# Release of Proinflammatory and Prothrombotic Mediators in the Brain and Peripheral Circulation in Spontaneously Hypertensive and Normotensive Wistar-Kyoto Rats

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*Background and Purpose:* We reported previously that stroke risk factors prepared the brain stem for the development of ischemia and hemorrhage and induced the production of tumor necrosis factor following an intrathecal injection of lipopolysaccharide, a prototypic monocyte-activating stimulus. This study evaluates whether blood or brain cells of hypertensive rats produce more proinflammatory and prothrombotic mediators than do blood or brain cells of normotensive rats.

*Methods:* Levels of tumor necrosis factor, platelet-activating factor, 6-ketoprostaglandin $F_{1\alpha}$, and thromboxane $B_2$ in the cerebrospinal fluid and blood of spontaneously hypertensive and normotensive Wistar-Kyoto rats were monitored before and after a challenge with lipopolysaccharide.

*Results:* Little or no activity from these mediators was found in the cerebrospinal fluid or blood of saline-injected control animals. Intravenous administration of lipopolysaccharide (0.001, 0.1, and 1.8 mg/kg) produced dose-dependent increases in blood levels of all mediators in hypertensive rats. In normotensive rats the levels were less than in hypertensive rats and were not clearly dose-related. When lipopolysaccharide was injected intracerebroventricularly, more tumor necrosis factor was measured in the cerebrospinal fluid than in the blood, suggesting local synthesis of this cytokine. Levels of tumor necrosis factor and platelet-activating factor in the cerebrospinal fluid were higher in hypertensive than in normotensive rats. The thromboxane $A_2$/prostacyclin ratio was not altered significantly between the two rat strains.

*Conclusions:* It is suggested that the higher incidence of brain stem ischemia and hemorrhage after the intrathecal injection of lipopolysaccharide in hypertensive rats than in normotensive rats might be related to the higher levels of the two cytotoxic factors tumor necrosis factor and platelet-activating factor produced in response to such challenge. (*Stroke* 1992;23:1643–1651)

KEY WORDS • platelet-activating factor • prostacyclins • tumor necrosis factor • rats

Risk factors for stroke increase the adhesion and emigration of mononuclear cells.[1] The increased mononuclear cell adhesion is regarded by some as the initial step in the atherosclerotic process and an important contributing factor for the initiation of vascular injury in hypertension.[1,2] In a previous study, we described that several established risk factors for stroke (hypertension, old age, diabetes, genetic stroke-proneness) prepared brain stem tissue for a modified Shwartzman reaction, including the development of ischemia and hemorrhage, when challenged with a prototypic activator of monocytes, lipo-

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polysaccharide, administered as a provocative dose intracisternally or intravenously.[3] Recent studies from this laboratory further suggested that the increased frequency of brain stem thrombosis and hemorrhage in rats with stroke risk factors after exposure to lipopolysaccharide[3] could have resulted from an exaggerated production of proinflammatory and prothrombotic mediators because hypertensive rats produced more tumor necrosis factor (TNF-$\alpha$) in the blood than normotensive rats when challenged with a provocative intravenous dose of lipopolysaccharide.[4] Moreover, administration of lipopolysaccharide into the cerebral ventricles of normotensive rats stimulated local generation of TNF-$\alpha$ into the cerebrospinal fluid.[4] The aim of the present study was to evaluate whether both blood and cells within the central nervous system of hypertensive rats

produce more TNF-$\alpha$, eicosanoids, and platelet-activating factor (PAF) than cells of normotensive rats. We chose TNF-$\alpha$ to represent the cytokine class of mediators since it activates the endothelium for leukocyte adherence and procoagulant processes.[5] Thromboxane B$_2$ (TXB$_2$), a mediator of leukocyte and platelet activation (measured relative to the stable metabolite of prostacyclin [PGI$_2$]), and PAF, a mediator of hemostasis and inflammation,[6] were chosen to reflect the level of activation of the hemostatic system and the potential risk of thrombosis. These variables were measured in serum and cerebrospinal fluid taken at several times over a dose range of lipopolysaccharide administered both intravenously and into the cerebral ventricles.

## Materials and Methods

Sixteen-week-old male spontaneously hypertensive rats *(SHR)* and age-matched male normotensive Wistar-Kyoto rats (WKY) were purchased from Taconic Farms, Inc., Germantown, N.Y., and housed at 22°C with a 12 hour/12 hour light/dark cycle with food and tap water provided ad libitum. The experiments reported herein were conducted according to the principles set forth in the "Guide for Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council (DHEW publication No. NIH 85-23, 1985).

