*Original Article*

# Oxidative Stress by Peripheral Blood Mononuclear Cells Is Increased in Hypertensives with an Extreme-Dipper Pattern and/or Morning Surge in Blood Pressure

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**Because oxidative stress and inflammation are known to play important roles in the pathogenesis of cardiovascular events that occur most frequently in the morning, we studied the association between reactive oxygen species (ROS) formation by polymorphonuclear leukocytes (PMNs) or mononuclear cells (MNCs) and morning blood pressure (BP) rhythm. A total of 31 hypertensives in whom ambulatory BP monitoring was performed participated in this study. They were first divided into three groups according to their nocturnal BP rhythm (non-dippers, dippers and extreme dippers), and then into two groups according to their morning BP change (surge-type and sustained-type). ROS formation by PMNs and MNCs was measured by gated flow cytometry. C-reactive protein and traditional risk factors such as age, gender, body mass index, hemoglobin $A_{1c}$, and total cholesterol were also measured. ROS formation by MNCs was significantly increased in extreme dippers (*vs.* dippers, $p<0.05$, $n=11$) and in morning BP surge-type hypertensives (*vs.* sustained-type, $p<0.05$, $n=13$). In patients who were both extreme dippers and morning BP surge-types, ROS formation by MNCs was significantly higher than that in other groups. These results suggest that both extreme dippers and morning BP surge-type hypertensives may suffer increased ROS formation by MNCs, and that increased ROS formation by MNCs may underlie morning strokes. (*Hypertens Res* 2005; 28: 755–761)**

***Key Words*: blood pressure, hypertension, leukocytes, oxidative stress, fluorescence**

## Introduction

It was recently reported that elderly hypertensive extreme dippers (ED) with marked nocturnal blood pressure (BP) decrease showed more advanced silent cerebrovascular disease than elderly hypertensive ED without marked nocturnal BP (*1*) and that a high morning BP surge was associated with increased stroke risk (*2*). The study of this circadian pattern has identified the hour of awakening, rather than the hour of the day, as being most closely related to the occurrence of vascular events (*3*, *4*). BP shows a diurnal variation, reaching its highest level during the morning and then declining to reach a trough value at about midnight. In the early morning, an abrupt and steep acceleration in BP occurs, coinciding with arousal and rising from overnight sleep (*5*).

It has been suggested that extreme dippers and morning BP surge hypertensives are at increased risk of cerebrovascular

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**Table 1. Baseline Characteristics and Blood Pressure Values of Studied Hypertensives**

| Parameter | Extreme dippers | Dippers | Non-dippers |
|---|---|---|---|
| Number | 11 | 6 | 14 |
| Age (years) | 62.8±11.6 | 63.0±6.6 | 66.4±9.2 |
| Gender (male/female) | 3/8 | 2/4 | 5/9 |
| Body mass index (kg/m$^2$) | 22.2±3.1 | 24.9±2.8 | 23.7±3.9 |
| Daytime SBP (mmHg) | 143.6±14.9 | 136.5±8.8 | 135.1±17.2 |
| Daytime DBP (mmHg) | 89.3±12.9 | 90.2±7.3 | 83.4±3.5* |
| Daytime MBP (mmHg) | 107.3±12.4 | 105.6±7.7 | 100.6±7.1 |
| Daytime HR (beats/min) | 74.0±9.0 | 76.5±10.8 | 68.5±7.4 |
| Nighttime SBP (mmHg) | 112.8±8.7 | 121.5±10.8 | 132.9±16.5 |
| Nighttime DBP (mmHg) | 74.2±8.2 | 78.5±5.0 | 74.3±9.0 |
| Nighttime MBP (mmHg) | 88.5±9.0 | 92.9±6.4 | 93.3±9.0 |
| Nighttime HR (beats/min) | 61.0±9.1 | 62.3±10.4 | 60.8±7.6 |
| 24-h SBP (mmHg) | 138.2±15.0 | 133.7±9.0 | 134.3±17.2 |
| 24-h DBP (mmHg) | 86.3±12.0 | 87.8±7.1 | 82.3±4.7 |
| 24-h MBP (mmHg) | 103.6±11.8 | 103.1±7.5 | 96.8±9.1 |
| 24-h HR (beats/min) | 71.7±8.6 | 73.5±10.6 | 66.8±7.0 |

SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; HR, heart rate. Values are means±SD. *$p<0.05$ *vs.* dippers.

