Agonist-stimulated release of von Willebrand factor and procoagulant factor VIII in rats with and without risk factors for stroke

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Abstract

Lipopolysaccharide (LPS)-induced (i.v. or i.c.v., 1.8 mg/kg) release of von Willebrand factor (vWF) was examined in spontaneously hypertensive (SHR) and normotensive Wistar-Kyoto (WKY) rats. SHR rats released significantly (P < 0.05) more vWF than WKY rats in response to LPS. LPS also inhibited factor VIII procoagulant activity (FVIII: c) which may indicate an increase in thrombin activity. Cultured cerebrovascular endothelial cells (EC) derived from both SHR and WKY rats, as well as human umbilical vein EC (HUVEC) cultures constitutively released vWF. Treatment with agonists including LPS, thrombin and tumor necrosis factor-α (TNFa) did not affect the in vitro secretion of vWF by cerebrovascular EC cultures but significantly upregulated vWF release by HUVEC cultures. Preincubation of cerebrovascular EC cultures with interleukin-1 (IL-1) ± TNFa or co-culturing in the presence of LPS-activated syngeneic monocytes had no effect on vWF secretion. The findings demonstrate that conditions of hypertension may affect endothelial cells and make them more responsive to agonist stimulation and thereby increase secretion of vWF, an important factor in hemostasis as well as thrombosis. The capacity of LPS to significantly affect the in vivo secretion of vWF in SHR and WKY rats but not cultured cerebrovascular EC indicates that observed elevations in plasma vWF were not derived from cerebrovascular EC. It is suggested that hypertension may function as a risk factor for thrombotic stroke by influencing factors involved in coagulation processes, such as vWF and factor VIII: c.

Key words: von Willebrand factor; Hypertension; Lipopolysaccharide; Endothelial cell; Stroke; Monocyte

1. Introduction

Although hypertension has been identified as a risk factor for stroke, the mechanism by which it functions as a predisposing agent is unclear [8]. It has been shown that rats with stroke risk factors, such as hypertension, diabetes and advanced age, have significantly higher incidences of stroke than control rats when challenged with a single dose of lipopolysaccharide (LPS) [12]. In addition, spontaneously hypertensive (SHR) rats injected (i.v. or i.c.v.) with LPS produced more TNFα in both blood and cerebrospinal fluid samples than normotensive (Wistar-Kyoto, WKY) rats [31]. Hypertension may function as a stroke risk factor by altering endothelium to render it more proinflammatory and procoagulant. In hypertensive animals, segments of the vasculature can upregulate the expression of adhesion receptors, such as ICAM-1 [32] and circulating blood cells, can bind and accumulate locally. Monocytes that accumulate in a perivascular location [3,32] expose the adjacent endothelium to the potential for periodic activation by cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNFα).
These cytokines are sufficient to "prepare" the vessel segment for a localized Shwartzman reaction [21] and a process related to this paradigm could underlie the increased probability of stroke due to thrombosis and/or hemorrhage that accompanies hypertension [12].

An important factor expressed by vascular endothelium which plays a critical role in hemostasis as well as thrombosis is von Willebrand factor (vWF) [28,36]. This factor is known to mediate platelet adhesion/aggregation and thrombus formation by interaction with platelets [36]. Endothelial cells (EC), megakaryocytes and platelets are the only cell types known to synthesize and secrete vWF [16,34]. EC can secrete vWF by both a constitutive pathway linked to synthesis and a regulatory pathway which can be induced by a variety of agonists and utilizes vWF stored in Weibel–Palade bodies [13,18,19,23,28,29,35].

In addition, vWF also functions as a carrier for the procoagulant factor (FVIII:c) in circulating blood [15]. FVIII:c normally circulates in plasma in an inactive form noncovalently bound to vWF which protects FVIII:c from enzymatic degradation and stabilizes its procoagulant activity [37]. Activation of coagulation includes dissociation of vWF/FVIII:c complex and activation of FVIII:c by serine proteases, such as thrombin [9]. Since thrombin can also inactivate FVIII:c [9] (by activating protein c) and enhance vWF secretion [13,23,29], an increase in the ratio between vWF and FVIII:c may be utilized as an indicator of thrombin generation and activation of hemostatic mechanisms [5].