### Surgical Procedures

The rats were anesthetized with 4% halothane in 100% oxygen. For intravenous injections, a sterile PE-50 catheter was inserted into the femoral vein under aseptic conditions. Lipopolysaccharide from *Escherichia coli* serotype 0111:B4 (phenol extracted; Sigma Chemical Co., St. Louis, Mo.) dissolved in sterile, pyrogen-free 0.9% (wt/vol) NaCl was injected intravenously through the femoral vein catheter in animals receiving systemic injections of lipopolysaccharide. Following the injection, the catheter was withdrawn and the femoral vein ligated. For injections into the cerebral ventricles, the rat was placed in a stereotaxic device (David Kopf Instruments, Tujunga, Calif.). A midline cut was then made on the scalp to expose the parietooccipital bone area, and the muscles attached to the occiput were gently separated to expose the atlantooccipital membrane. A stainless steel guide cannula was placed on the parietal skull (coordinates from bregma: anteroposterior, 0.8 mm; lateral, 1.2 mm) and fixed with glue. Lipopolysaccharide dissolved in sterile, pyrogen-free 0.9% NaCl was injected in a total volume of 10 $\mu$l by means of a premeasured 30-gauge cannula (7.5 mm) over a period of 5 minutes. Two additional minutes were allowed for dispersal of the injected fluid before withdrawal of the cannula. Control animals received 10 $\mu$l of sterile, pyrogen-free 0.9% NaCl through the intracerebroventricular needle.

In all experiments, at a designated single time (15, 120, or 240 minutes), the rats were anesthetized with halothane and mounted in a stereotaxic device. A sterile 30-gauge needle attached to a sterile syringe was inserted into the intracisternal space through the atlantooccipital membrane. Clear cerebrospinal fluid in a volume of 150 $\mu$l was withdrawn and frozen immediately in a sterile Eppendorf vial on dry ice. Thereafter, a laparotomy was performed and a sterile 23-gauge but-

terfly needle was inserted into the abdominal aorta. Blood was withdrawn through sterile syringes to allow simultaneous measurement of all the mediators in each animal. Blood samples were collected in separate syringes as follows: 1) 4 ml blood was withdrawn into a cooled syringe containing 12.9 $\mu$M buffered sodium citrate, the blood was centrifuged, and the plasma was used for measurement of PAF; 2) 2.5 ml blood was withdrawn into a syringe containing 350 $\mu$M indomethacin and 100 units heparin for assay of TXB$_2$ and 6-ketoprostaglandin F$_{1\alpha}$ (6-keto-PGF$_{1\alpha}$); and 3) 2 ml blood was withdrawn into a sterile plastic tube and allowed to clot for assay of TNF-$\alpha$.

### Tumor Necrosis Factor Assay

The serum or cerebrospinal fluid samples were stored at $-20$°C for 1–2 weeks until assayed using the L929 cell lysis bioassay as described elsewhere.[7] In brief, L929 cells obtained from Dr. Stefanie Vogel, Department of Microbiology, Uniformed Services University of the Health Sciences, were grown in supplemented Eagle's minimum essential medium with 10% fetal bovine serum in 150-ml tissue culture flasks; $8 \times 10^5$ cells were seeded into 50 ml medium. L-cells were plated at $4 \times 10^5$ cells/ml in a 100-$\mu$l well and incubated overnight. Prior to the experiment, the medium was aspirated and 50 $\mu$l Eagle's minimum essential medium with 10% fetal bovine albumin was added to the well. Test samples (50 $\mu$l serum or cerebrospinal fluid) underwent twofold serial dilutions by sequential transfer of 50 $\mu$l across the wells. In addition, 50 $\mu$l actinomycin D/well was added, to a final concentration of 0.4 $\mu$g/well. Incubation after 18 hours at 37°C was terminated by aspiration of the supernatants and washing with 200 $\mu$l normal saline/ well. After aspiration of the saline, the cells were stained by 50 $\mu$l 0.05% crystal violet in 20% ethanol for 10 minutes. The wells were scored visually, and TNF-$\alpha$ units were calculated as the reciprocal of the highest dilution giving 50% lysis of a well. To convert to units per milliliter, this value was multiplied by 20.