**Table 2. Laboratory Findings of Studied Hypertensives**

| Risk factors | Extreme dippers | Dippers | Non-dippers |
|---|---|---|---|
| Biochemical analysis | | | |
| Glucose (mg/dl) | 98.8±10.0* | 84.8±4.2 | 94.0±7.3* |
| $HbA_{1c}$ (%) | 5.1±0.4 | 5.3±0.3 | 5.3±0.3 |
| Total cholesterol (mg/dl) | 183.3±28.0 | 186.2±26.7 | 199.2±34.9 |
| Triglycerides (mg/dl) | 116.7±34.7 | 102.6±61.0 | 110.9±46.9 |
| HDL cholesterol (mg/dl) | 53.0±6.2 | 53.6±14.3 | 57.5±14.3 |
| LDL cholesterol (mg/dl) | 107.0±26.5 | 112.1±22.0 | 119.5±33.3 |
| CRP (mg/dl) | 0.18±0.16 | 0.06±0.05 | 0.12±0.07 |
| Oxidative stress | | | |
| PMN (arbitrary units) | 238.7±6.5 | 214.0±54.2 | 226.8±11.2 |
| MNC (arbitrary units) | 105.2±10.1* | 77.7±6.9 | 83.0±14.6 |

$HbA_{1c}$, hemoglobin $A_{1c}$; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CRP, C-reactive protein; PMN, polymorphonuclear leukocyte; MNC, mononuclear cells. Values are means±SD. *$p<0.05$ *vs.* dippers.

events (*2*), and that oxidative stress is one of the causes of cerebrovascular stroke (*6*–*8*). Hypertension is known to increase oxidative stress in polymorphonuclear leukocytes (PMNs) and mononuclear cells (MNCs) (*9*, *10*). PMNs are one of the main types of inflammatory cells. Once activated, PMNs release reactive oxygen species (ROS), including hydrogen peroxide, contributing to endothelial damage and cardiovascular disease (*11*, *12*). MNCs are crucial cells in the genesis of atherosclerotic lesions. When ROS formation is increased, MNCs have been shown to induce adhesion to the endothelium, which results in vascular disease (*13*). It has not been clearly identified whether PMNs contribute to brain damage after stroke (*14*, *15*). The vascular endothelium is known to promote inflammation through the up-regulation of adhesion molecules such as intercellular adhesion molecule-1, E-selectin, and P-selectin (*16*). Therefore, early oxidative stress by leukocytes may well be implicated in cerebral damage.

The ED and morning BP surge hypertensives are at particularly high risk for vascular events, but it is not clear whether this risk is related to oxidative stress. In the present study, we have examined the relationship between nocturnal BP, morning BP surge and ROS formation by PMNs and MNCs, and also which component of diurnal BP rhythm was more

