blood pressure. (*Hypertens Res* 2005; 28: 107–112)

Key Words: blood pressure, calcium channel blockers, leukocytes, neutrophils, oxidative stress

Introduction

Although the molecular pathogenesis of essential hypertension is still not known in detail, increased oxidative stress has been reported in experimental models of hypertension (*1*) and in patients with essential hypertension (*2*). If enhanced oxidative stress is involved in the pathogenesis of atherosclerosis in hypertensive patients (*3*), an adequate antioxidant supply should be included in the treatment of such patients, and thus it is essential to determine whether an antihypertensive drug has an antioxidative action *in vivo*.

The long-acting calcium channel blockers (CCBs), which are widely used in the clinical setting, have been shown to prevent atherosclerosis, as indicated by some clinical and experimental reports (*4*–*6*). It has been reported that a long-acting CCB, amlodipine, has an antioxidative action *in vivo* (*7*). However, the subcellular signaling mechanisms of this
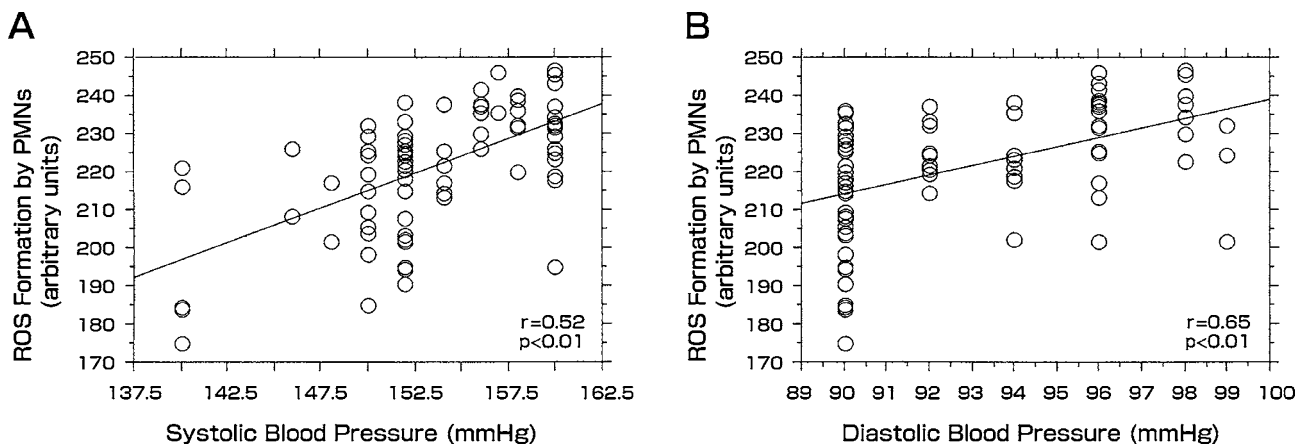
A

B

Fig. 1. *The relationship between systolic blood pressure and ROS formation by PMNs (A) and diastolic blood pressure and ROS formation by PMNs (B) at baseline.*

Table 1.   Baseline Characteristics of Hypertensive Subjects

	Benidipine (*n*=40)	Control (*n*=40)	*p*
Age (yaers)	62±9	64±12	0.40
Sex (M/W)	19/21	17/23	0.21
Body mass index (kg/m^2)	24±4	25±3	1.00
Systolic arterial pressure (mmHg)	153±7	153±3	1.00
Diastolic arterial pressure (mmHg)	93±5	93±3	0.28
PMN oxidative stress (arbitrary units)	219±18	223±15	0.28
Hemoglobin A$_{1C}$ (%)	5.3±0.4	5.5±1.1	0.28
Triglyceride (mg/dl)	118±72	114±54	0.77
HDL-cholesterol (mg/dl)	50±11	54±13	0.14
LDL-cholesterol (mg/dl)	121±46	113±31	0.36

M/W, men/ women; PMN, polymorphonuclear cell; HDL, high density lipoprotein; LDL, low density lipoprotein.

effect are not well understood.