### Platelet Activating Factor Assays

*Plasma samples.* The PAF assay used for plasma samples has been described in detail by Yue and coworkers.[8] In brief, lipids were extracted from the sample by the method of Bligh and Dyer[9] in the presence of 2% acetic acid in methanol. The extracted samples were sent to Dr. Tian-Li Yue, SmithKline Beecham Laboratories, for PAF bioassay. The extracted samples were spotted onto thin-layer chromatography plates (LK50, Whatman Inc., Clifton, N.J.). The solvent system used was chloroform/methanol/water (65/35/6, vol/vol/vol). The PAF and lyso-PAF standards were spotted onto the outer lanes of the thin-layer chromatography plates between the experimental samples for use as references for later identification. The plates were sprayed with 0.2% dichlorofluorescein in 50% ethanol to identify the resolved lipids. The lipid area on the plates with a retention factor value corresponding to that of the PAF standard was scraped off and extracted. After removal of the organic solvent by evaporation with N$_2$, the purified samples were dissolved in 0.25% bovine serum albumin in saline. The PAF activity was assessed by platelet [$^3$H]serotonin release as described previously.[8] The amount of PAF was calculated from a calibra-
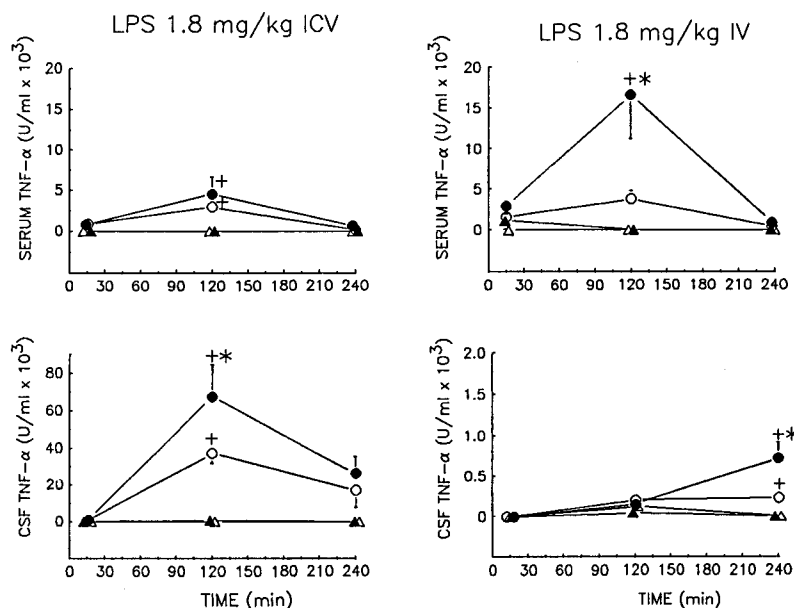
FIGURE 1. *Graphs of time courses of serum (upper row) and cerebrospinal fluid (CSF, lower row) levels of tumor necrosis factor (TNF-α) after intracerebroventricular (ICV, left column) or intravenous (IV, right column) injection of lipopolysaccharide (LPS) at 1.8 mg/kg in spontaneously hypertensive and normotensive Wistar-Kyoto rats. ●, Hypertensive rats injected with LPS; ▲, hypertensive rats injected with sterile saline; ○, normotensive rats injected with LPS; △, normotensive rats injected with sterile saline. Values indicate mean±SEM for 5–8 rats at 15 minutes, 5–12 rats at 120 minutes, and 4–6 rats at 240 minutes. Two-way analysis of variance revealed significant time×treatment interaction for effect of ICV LPS on CSF TNF-α concentration (F=8.51002, p=0.00085) and serum TNF-α concentration (F=4.56473, p=0.01358). Time×treatment interaction for effect of IV LPS on serum TNF-α concentration was significant at F=3.22826 (p=0.04455) and on CSF TNF-α concentration at F=11.81567 (p=0.00015). [+]p<0.05 ○ versus △ and ● versus ▲, by Student-Newman-Keuls test; [*]p<0.05 ● versus ○.*

tion curve of standard PAF constructed for each test. The specificity of [³H]serotonin release was confirmed by the inhibitory effect of the specific PAF antagonist BN50739.[8]

*Cerebrospinal fluid samples.* A 100-μl aliquot of cerebrospinal fluid was used for the PAF radioimmunoassay using a commercial PAF radioimmunoassay kit (NEK-062, New England Nuclear–Du Pont, Boston, Mass.). The sensitivity of the assay was 50 pg.

### Eicosanoid Assays

The $TXB_2$ levels were assayed by radioimmunoassay (New England Nuclear–Du Pont). The stable $PGI_2$ metabolite 6-keto-$PGF_{1α}$ was assayed directly by radioimmunoassay using specific antibodies purchased from L. Levine, Brandeis University, Waltham, Mass.

### White Blood Cell Counts

In a parallel study, 30 rats were anesthetized with 4% halothane in 100% oxygen. When surgical anesthesia was reached (usually within 3–5 minutes), a laparotomy was performed and a sterile 23-gauge butterfly needle was inserted into the abdominal aorta. A blood sample (1 ml) was then withdrawn into a heparinized sterile syringe. The blood samples were collected in the morning before 10 AM. The total leukocyte count of the freshly drawn blood was measured with an automatic cell counter (System 9000, Baker Instruments). The differential leukocyte counts for neutrophils, monocytes, and lymphocytes were made from a blood smear stained with Giemsa-Wright stain.

### Statistical Analysis

Data in the text and figures are mean±SEM for the given number of rats. The dose–response and time–response relations were analyzed by two-way analysis of

variance (ANOVA) using the css/pc statistical package for microcomputers (Complete Statistical System, Tulsa, Oklahoma, StatSoft 1987). When ANOVA revealed significant differences in the test parameters, the Student-Newman-Keuls test was used to analyze the differences between treatment groups.

## Results

Little or no TNF-α activity was found in the cerebrospinal fluid or serum of control rats. Time course studies at the 1.8 mg/kg intravenous dose revealed a significant increase in the serum TNF-α level 120 minutes after lipopolysaccharide administration in SHR (Figure 1); at 240 minutes the serum TNF-α level had returned to the control value (Figure 1). Therefore, the 120-minute time point was chosen for the dose–response studies. Intravenous administration of lipopolysaccharide at doses of 0.1 and 1.8 mg/kg increased the serum TNF-α levels in a dose-dependent manner in SHR, whereas in WKY the TNF-α levels were significantly less and not clearly dose-related (Table 1). The cerebrospinal fluid levels of TNF-α were not affected by intravenous administration of lipopolysaccharide at 120 minutes but were increased at 240 minutes (Figure 1). Again, the increase in the TNF-α concentration was greater in SHR than in WKY (Figure 1).