In the present paper, we investigated the hypothesis that hypertension may increase the probability of local thrombosis or hemorrhage by enhancing the sensitivity of endothelium to factors capable of generating a procoagulant state. We have examined how LPS and other potentially relevant physiologic factors on the secretion of vWF by cultured EC derived from SHR and WKY rats. In addition, we examined the effect of LPS and other potentially relevant physiologic factors on the secretion of vWF by cultured EC derived from SHR and WKY rats. Finally, the effect of LPS on FVIII:c levels was also compared in these two strains of animals to compare their procoagulant potential.

2. Materials and methods

2.1. Animals

SHR and normotensive WKY rats (10–16 weeks old) were obtained from Taconic Farms (Germantown, NY). All rats were housed for 3–5 days after delivery with food and water ad libitum at 20–22°C, 50% humidity and 12/12 h light/dark cycles. The experiments described here were performed in accordance with guidelines put forth in the Guide for the Care and Use of Laboratory Animals (DHEW Publication NIH 85–23, 1985).

2.2. Surgical procedures

I.v. and i.c.v. injections of the LPS (E.coli LPS 0111:B4, phenol extract; Sigma, St. Louis, MO) were performed as described by Sirin et al. [31]. The proper position of the i.c.v. cannula was ascertained at the end of the experiment by an injection of methylene blue (5 μl) into the ventricular space. The rats were allowed to recover in single cages with food and water and regained consciousness within 10 min of the end of the procedure. Each animal received only one injection of LPS. Arterial blood samples from rats anesthetized with phenobarbital (50 mg/kg i.p.) were collected at indicated time points after LPS injection. A laparotomy was performed and blood was withdrawn via a sterile syringe (containing 0.1 M sodium citrate) from the abdominal aorta. All samples were centrifuged at 2000 × g, 5 min and the plasma was removed and stored at −70°C.

2.3. vWF assays

The vWF concentration in the plasma of WKY and SHR rats and in the conditioned media of all cultured EC was measured by an ELISA kit (Asserachrom, American Bioproducts, Parsippany, NJ). This assay was previously shown to cross-react with the rat vWF [6]. The lower limit of sensitivity of the assay was 10 ng/ml, assuming a vWF concentration in human plasma of 10 μg/ml [15]. EC were grown to confluence, washed three times with phenol red-free and calcium-free M199 media (Biofluids, Rockville, MD), supplemented with 3 mM CaCl2, 2 mM glutamine, 20 mM hepes, antibiotic–antimycotic mixture and 2% BSA and incubated with 50 μl of either media alone or indicated concentrations of LPS, calcium ionophore A23187, thrombin, phorbol myristic acid (PMA), IL-1, TNFα for fixed periods of time at 37°C in a humidified atmosphere in 5% CO2. In indicated experiments, EC cultures contained cycloheximide (1 μg/ml). Media for vWF determinations were removed from each well and immediately frozen at −70°C.

2.4. Factor VIII:c assay (COATEST)

The coagulating activity of FVIII:c was assessed by a two-stage assay (COATEST) kit based upon the stimulatory effect of FVIII:c on the generation of Xa in the presence of IX, X, phospholipid and calcium, which results in the formation of free-nitrosoxime (measured at 405 nm) and reflects the FVIII:c activity [26]. Briefly, 100 μl samples of either test plasma or standard (pooled normal human plasma) were incubated (5 min, 37°C) with 20 μl of a mixture of factor IXa, X and phospholipid followed by 100 μl of 0.025 M CaCl2. After a second 5-min incubation, 200 μl of the chromogenic and highly selective factor Xa substrate S-2222 [1] was added and, after a 5-min incubation, the reaction was stopped by the addition of 100 μl of 50% acetic acid. The volume was adjusted to 1 ml and the FVIII:c activity was assessed by measuring the absorbance at 405 nm. No differences were found when human and rat standard curves were compared for the COATEST activity.