Polymorphonuclear cells (PMNs) are one of the main types of inflammatory cells. Once activated, PMNs release reactive oxygen species (ROS), including hydrogen peroxide and mediators of proteolytic tissue degradation, contributing to oxidative stress, inflammation, endothelial damage, and atherosclerosis in the long run (*8*, *9*). PMN infiltration has also been observed in the culprit lesions in acute coronary syndromes (*10*). ROS formation by PMNs not only reflects intravascular oxidative stress, but can also be a link between oxidative stress and inflammation *in vivo*.

The objective of the present study was to determine the effect of benidipine on oxidative stress in PMNs in patients with essential hypertension.

Methods

Study Protocol

The study was approved by the Institutional Review Board of

Osaka City University Hospital. Written, informed consent was obtained from each subject.

To examine whether benidipine or placebo inhibited a pressure-induced increase in ROS formation by PMNs, mildly hypertensive patients who visited the University Hospital were recruited into this study. Mild hypertension was defined as a systolic blood pressure of 140–160 mmHg, a diastolic blood pressure of at least 90–100 mmHg, or both, as measured with the subject in a sitting position, on at least three different occasions in the outpatient clinic of the hospital. None of the subjects were on any medications; they were not taking non-steroidal anti-inflammatory drugs, vitamin E, or other antioxidants. Fasting blood samples were collected at baseline in tubes with heparin as an anticoagulant.

After a 4-week run-in period, the subjects were given 4 mg benidipine or placebo per os for 6 months in a randomized, double-blind fashion. Six months later, another fasting blood sample was collected as above. No subjects were lost to follow-up during this period. ROS formation by PMNs was measured at baseline, and again 6 months later. Degree of
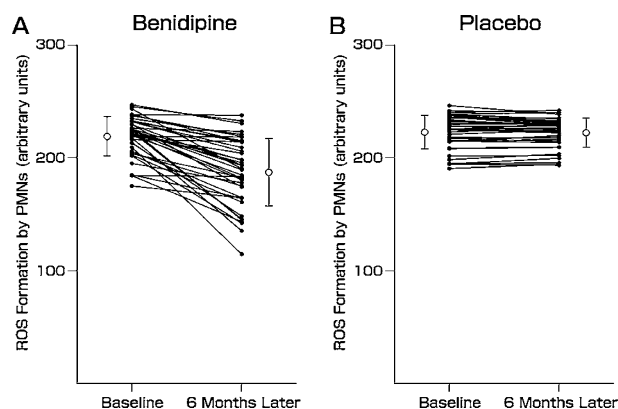
Fig. 2. *ROS formation by PMNs before and after 6 months of treatment with benidipine 4 mg (A) or placebo (B). ROS formation by PMNs was measured by flow cytometry. The open circle and bars indicate the mean±SD of each value.*

obesity was estimated by the body mass index. Venous blood was used for measurement of plasma insulin, plasma glucose, hemoglobin A_{1C}, plasma cholesterol, triglyceride and high density lipoprotein (HDL)-cholesterol concentration.

Assay of ROS Formation by PMNs

ROS formation by PMNs was measured using a gated flow cytometry technique described previously (*11*, *12*), with some modifications (*13*). Fresh blood (1 ml) from participants, collected into preservative-free heparin (10 U/ml of blood), was pre-incubated for 15 min with 2′,7′-carboxydichlorofluorescin diacetate bis-acetoxymethyl ester (CDCFH bis-AM ester; Molecular Probe Co., Eugene, USA) (100 μmol/l) in a 37°C water bath with gentle horizontal shaking. CDCFH diacetate bis-AM ester is a compound that is converted into a non-fluorescent derivative (CDCFH) by cellular esterases after incorporation into cells. CDCFH is rapidly oxidized to the fluorescent carboxydichlorofluorescein (CDCF) in the presence of intracellular hydrogen peroxide. The fixed samples were kept on ice until flow cytometric analysis on the same day. The ROS formation by PMNs was measured as fluorescence intensity by gated flow cytometry.