When lipopolysaccharide was injected intracerebroventricularly, TNF-α activity in the cerebrospinal fluid was increased after the 1.8 mg/kg dose (Figure 1, Table 1). The maximum level of TNF-α in the cerebrospinal fluid was achieved 120 minutes after intracerebroventricular administration of lipopolysaccharide, and the TNF-α level was still elevated at 240 minutes (Figure 1). After intracerebroventricular administration of lipopolysaccharide, in SHR TNF-α levels in the cerebrospinal fluid were much higher than in the serum

TABLE 1.  **Dose–Response Effect of LPS on TNF-α Levels at 120 Minutes in Serum and CSF in SHR and Normotensive WKY**

| Treatment | Serum TNF-α (units/ml×10³) | | | | CSF TNF-α (units/ml×10³) | | | |
|---|---|---|---|---|---|---|---|---|
| | SHR | | WKY | | SHR | | WKY | |
| | Mean±SEM | n | Mean±SEM | n | Mean±SEM | n | Mean±SEM | n |
| Intracerebroventricular administration | | | | | | | | |
| Saline | 0±0 | 18 | 0±0 | 18 | 0±0 | 16 | 0±0 | 15 |
| LPS (mg/kg) | | | | | | | | |
| 0.001 | 1.20±0.63 | 8 | 1.28±0.61 | 8 | 3.12±1.56 | 8 | 1.71±0.39 | 6 |
| 0.1 | 0.75±0.38 | 6 | 1.12±0.8 | 6 | 7.15±4.13 | 6 | 5.18±2.26 | 5 |
| 1.8 | 4.55±2.06 | 9 | 2.94±0.63 | 10 | 67.58±16.75* | 10 | 37.24±5.39 | 11 |
| Intravenous administration | | | | | | | | |
| Saline | 0.65±0.60 | 16 | 0±0 | 13 | 0.01±0.01 | 19 | 0.07±0.04 | 19 |
| LPS (mg/kg) | | | | | | | | |
| 0.001 | 0.11±0.04 | 13 | 0.02±0.02 | 14 | 0.16±0.04 | 7 | 0.11±0.03 | 8 |
| 0.1 | 8.16±2.86 | 8 | 5.63±3.73 | 5 | 0.42±0.07 | 8 | 0.26±0.04 | 5 |
| 1.8 | 15.15±4.58* | 12 | 3.35±0.87 | 11 | 0.15±0.06 | 11 | 0.19±0.05 | 11 |

LPS, lipopolysaccharide; TNF-α, tumor necrosis factor; CSF, cerebrospinal fluid; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.
*$p<0.05$ different from WKY by Student-Newman-Keuls test.

(67,584±16,748 units/ml compared with 4,551±2,056 units/ml), implying that the TNF-α in the cerebrospinal fluid was produced locally in the brain. The increase in cerebrospinal fluid TNF-α activity in response to the 1.8 mg/kg dose of lipopolysaccharide was significantly greater in SHR than in WKY (Figure 1, Table 1).

Intravenously administered lipopolysaccharide (0.001, 0.1, and 1.8 mg/kg) stimulated PAF release into the plasma in SHR in a dose-related manner (Table 2). Again, the maximum increase in PAF activity was reached 120 minutes after lipopolysaccharide injection while no significant increase was observed at 15 minutes or 240 minutes (Figure 2). In WKY, the increase in plasma PAF activity in response to intravenously administered lipopolysaccharide was significantly less than in SHR. In fact, plasma PAF activity was significantly increased compared with the control rats only after the 1.8 mg/kg dose of lipopolysaccharide (Figure 2, Table

2). The cerebrospinal fluid level of PAF was elevated compared with the control value 15 minutes and 120 minutes after the 1.8 mg/kg dose of lipopolysaccharide in SHR and after 120 minutes in WKY (Figure 2).

When lipopolysaccharide was injected into the cerebral ventricles, cerebrospinal fluid PAF levels were increased, with the maximum reached 15 minutes after lipopolysaccharide administration (Figure 2). At 120 minutes after the 1.8 mg/kg dose, cerebrospinal fluid levels of PAF were significantly higher in SHR than in WKY (Figure 2, Table 2). In SHR, plasma PAF was significantly increased 120 minutes after the 1.8 mg/kg dose of lipopolysaccharide, whereas in WKY plasma PAF levels were not altered (Figure 2, Table 2).