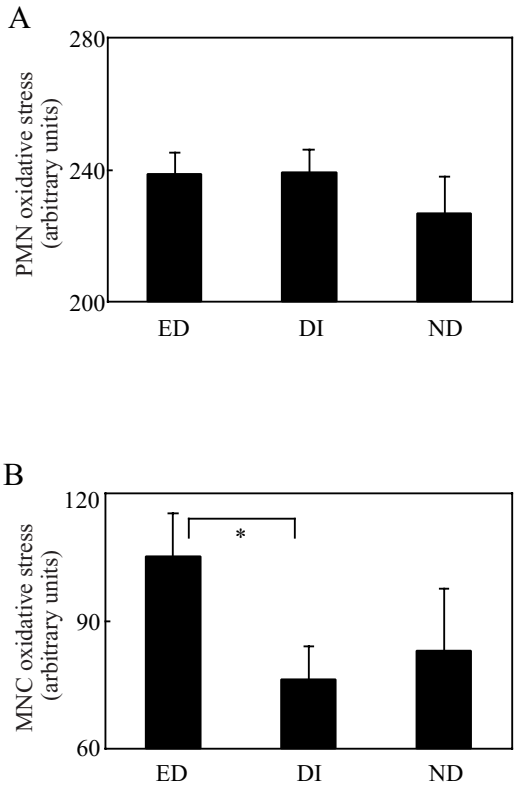


**Fig. 1.** *Fluorescence intensities of CDCFH-treated PMN (A) and MNC (B) obtained from fresh blood samples were analyzed using flow cytometry in non-dippers (ND), dippers (DI) and extreme dippers (ED). Values (arbitrary units) are the means ± SD.* *$p < 0.05$.

closely related to ROS formation by PMNs and MNCs.

## Methods

### Subjects

The study protocol was approved by the Osaka City University Institutional Review Board, and written informed consent was obtained from all subjects. The study was a prospective study of 31 hypertensives who visited Osaka City University Hospital. We selected hypertensives (BP >130/85 mmHg) with a dipper (DI), non-dipper (ND) or ED pattern who also had a morning BP surge-type or morning BP sustained-type pattern. we categorized the 31 hypertensives in these two ways balanced for gender, age, and body mass index. All subjects had either stopped their antihypertensive agents for 4 weeks, or had never been treated for hypertension. Except for hypertension, individuals enrolled in the study were determined to be healthy on the basis of their medical history, physical examinations, and results of routine laboratory tests.

### Ambulatory Blood Pressure Monitoring

Ambulatory BP monitoring was performed with a noninvasive recorder (ABPM630; Nippon Colin, Komaki, Japan) on a day of normal activity. Ambulatory BP readings were obtained at 30-min intervals from 6 AM to 10 PM, and at 60-min intervals from 10 PM to 6 AM. Average daytime (awake period), average nighttime (asleep period, defined as the period from falling asleep to awakening rather than time in bed), and average 24-h systolic and diastolic BP (SBP and DBP) were evaluated. Patients were arbitrarily defined as ND when their nighttime SBP and DBP pressure fall was <10%, as DI when their nighttime SBP and DBP fall was >10% and as ED when their nighttime SBP and DBP fall was >20%. We subclassified the patients according to the extent of the sleep-trough morning BP surge as follows: the top decile of sleep-trough morning BP surge (>30 mmHg, surge type) *vs.* all others (<30 mmHg, sustained type). Patients were asked to define the quality of their sleep and only those who reported a normal sleep or a sleep like that on the previous nights were included in the study. Ambulatory hypertension was defined as daytime SBP >135 mmHg or DBP >85 mmHg. All patients studied had recordings of good technical quality.

### Biochemical Analysis

Blood samples were drawn after a fasting period of 12 h. Venous blood was used for measurement of plasma glucose, plasma hemoglobin $A_{1c}$, plasma cholesterol, triglyceride, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, and plasma C-reactive protein (CRP). Serum CRP was measured by a highly sensitive microparticle enzyme immunoassay.