Statistical Methods

All values were expressed as the mean±SD, unless otherwise specified. Statistical analysis of the results for two group comparisons was performed by Student's *t*-test for continuous data, and the χ^2 test for categorical data. Comparison of measurements at baseline and 6 months later was carried out by the paired *t*-test with a 2-sided *p* value and a 95% confidence interval (CI). The relationship between ROS formation by PMNs and relevant covariates was examined by determi-

nation of Pearson's correlation coefficients (SAS). Correction of the relationship by the other covariates was performed by multivariate analysis.

Results

Baseline Characteristics

During the 4-week run-in period, of the 100 subjects who underwent randomization, 16 withdrew because they became normotensive. Four subjects also withdrew because their systolic blood pressure rose above 160 mmHg or diastolic blood pressure rose above 100 mmHg.

There was a significant relationship between systolic arterial pressure and ROS formation by PMNs in the hypertensive participants ($n=80$, $r=0.52$, $p<0.01$) (Fig. 1A). There was also a significant relationship between diastolic arterial pressure and ROS formation by PMNs in the hypertensive participants ($n=80$, $r=0.65$, $p<0.01$) (Fig. 1B). Since ROS formation by PMNs is related to blood pressure and hemoglobin A_{1C} (*13*), these relationships were corrected by hemoglobin A_{1C} with multivariate analysis. When corrected by hemoglobin A_{1C}, there was still a significant relationship between systolic or diastolic arterial pressure and ROS formation by PMNs in the hypertensive participants ($n=80$, $r=0.58$, $p<0.01$; $n=80$, $r=0.47$, $p<0.01$).

Baseline characteristics of the benidipine group ($n=40$) and the placebo group ($n=40$) are summarized in Table 1. No difference in ROS formation by PMNs was observed between the benidipine and placebo groups. There was a significant relationship between systolic arterial pressure and ROS formation by PMNs in the benidipine group ($r=0.61$, $p<0.01$) and in the placebo group ($r=0.58$, $p<0.01$). There was also a significant relationship between diastolic arterial pressure and ROS formation by PMNs in both the benidipine group ($r=0.44$, $p<0.01$) and the placebo group ($r=0.52$, $p<0.01$).

ROS Formation by PMNs

After administration of 4 mg benidipine, ROS formation by PMNs fell by 32 arbitrary units ($n=40$, $p<0.01$) (Fig. 2, Table 2). After administration of placebo, ROS formation by PMNs did not decrease ($n=40$, $p=0.31$) (Fig. 2, Table 2). The decrease in ROS formation by PMNs after administration of placebo by a mean of 31 arbitrary units (range, 24−39 arbitrary units; 95% CI) ($n=40$, $p<0.01$). The decrease in systolic and diastolic arterial pressure after administration of 4 mg benidipine was significantly different from that after administration of placebo: the difference was 9 (7−11) mmHg for systolic ($n=40$, $p<0.01$) and 5 (3−7) mmHg for diastolic ($n=40$, $p<0.01$) arterial pressure (Table 2).

There was a significant relationship between the decrease in systolic arterial pressure and the decrease in ROS formation by PMNs in the benidipine group ($r=0.58$, $p<0.01$) (Fig. 3A), but not in the placebo group ($r=-0.08$, $p=0.61$). There