The PGI₂ and thromboxane A₂ (TXA₂) levels were measured in the plasma of SHR and WKY mentioned above. At 120 minutes after a challenge with intravenous lipopolysaccharide at doses of 0.1 and 1.8 mg/kg,

TABLE 2.  **Dose–Response Effect of LPS on PAF Levels at 120 Minutes in Plasma and CSF in SHR and Normotensive WKY**

| Treatment | Plasma PAF (ng/ml) | | | | CSF PAF (ng/ml) | | | |
|---|---|---|---|---|---|---|---|---|
| | SHR | | WKY | | SHR | | WKY | |
| | Mean±SEM | n | Mean±SEM | n | Mean±SEM | n | Mean±SEM | n |
| Intracerebroventricular administration | | | | | | | | |
| Saline | 0.27±0.01 | 16 | 0.25±0.02 | 13 | 2.60±0.38 | 15 | 1.96±0.38 | 9 |
| LPS (mg/kg) | | | | | | | | |
| 0.001 | 0.54±0.13 | 7 | 0.28±0.01 | 7 | ND | ... | ND | ... |
| 0.1 | 0.36±0.03 | 4 | 0.27±0.02 | 4 | 6.08±1.58 | 5 | 5.48±2.10 | 4 |
| 1.8 | 8.49±3.68* | 10 | 0.91±0.22 | 10 | 5.58±0.54* | 4 | 3.40±0.91 | 4 |
| Intravenous administration | | | | | | | | |
| Saline | 0.21±0.03 | 19 | 0.27±0.05 | 17 | 2.02±0.34 | 6 | 2.76±0.29 | 11 |
| LPS (mg/kg) | | | | | | | | |
| 0.001 | 0.41±0.07* | 5 | 0.24±0.02 | 7 | ND | ... | ND | ... |
| 0.1 | 0.48±0.03* | 7 | 0.36±0.03 | 6 | 1.70±0.23 | 4 | 2.13±0.12 | 7 |
| 1.8 | 0.75±0.05* | 12 | 0.55±0.05 | 16 | 4.36±0.75 | 6 | 4.65±0.83 | 8 |

LPS, lipopolysaccharide; PAF, platelet-activating factor; CSF, cerebrospinal fluid; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; ND, not determined.
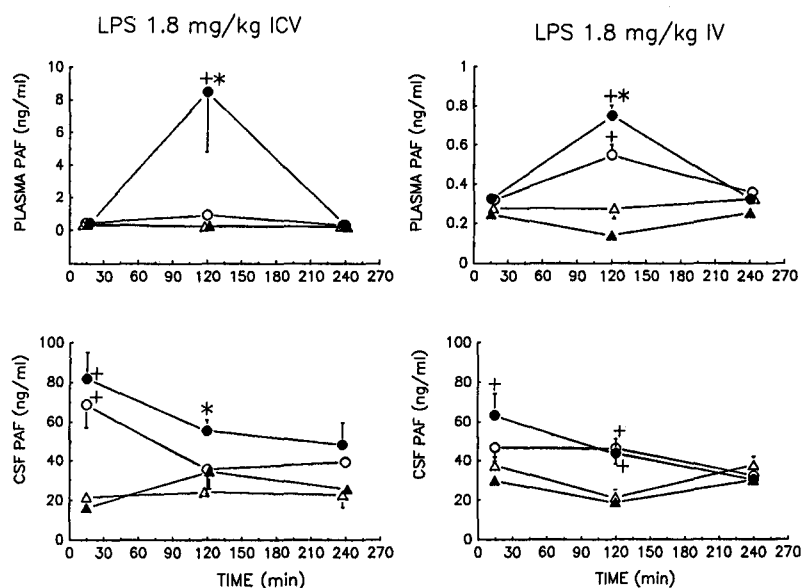*$p<0.05$ different from WKY by Student-Newman-Keuls test.

FIGURE 2. *Graphs of time courses of plasma (upper row) and cerebrospinal fluid (CSF, lower row) levels of platelet-activating factor (PAF) after intracerebroventricular (ICV, left column) or intravenous (IV, right column) injection of lipopolysaccharide (LPS) at 1.8 mg/kg in spontaneously hypertensive and normotensive Wistar-Kyoto rats.* ●, *Hypertensive rats injected with LPS;* ▲, *hypertensive rats injected with sterile saline;* ○, *normotensive rats injected with LPS;* △, *normotensive rats injected with sterile saline. Values indicate mean±SEM for 5–15 rats at 15 minutes (except four rats for IV saline), 10–12 rats for plasma PAF and 4–8 rats for CSF PAF at 120 minutes, and 3–5 rats at 240 minutes. Analysis of variance revealed significant treatment effect for ICV LPS on CSF PAF concentration (F=8.21636, p=0.00598); time×treatment interaction was not significant (F=1.97685, p=0.14629). Significant time× treatment interaction was also found for effect of IV LPS on plasma PAF concentration (F=20.16105, p=0.00001) and CSF PAF concentration (F=4.92271, p=0.01122).* +*p<0.05* ○ *versus* △ *and* ● *versus* ▲, *by Student-Newman-Keuls test;* *p<0.05* ● *versus* ○.

the amounts of 6-keto-PGF$_{1\alpha}$ and TXB$_2$ were significantly increased in the plasma of both SHR and WKY (Table 3). After intracerebroventricular challenge, levels of 6-keto-PGF$_{1\alpha}$ in the plasma of SHR were increased in a dose-dependent manner whereas in WKY no significant changes were found (Table 3). The plasma level of TXB$_2$ in SHR was elevated significantly after the 1.8 mg/kg dose of intracerebroventricularly administered lipopolysaccharide. In WKY, intracerebroventricular lipopolysaccharide had no significant effect on plasma TXB$_2$. The level of 6-keto-PGF$_{1\alpha}$ in plasma was significantly higher in SHR than in WKY

after the 0.1 mg/kg and 1.8 mg/kg doses of lipopolysaccharide, while the difference in TXB$_2$ level was significantly higher in SHR only after the 1.8 mg/kg dose (Table 3). However, at no time or dose was the TXA$_2$/PGI$_2$ ratio altered.