### Oxidative Stress

The generation of ROS by PMNs and MNCs was measured using a gated-flow cytometry technique as described by Bass *et al*. (*17*), with some modifications (*18*). Fresh blood (1 ml) from patients was preincubated for 15 min with 2′,7′-carboxy dichlorofluorescein diacetate bis-acetoxymethyl ester (CDCFH diacetate bis-AM ester; 100 μmol/l). CDCFH diacetate bis-AM ester is a nonpolar compound that is converted into a nonfluorescent polar derivative (CDCFH) by cellular esterases after incorporation into cells. CDCFH is membrane-impermeable and is rapidly oxidized to a highly fluorescent carboxydichlorofluorescein (CDCF) in the presence of intracellular ROS. The ROS production by PMNs and MNCs was measured as fluorescence intensity by gated flow cytometry (FACSCalibur; Becton Dickinson, Sunnyvale, USA). Through two-dimensional analysis and gating by flow cytometry, discrete populations representing lymphocytes, MNCs and PMNs could be identified.

**Table 3. Clinical and Laboratory Characteristics of Surge Type and Sustained Type Hypertensives**

| Risk factors | Surge type | Sustained type |
|---|---|---|
| Characteristics | | |
| Number | 13 | 18 |
| Age (years) | 63.7±12.1 | 63.1±9.6 |
| Gender (male/female) | 4/9 | 6/12 |
| Body mass index (kg/m$^2$) | 24.4±3.6 | 22.8±3.4 |
| 24-h MBP (mmHg) | 101.4±6.8 | 99.8±8.3 |
| Morning SBP − lowest SBP (mmHg) | 38.7±6.4* | 23.7±4.3 |
| Biochemical analysis | | |
| Glucose (mg/dl) | 94.7±9.3 | 93.3±9.2 |
| $HbA_{1c}$ (%) | 5.3±0.3 | 5.2±0.4 |
| Total cholesterol (mg/dl) | 188.2±23.4 | 194.1±37.6 |
| Triglycerides (mg/dl) | 119.8±40.5 | 104.5±50.0 |
| HDL cholesterol (mg/dl) | 51.3±5.9 | 58.5±15.1 |
| LDL cholesterol (mg/dl) | 113.0±20.4 | 114.8±35.5 |
| CRP (mg/dl) | 0.16±0.14 | 0.12±0.05 |
| Oxidative stress | | |
| PMN (arbitrary units) | 223.8±39.5 | 203.6±48.3 |
| MNC (arbitrary units) | 101.5±16.2* | 74.5±17.5 |

Values are means±SD. *$p<0.05$ *vs.* sustained type. Abbreviations are the same as in Table 2.

### Statistical Analysis

Data obtained in individual subjects were averaged separately for the groups. Between-group differences were analyzed using ANOVA for repeated measures. Differences between individual ROS formations were also assessed by post hoc analysis using a *t*-test with a Tukey-Kramer correction. All values are expressed as the means±SD. Values of $p<0.05$ were considered to indicate statistical significance.

## Results

### Extreme Dippers, Dippers and Non-Dippers

Subjects were divided into ED ($n=11$), DI ($n=6$) and ND ($n=14$) groups. Baseline characteristics and BP values of the groups are shown in Table 1. Age, gender distribution, and body mass index were similar in the three groups. For daytime parameters, the DBP of ND was significantly lower than that of DI. There were no significant differences in daytime SBP, mean BP and heart rate between ND and DI. Nighttime and 24-h parameters of ND were not significantly different from those of DI. All parameters of BP and heart rate in ED were similar to those of DI.

Laboratory findings are shown in Table 2. Although blood glucose was within the physiological range in all groups, blood glucose levels of the ED and ND groups were higher than those of the DI group. There was no difference in hemoglobin $A_{1c}$ levels among the three groups. Total cholesterol, triglyceride, HDL cholesterol, LDL cholesterol, and CRP did not differ among the three groups. The relationship among the groups and oxidative stress (PMNs, MNCs) is shown in Fig. 1. ROS formation by PMNs was similar in all groups (Fig. 1A). On the other hand, oxidative stress of MNCs in the ED group was significantly higher than that of the DI group, although there was no difference in MNC oxidative stress between the DI and ND groups (Fig. 1B).