Table 2. The Changes in Measurements of Hypertensive Subjects

	Benedipine ($n=40$)			Control ($n=40$)			Inter-group
	Difference	95% CI	p	Difference	95% CI	p	p
Body mass index (kg/m^2)	−0.1	(−0.3 to 0. 1)	0.38	0	(−0.2 to 0.1)	0.67	0.62
Systolic arterial pressure (mmHg)	−10	(−12 to −7)	<0.01	−0.5	(−1.5 to 0.5)	0.34	<0.01
Diastolic arterial pressure (mmHg)	−5	(−7 to 4)	<0.01	−0.3	(−1.0 to 0.5)	0.43	<0.01
PMN oxidative stress (arbitrary units)	−32	(−40 to −24)	<0.01	−0.6	(−1.7 to −0.6)	0.31	<0.01
Hemoglobin A$_{1C}$ (%)	−0.02	(−0.08 to 0.05)	0.58	0.01	(−0.09 to 0.12)	0.74	0.55
Triglyceride (mg/dl)	2	(−15 to 19)	0.81	−0.5	(−16 to 15)	0.95	0.83
HDL-cholesterol (mg/dl)	0.2	(−0.21 to 2.4)	0.89	−1.6	(−3.3 to 0.2)	0.08	0.2
LDL-cholesterol (mg/dl)	1.1	(−6.4 to 8.5)	0.77	−3.7	(−9.5 to 2.1)	0.21	0.28

CI, confidence interval; PMN, polymorphonuclear cell; HDL, high density lipoprotein; LDL, low density lipoprotein.

was also a significant relationship between the decrease in diastolic arterial pressure and the decrease in ROS formation by PMNs in the benidipine group ($r=0.47$, $p<0.01$) (Fig. 3B), but not in the placebo group ($r=−0.09$, $p=0.59$). After correction by hemoglobin A$_{1C}$, there was still a significant relationship between the decrease in systolic or diastolic arterial pressure and the decrease in ROS formation by PMNs in the hypertensive participants (for systolic arterial pressure: $n=40$, $r=0.65$, $p<0.01$; for diastolic: $n=40$, $r=0.50$, $p<0.01$).

Other Traditional Risk Factors

As additional risk factors, age, sex, body mass index, systolic arterial pressure, diastolic blood pressure, hemoglobin A$_{1C}$, triglyceride, HDL-cholesterol, and low density lipoprotein (LDL)-cholesterol were measured at baseline, and 6 months later, in both the benidipine and placebo groups. These factors were similar in the benidipine and placebo groups at baseline (Table 1), and none of them had changed significant at 6 months (Table 2).

Discussion

In the present study, we found that benidipine, but not placebo, inhibited ROS formation by PMNs. This may be partly explained by a decrease in systolic or diastolic arterial pressure. It has been reported that arterial pressure increases oxidative stress *in vitro* (*14*) and that ROS formation by PMNs is increased in spontaneously hypertensive rats (*15*, *16*) and in patients with essential hypertension (*17*). A significant relationship between blood pressure and ROS formation by PMNs has been observed in apparently healthy subjects (*13*), and more clearly in patients with essential hypertension (Fig. 1). In the present study, we showed that there was a significant relationship between the decrease in systolic or diastolic arterial pressure and the decrease in ROS formation by PMNs in hypertensive subjects (Fig. 3). Even when corrected by hemoglobin A$_{1C}$, a significant relationship was still observed.

Therefore, increased PMN oxidative stress in hypertensive patients may be, at least in part, a consequence of increased blood pressure. However, we cannot completely rule out the possibility that benidipine itself may act as an antioxidative agent. It has been reported that the influx of calcium is an important step in ROS formation by PMNs (*18*) and that benidipine significantly reduces the hydrogen peroxide-induced myocardial damage and inhibits the increase in tissue lipid peroxidation (*19*).

Although the clinical significance of decreasing ROS formation by PMNs is still speculative, Ito *et al*. have recently linked the increased ROS formation by PMNs to altered endothelial vascular reactivity and damage in an experimental model of hypertension (*20*). It has also been reported that leukocyte count is associated with carotid intima-media thickness (*21*) and that PMN count is related to ROS formation by PMNs (*22*). Thus, a relationship between carotid intima-media thickness and ROS formation by PMNs is suggested. In the Prospective Randomized Evaluation of the Vascular Effects of Norvasc Trial, the long-acting calcium channel blocker amlodipine provided significant clinical benefits when compared with placebo, including a marked reduction in cardiovascular morbidity, and a reduction in the progression of carotid atherosclerosis (*5*).