In parallel studies, the white blood cell counts in SHR and WKY ranged from $3.3\times10^3$ to $7.4\times10^3$ cells/μl. The percentages of neutrophils, lymphocytes, and monocytes in blood from 18 WKY were 19±3%, 78±3%, and 3±1%, respectively. The corresponding values in 20 SHR were 28±3%, 65±3%, and 7±1%. The total white blood cell counts and neutrophil counts were not differ-

TABLE 3. **Dose–Response Effect of LPS on Plasma Eicosanoid Levels at 120 Minutes in SHR and Normotensive WKY**

| | 6-Ketoprostaglandin F$_{1\alpha}$ (pg/ml) | | | | Thromboxane B$_2$ (pg/ml) | | | |
| | SHR | | WKY | | SHR | | WKY | |
| Treatment | Mean±SEM | n | Mean±SEM | n | Mean±SEM | n | Mean±SEM | n |
|---|---|---|---|---|---|---|---|---|
| Intracerebroventricular administration | | | | | | | | |
| Saline | 28±8 | 12 | 15±1 | 12 | 20±3 | 12 | 22±5 | 9 |
| LPS (mg/kg) | | | | | | | | |
| 0.001 | 65±24 | 8 | 118±45 | 5 | 10±3 | 7 | 18±7 | 5 |
| 0.1 | 89±21* | 4 | 10±9 | 4 | 5±1 | 4 | 4±1 | 4 |
| 1.8 | 136±55*† | 12 | 69±9 | 14 | 118±35*† | 10 | 38±5 | 10 |
| Intravenous administration | | | | | | | | |
| Saline | 16±2 | 12 | 16±1 | 12 | 16±3 | 12 | 17±4 | 12 |
| LPS (mg/kg) | | | | | | | | |
| 0.001 | 17±6 | 6 | 13±2 | 7 | 5±3 | 6 | 5±1 | 6 |
| 0.1 | 134±65*† | 8 | 34±11 | 4 | 88±22† | 8 | 82±33† | 4 |
| 1.8 | 143±35† | 12 | 117±27† | 10 | 37±6 | 12 | 28±8 | 15 |

LPS, lipopolysaccharide; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.
*p<0.05 different from WKY by Student-Newman-Keuls test.
†p<0.05 different from saline by Student-Newman-Keuls test.

ent in SHR and WKY. However, the blood of SHR contained significantly fewer lymphocytes ($p < 0.05$, Student's two-tailed test for independent samples) and significantly more monocytes ($p < 0.001$, Student's two-tailed $t$ test) than the blood of WKY.

## Discussion

The present study demonstrates that hypertensive rats produced more TNF-$\alpha$ in circulating blood and cerebrospinal fluid in response to provocative doses of lipopolysaccharide than normotensive rats. The excessive production of TNF-$\alpha$ in hypertensive rats could prepare vessels for ischemic and inflammatory tissue damage via increased adhesion of monocytes and neutrophils on vascular endothelium and could transform the endothelial surface from an actively anticoagulant to a procoagulant state.[5] The specific endothelial changes under the influence of TNF-$\alpha$ include synthesis and surface expression of tissue factor,[10] synthesis and expression of PAF,[11] release of interleukin-1 (IL-1),[12] release of factor VIII/von Willebrand factor,[12] and enhanced adhesion of monocytes and granulocytes[5] via expression of adhesion receptor molecules.[5] In addition, anticoagulant mechanisms such as the thrombomodulin–protein C–protein S system are inhibited.[10] The local procoagulant process could become deviation-amplifying as more monocytes and other cells are recruited[13,14] and the endothelium both generates and responds to IL-1 and thrombin.[5] The overall effect of these interactions is to render the endothelial cell surface procoagulant, increase the adhesion of platelets and leukocytes, and increase local permeability. These disturbances could predispose to local vessel occlusion and circulatory impairment.

A previous study from this laboratory suggested local production of TNF-$\alpha$ by brain tissue of normotensive Sprague-Dawley rats since TNF-$\alpha$ activity in the cerebrospinal fluid was higher than in the blood after intracerebroventricular administration of lipopolysaccharide.[4] In the present study the previous findings were confirmed in SHR. Moreover, hypertensive rats produced more TNF-$\alpha$ in the cerebrospinal fluid in response to lipopolysaccharide than normotensive rats. Perivascular macrophages derived from bone marrow could account for the production of TNF-$\alpha$ in the brain since monocytes/macrophages are the major source for TNF-$\alpha$.[5] However, several other cell types in the central nervous system are capable of TNF-$\alpha$ expression; the ameboid microglia share many features with macrophages, including production of IL-1 and TNF-$\alpha$.[15-17] Astrocytes may also be capable of transforming into macrophage-like cells[18] and have been shown to release cytokines in vitro.[16,17] The TNF-$\alpha$ released into the cerebrospinal fluid could account for the ischemic and hemorrhagic lesions found in the brain stems of hypertensive rats following a provocative dose of lipopolysaccharide.[3] This assumption is supported by the recent study that demonstrated hemorrhagic necrosis of the neuropil after administration of recombinant TNF-$\alpha$.[19]