2.5. EC preparation and culture

Cerebrovascular EC were isolated following the procedures described by Doron et al. [6]. Five WKY and SHR rats (16–22 weeks old) were utilized for each preparation, which were ultimately suspended in M199 (Biofluids) containing 20% heat-inactivated FCS, 2 mM glucose, 90 μg/ml heparin (Calbiochem, La Jolla, CA), 20 μg/ml EC growth supplement (Sigma), 1× antibiotic-antimycotic mixture (Gibco). The freshly isolated EC were plated (~1×104/100 μl/well) on 96-well microtiter plates precoated with Matrigel (Collaborative Research, Bedford, MA) as previously described [6]. HUVEC were obtained from Clonetics (San Diego, CA).
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2.6. Monocyte preparation

Peripheral blood monocytes were prepared from blood (obtained by i.c. puncture of SHR and WKY rats) by density gradient centrifugation using HISTOPAQUE (Sigma). All monocyte preparations were phenotypically characterized by staining with monocyte/macrophage specific antibodies (Mac-1 and ED-1; Accurate Chemical and Scientific). Monocyte preparations were cultured (2 h) in the presence or absence of LPS (10 ng/ml) and added to EC monolayers at indicated concentrations. Monocyte culture supernatants were also concomitantly harvested and frozen (-70°C) until use.

2.7. Statistical analysis

Statistical analysis consisted of one-way ANOVA followed by Bonferroni correction. Statistical significance was accepted at \( P < 0.05 \); values are presented as mean ± S.E.M. or S.D., as indicated. When ANOVA revealed significant differences, Student-Newman-Keuls test was used to analyse differences within individual groups.

3. Results

3.1. Effect of LPS i.v.

The effect of LPS on vWF plasma levels was examined following i.v. injection. SHR rats injected (i.v.) with a single dose (1.8 mg/kg) of LPS exhibited a maximum response (vWF release) of 46% increase above background (\( P < 0.01 \)) at a time period of 2 h after injection (Fig. 1). The vWF levels at 15 min or 4 h after injection were not significantly different from controls (injected i.v. with sterile saline) (Fig. 1). A similar time course of vWF release was observed with WKY rats.

To directly compare the responsiveness of SHR and WKY rats, vWF levels in plasma were measured at 2 h after i.v. injection of different doses (0.1 and 1.8 mg/kg) of LPS. The results showed that SHR rats released significantly (\( P < 0.05 \)) more vWF than WKY rats when injected with 1.8 mg/kg LPS. No elevation in the levels of released VWF were observed at the lower dose of LPS (0.1 mg/kg) in either SHR or WKY rats (Fig. 2A).

3.2. Effect of LPS i.c.v.

LPS injected i.c.v. also elevated vWF levels (Fig. 2B). Similar to observations with i.v. injections, significant differences in the levels of vWF release between SHR and WKY rats were only observed at the higher concentration of LPS (1.8 mg/kg). SHR rats produced more vWF (23% increase above background; \( P < 0.05 \)) as compared with WKY rats (11% increase above background). The increased plasma levels of VWF in SHR rats injected with this concentration of LPS were cultivated under conditions similar to those described above for cerebrovascular EC.

Confluent cerebrovascular EC and HUVEC cultures exhibited characteristic cobblestone appearance and cell viability were > 95% as determined by the trypan blue exclusion technique. All cell cultures contained > 90% EC as determined by immunocytochemical staining using antibody to vWF (FVIIIIR: Ag) (Accurate Chemical and Scientific, Westbury, NY).
considerably lower than those observed after i.v. injection of LPS.

3.3. Effect of LPS i.v. on FVIII:c

It was observed that LPS injected i.v. at a concentration of 1.8 mg/kg reduced the FVIII:c activity in both SHR and WKY rats (Fig. 3A). No significant differences in the magnitude of the reduction of FVIII:c activity were observed in both rat strains. The decreased levels of FVIII:c along with the significantly increased levels of vWF released in both strains of rats resulted in increases in the vWF/VIII:c ratio in both SHR and WKY rats (Fig. 3B).

3.4. Release of vWF by cerebrovascular EC cultures

Experiments performed on cultured cerebrovascular EC indicated that the concentration of vWF in supernatant medium gradually increased. This time-dependent increase could be detected as early as 1 h after culture. Although the total amount of vWF released varied from one culture to another, no significant differences were consistently observed between cerebrovascular EC cultures from SHR and WKY rats. The release of vWF could be inhibited by preincubation of EC with cycloheximide (results not shown).