In the Systolic Hypertension in Europe study, a calcium antagonist dramatically reduced cardiovascular events in elderly diabetics when compared with placebo (*23*). The Hypertension Optimal Treatment study showed that, when using a calcium antagonist-based regimen, the degree of blood pressure lowering determines the degree of cardiovascular event reduction (*24*). The results of the Valsartan Antihypertensive Long-term Use Evaluation (VALUE) Trial of Cardiovascular Events in Hypertension showed that blood pressure reduction by 5 mg amlodipine decreased the incidence of myocardial infarction significantly better than that of 80 mg valsartan (*25*). Blood pressure reduction by a calcium antagonist may play a role in reducing cardiovascular events also in Japanese (*26*). We have already demonstrated that blood pressure reduction by β-blockers decreases ROS formation by PMNs.
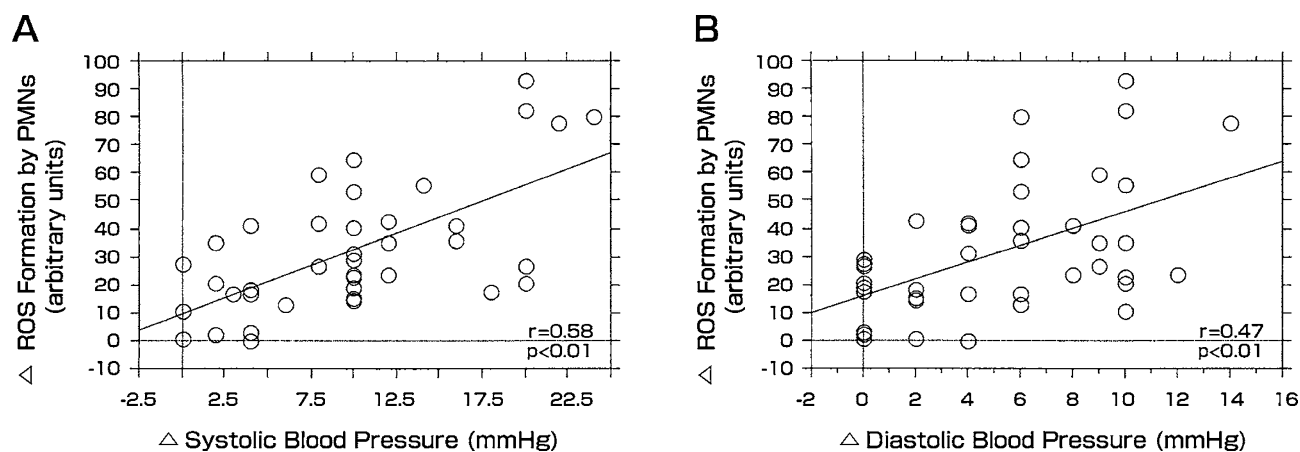
Fig. 3. *The relationship between changes (Δ) in systolic blood pressure and those in ROS formation by PMNs (A) and changes (Δ) in diastolic blood pressure and those in ROS formation by PMNs (B) after administration of benidipine for 6 months.*

Thus, the reduction of ROS formation by PMNs may result from a reduction of blood pressure, and may be a mechanism for cardiovascular event reduction, since ROS formation by PMNs is one of the major components of leukocyte oxidative stress (*13*), and leukocyte oxidative stress would be an additional systemic source of oxidative stress (*27*).

In summary, we observed a significant relationship between systolic or diastolic blood pressure and ROS formation by PMNs in hypertensive patients, and found that benidipine decreased ROS formation by PMNs of hypertensive patients, at least in part by decreasing blood pressure. ROS formation by PMNs may be a marker of long-term antihypertensive treatment and intravascular oxidative stress in humans.

Acknowledgements

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