The release of hemostatic and proinflammatory mediators was exaggerated in rats with the stroke risk factor hypertension as evidenced by the increased formation of PAF in the circulating blood and cerebrospinal fluid after systemic and central nervous system administration of provocative doses of lipopolysaccharide in SHR compared with normotensive WKY. Direct evidence for PAF release in stroke has been recently provided by studies demonstrating increased levels of PAF in blood from patients with ischemic stroke.[20] PAF is a lipid mediator that induces platelet aggregation, increases vascular permeability, and acts as an endothelial adhesion receptor for white blood cells.[6] Mononuclear cells may act as the primary source for PAF in response to lipopolysaccharide,[21] but PAF can be produced by many other cell types including endothelial cells, neutrophils, glial cells, and neurons.[22-24] Interestingly, TNF-$\alpha$ can induce PAF release from endothelial cell cultures.[11] This may bear relevance to our results as the maximum levels of PAF in circulating blood were found 120 minutes after lipopolysaccharide administration, coinciding with the peak levels of circulating TNF-$\alpha$.

Plasma levels of PGI$_2$ and TXA$_2$ were significantly increased in both hypertensive and normotensive rats after the systemic injection of lipopolysaccharide. After intracerebroventricular challenge, levels of both PGI$_2$ and TXA$_2$ were significantly higher in hypertensive rats' plasma than in that of normotensive rats. The elevation in eicosanoid release peaked 120 minutes after the challenge and might thus be related to the increased levels of TNF-$\alpha$ or PAF mentioned above. At no time or dose was the TXA$_2$/PGI$_2$ ratio altered, suggesting that the elevation of these eicosanoids remained balanced and did not contribute to an increased hemostatic potential. The arterial TXA$_2$/PGI$_2$ ratio may not reflect the changes in these eicosanoids at the tissue level, however.

Blood pressure was not monitored in this study since we wanted to avoid any possibility of activation of blood components by surgical implantation of catheters. The distinct mechanisms of TNF-$\alpha$ and PAF release were not in the scope of this study and can, in our opinion, be dissected out only at a more defined cellular level using isolated cell preparations rather than an in vivo preparation. In fact, increased TNF-$\alpha$ release in tissues from hypertensive rats has been confirmed in our preliminary studies in which TNF-$\alpha$ release has been stimulated by in vitro incubation of carotid artery rings from hypertensive and normotensive rats with lipopolysaccharide.[25] In a previous study from this laboratory,[3] a depression of mean arterial pressure reaching a minimum of $90 \pm 8$ mm Hg in nine normotensive rats and $95 \pm 11$ mm Hg in 11 SHR was recorded 1 hour after the intravenous injection of 1.8 mg/kg lipopolysaccharide. Thus, it could be conceivable that the more robust decrease in blood pressure in SHR compared with normotensive rats could contribute to the increase in plasma PAF. Conversely, the difference in the hemodynamic effects of lipopolysaccharide between SHR and normotensive rats could also reflect the increased PAF release in hypertensive animals since intravenous administration of PAF is known to produce a more pronounced hypotensive response in SHR than in normotensive WKY.[26] Moreover, it is important to note that blood pressure never reached the shock level, even at the highest lipopolysaccharide dose, and when organs such as the lungs, kidneys, and small intestine were studied for histopathological signs of shock, they were found to be normal after lipopolysaccharide administration in both SHR and normotensive rats.[3] In our recent (unpub-

lished) studies we found increased levels of TNF-α and PAF in the cerebrospinal fluid and plasma of aged normotensive rats after lipopolysaccharide challenge using a protocol similar to that used in the present study.

Increased counts and activities of leukocytes, monocytes, and lymphocytes in particular have been proposed to contribute to the risk of organ injury in SHR compared with normotensive WKY.[27] We could not find differences in the total white blood cell counts between these rat strains. In agreement with previous literature reviewed recently by Dzielak,[28] we found that the lymphocyte counts in hypertensive rats were significantly lower than those in normotensive rats, whereas the monocyte counts in hypertensive rats were elevated and could have contributed to the increased serum TNF-α levels because monocytes have been reported to be the primary source of TNF-α and PAF in the blood.[21] However, the number of white blood cells, including monocytes and lymphocytes, in SHR and normotensive rats reported in our present study as well as in the study reported by Schmid-Schönbein and coworkers[27] falls within the normal range of white blood cell counts in laboratory rats.[29] Preliminary studies in our laboratory have also demonstrated that isolated carotid artery rings from SHR produce more TNF-α than rings from normotensive rats. Increased expression of immunoreactive TNF-α has also been reported in human atherosclerotic arteries.[30] Therefore, it seems unlikely that the elevated serum levels of TNF-α in SHR could be entirely due to increased numbers of circulating monocytes. Increased levels of TNF-α and PAF in the cerebrospinal fluid of hypertensive rats compared with normotensive rats further support this view.