### Surge-Type and Sustained-Type Hypertensives

Subjects were also divided into a surge-type ($n=13$) and a sustained-type ($n=18$) group. Clinical and laboratory characteristics of the surge- and sustained-type subjects are shown in Table 3. Although the characteristics and biochemical analysis were similar in both groups, oxidative stress in the surge-type subjects was significantly higher than that of the sustained-type subjects (Fig. 2).

### Involvement of Extreme Dippers and/or Surge-Type Hypertensives

To determine whether ED or surge-type hypertensives are more prone to enhanced oxidative stress by leukocytes, we analyzed ROS formation by PMNs and MNCs by comparing three groups of hypertensives: non-ED and non-surge type (1 risk factor), ED or surge-type (2 risk factors), and ED and surge-type (3 risk factors) (Fig. 3). Although ROS formation by PMNs did not differ among the groups, ROS formation by MNCs of the 2-risk-factor group was higher than that of the 1-risk-factor group.

Furthermore, ROS formation by MNCs was significantly greater in patients in the 3-risk-factor group compared with the 1- or 2-risk-factor groups.

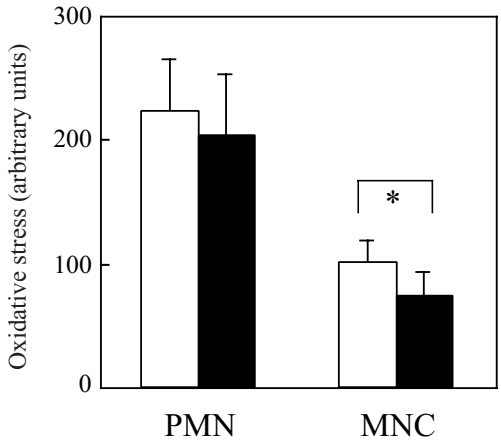


**Fig. 2.** *Fluorescence intensities of CDCFH-treated PMN and MNC obtained from fresh blood samples were analyzed using flow cytometry in surge type (white columns) and sustained type (black columns) morning BP hypertensives. Values (arbitrary units) are the means±SD.* *p < *0.05.*

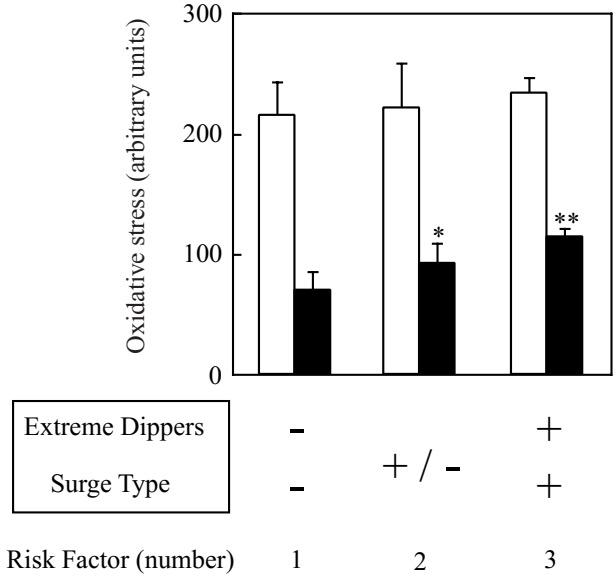


**Fig. 3.** *Fluorescence intensities of CDCFH-treated PMN (white columns) and MNC (black columns) obtained from fresh blood samples were analyzed using flow cytometry in extreme dippers and/or surge-type hypertensives. Values (arbitrary units) are the means±SD.* *p < *0.05* vs. *the 1-risk-factor group;* **p < *0.05* vs. *the 1- or 2-risk-factor group.*

## Discussion

This study showed that ROS formation by MNCs, but not by PMNs, was significantly increased in ED and surge-type morning BP hypertensives. Both ED and surge-type morning BP hypertensives are known to be at increased risk for vascular events (*1*, *2*). PMNs have not been clearly identified as contributing to brain damage (*14*, *15*), but MNCs have been found in damaged brain tissues (*19*), which is consistent with our previous findings that ROS formation by PMNs and MNCs are differently regulated; ROS formation by PMNs is related with BP (*20*) and hemoglobin $A_{1C}$, but not with blood glucose or CRP (*9*). ROS formation by MNCs is related to CRP (*9*). Oxidative stress is known to relate to stroke (*21*). Our findings may explain the mechanism of the increased risk of vascular events associated with these BP characteristics, through an increase in ROS formation by MNCs.