The present data demonstrate that hypertensive rats, when provoked by a conventional stimulus, release more TNF-α and undergo a more intense activation of hemostatic and proinflammatory mechanisms than risk factor–free control rats, probably as a result of a more vigorous interaction of monocytes and perivascular macrophages with the endothelium. These results are compatible with the following model: Risk factors for stroke are initially associated with a change in the endothelium. This change would probably involve the expression of monocyte receptors that enable the monocytes to adhere to vessel walls and undergo transendothelial migration in both large and small vessels, leading to local deposits of monocyte clusters in segments of the blood vessels. Our recent studies using immunofluorescent markers for monocytes and their adhesion receptors in vivo lend support to this hypothesis as the carotid endothelium in SHR and stroke-prone SHR but not normotensive WKY has been found to express the intercellular adhesion molecule-1 and to demonstrate increased numbers of adherent monocytes.[25] These monocyte clusters could then periodically signal the vessel endothelium via release of prothrombotic, proinflammatory, and chemotactic mediators such as TNF-α, PAF, and perhaps IL-1 to convert the endothelium to a procoagulant state and, in effect, prepare those vessel segments in a manner similar to the localized Shwartzman paradigm.[31-33] Complement activation or any stimulus leading to activation of the coagulation system could then precipitate a localized reaction of this pre-

pared vessel segment and lead to a local thrombosis or hemorrhage. This could represent a general mechanism through which risk factors for stroke operate to increase stroke likelihood.

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# *Editorial Comment*

An increasing body of evidence serves to demonstrate that leukocytes during shock, ischemia, and reperfusion are directly involved in organ dysfunction. In the brain the current evidence for a cytotoxic effect by leukocytes during experimental ischemia or in the presence of arterial lesion is challenging[1-5] and includes preliminary evidence in humans.[6-8] While activation of circulating leukocytes may lead to complications in the microcirculation of many organs, including the brain, the origin of the activation in selected forms of infarction and its exact manifestation are unresolved. Equally challenging questions are why persons with hypertension may be at risk for ischemia and hemorrhage and whether there may be a link between leukocyte entrapment in the microcirculation during ischemia and the conditions in chronic arterial hypertension.

The above report provides evidence for transiently increased production of tumor necrosis factor (TNF-$\alpha$) and platelet-activating factor (PAF) in the cerebrospinal fluid and plasma of normotensive and spontaneously hypertensive rats after stimulation with lipopolysaccharide. The enhanced production in hypertensive rats is detectable after intracerebroventricular and intravenous lipopolysaccharide administration, especially at doses of 100 $\mu$g/kg body wt and above. The authors also show enhanced plasma levels of eicosanoids and thromboxane $B_2$ in spontaneously hypertensive rats after endotoxin infusion. The significance of these important findings lies in the fact that these chemical mediators stimulate inflammatory cells. PAF, thromboxane, and TNF-$\alpha$ activate neutrophils and monocytes and the endothelium and are prothrombotic. They could thus play a role in the expression of selectins on the cerebral vascular endothelium and the depression of endothelium-derived relaxing factor release[9] and may serve as a trigger mechanism for leukocyte infiltration, microvascular obstruction, oxygen free radical production, lipid and protein peroxidation, thrombus formation, and

eventual infarction. The enhanced production of these inflammatory mediators in hypertensive rats is an important lead to understanding the high propensity for organ injury in this rat strain. It is also in line with the poor compensatory response by spontaneously hypertensive rats to a cardiovascular challenge.[10]

Leukocytes may be trapped in the microcirculation by a blood pressure reduction and/or cytoplasmic stiffening and expression of membrane adhesion proteins. Hypertensive rats show signs of activation of circulating leukocytes under resting conditions, and thus production of proinflammatory agents in response to a secondary stimulus may lead to a condition that favors microvascular entrapment of leukocytes, even in the presence of normal or elevated perfusion pressures.

This study raises several questions. Are there stimuli other than endotoxin administration, such as ischemia, that may lead to an enhanced expression of inflammatory mediators in hypertensive rats? What could be the source of the enhanced intracerebroventricular TNF-$\alpha$ and PAF production in hypertensive rats? Are resident cells in the brain participating, and to what degree? Is the enhanced production of TNF-$\alpha$, thromboxane, prostaglandin, or PAF purely the result of endotoxin stimulation or are elevated blood pressure and/or a genetic factor involved? In light of the fact that endotoxin per se stimulates neutrophils and monocytes, a high sensitivity of hypertensive rats to endotoxin may accompany the increased release of inflammatory mediators. Equally important is the question of whether the enhanced expression of thromboxane, TNF-$\alpha$, or PAF may occur chronically in hypertensive rats, even though the production may be localized and the levels may remain low. It will be interesting to monitor in the future other proinflammatory and prothrombotic mediators in hypertensive rats and determine their effects on leukocyte or platelet adhesion, oxygen free radical production, and degranulation. This is especially interesting since

other enzyme systems known to affect the hypertensive state may be affected by activated leukocytes.[11,12]

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