Although ROS generation has been reported to be enhanced in circulating PMNs in patients with essential hypertension (*9*, *22*, *23*), it is not known which patterns in diurnal BP rhythm are related to ROS formation by leukocytes (PMNs, MNCs). Using a flow cytometric method, we examined which type of oxidative stress by leukocytes was increased in the ED, DI and ND groups. The differences among these groups were more evident for ROS formation by MNCs than for that by PMNs (Fig. 1). In the present study, ROS formation by MNCs in ED was significantly higher than that in DI. We postulated that dynamic change in nocturnal BP, a known risk factor for vascular disease, may cause ROS formation by MNCs in hypertensive patients in the ED group, and therefore that ROS formation by MNCs might be an indicator for vascular disease. However, baseline BP level itself seems to play a less important role than change in nocturnal BP in ROS formation by MNCs, since there is no significant relationship between baseline BP and ROS formation by MNCs (*9*).

There is no consensus definition of the morning BP surge. We defined it as the sleep-trough morning BP surge (morning SBP minus lowest SBP during the night). Kario *et al*. (*2*) reported that sleep-trough morning BP surge gave a more clinically relevant definition of the morning BP surge. In our study, hypertensive patients were divided into two groups: a group of surge-type and a group of sustained-type subjects, and were matched for characteristics and biochemical analysis. ROS formation by PMNs in the surge-type subjects was similar to that in the sustained-type subjects, but ROS formation by MNCs was significantly higher in the surge-type than in the sustained-type subjects. In this model, being classified as a surge-type subject was significantly associated with enhanced ROS formation by MNCs. Both ischemic and hemorrhagic strokes showed a greater tendency to cluster in the morning period (6 AM to noon) in the morning BP surge-type than in the morning BP sustained-type subjects. It is thought that an excessive morning BP surge may trigger strokes through some hemodynamic mechanism, such as increased shear stress on the atherosclerotic cerebral vessels, but there are several other factors that change during the morning hours. These include an increase of sympathetic nervous activity (*24*, *25*), and other related acute risk factors such as platelet hyperactivity, hypercoagulability and hypofibrinoly-

sis, blood viscosity, and increased vascular spasm (*26–28*). This potentiation of acute risk factors might also be greater in the surge-type than in the sustained-type subjects, and may contribute to triggering morning strokes. MNC oxidative stress might underlie the risk of the morning strokes.

It has been reported that ED and morning BP surge-type overlapped significantly (*2*). This overlap may explain why the ED group not only had frequent sleep-onset ischemic strokes, but also had more strokes in the morning, which would be predominantly associated with an excessive morning BP surge. The present study showed that patients who are both extreme dippers and morning BP surge-types show a significantly greater increase in ROS formation by MNCs than those with only one of these risk factors. These findings suggest that increased ROS formation by MNCs might have played a role in the strokes.

It is important to note that, because we measured ROS formation by PMNs and MNCs only by FACS, and because the study was performed only in hypertensives, rather than normotensives, there are limits to the extensibility of the results.

In conclusion, using a flow cytometric method, we found significantly enhanced MNC oxidative stress in extreme dippers or morning BP surge-type patients. MNC oxidative stress may increase further with multiple risk factors. We have identified a possible cellular mechanism, oxidative stress, to explain why strokes tend to occur in ED and morning BP surge-type patients.

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