The ability of various agonists to affect the release of vWF by cultured cerebrovascular EC was examined (Table 1). In all experiments, the rates of vWF release by EC treated with these factors were not significantly different from control EC cultures incubated in media alone. Additional concentrations of LPS (0.1–100 ng/ml), thrombin (1–10 U/ml), PMA (10–100 ng/ml), and TNF + IL-1 8.22 ± 1.61 resulted in increases in the vWF/VIII:c ratio in both SHR and WKY rats (Fig. 3B).

Table 1
Effects of various substances on release of vWF in SHR and WKY EC cultures

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Media</td>
<td></td>
</tr>
<tr>
<td>LPS (100 ng/ml)</td>
<td>6.78 ± 1.03</td>
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<tr>
<td>PMA (100 ng/ml)</td>
<td>ND</td>
</tr>
<tr>
<td>Thrombin (10 U/ml)</td>
<td>ND</td>
</tr>
<tr>
<td>SHR</td>
<td></td>
</tr>
<tr>
<td>Media</td>
<td></td>
</tr>
<tr>
<td>LPS (100 ng/ml)</td>
<td>6.65 ± 1.57</td>
</tr>
<tr>
<td>PMA (100 ng/ml)</td>
<td>ND</td>
</tr>
<tr>
<td>Thrombin (10 U/ml)</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Values for vWF are presented as ng/10^5 cells (mean ± S.D.) obtained from three separate wells of a representative experiment out of five experiments with similar results.

* Significant difference of treatment (P < 0.05, ANOVA) from media alone.

Fig. 3. Effect of LPS (1.8 mg/kg) injected i.v. on serum FVIII:c (A) and vWF/FVIII:c (B) ratios. SHR and WKY rats were injected i.v. with sterile saline or 1.8 mg/kg LPS. FVIII:c and vWF levels were assessed as described in Materials and methods. Values (mean ± S.E.M.) represent levels of FVIII:c in plasma at 2 h postinjection.
TNFα (1–200 U/ml) and IL-1 (1–50 U/ml) had no effect on the constitutive release of vWF. Increasing the incubation time period to 8–24 h did not result in any stimulation in vWF levels above controls (results not shown). Preincubation of EC cultures with cycloheximide inhibited vWF release under all conditions (results not shown).

3.5. Release of vWF by HUVEC cultures

Confluent HUVEC cultures constitutively released vWF into culture medium at a rate similar to that observed with rat cerebrovascular EC (Table 2). In addition, pretreatment with cycloheximide also inhibited vWF release in a similar manner. However, unlike rat cerebrovascular EC, treatment of confluent HUVEC cultures with various agonists for different time periods had significant effects on the levels of vWF release (Table 2). HUVEC cultures incubated for 1 or 4 h with LPS (10 ng/ml), thrombin (1 U/ml) or PMA (100 ng/ml) exhibited significantly higher levels of vWF release than observed in control cultures incubated in media alone (Table 2). Incubation with greater concentrations did not cause higher vWF levels; incubation for longer periods of time did not result in vWF levels different from controls (Table 2). No significant elevation of vWF levels above controls were observed in HUVEC cultures incubated with A23187 (Table 2). The addition of thrombin (10 U/ml) or PMA (100 ng/ml) to HUVEC cultures preincubated with cycloheximide resulted in enhanced release of vWF at levels comparable to those observed with HUVEC not preincubated with cycloheximide (results not shown).

3.6. Modulated release of vWF by cerebrovascular EC and HUVEC cultures

Attempts to boost the agonist-mediated release of vWF by cerebrovascular EC (as well as HUVEC) cultures was performed by preincubation with IL-1 in the presence or absence of TNFα (Table 3). The results indicate that pretreatment had no effect on either constitutive or thrombin-induced vWF release nor did it alter the already significant thrombin-stimulated release of vWF in HUVEC cultures. No response was observed with rat cerebrovascular EC.

The ability of monocytes to mediate enhanced vWF release by HUVEC was examined by incubating HUVEC with various concentrations of monocytes. Although resting monocytes did not significantly affect the secretion of vWF, incubation with LPS-activated monocytes dose-dependently enhanced the secretion of vWF after 1 h incubation (Table 4). Supernatants from resting and LPS-activated Mo were present at a concentration of 25% (v/v) and were added at start of experiment. Significant difference of treatment (P < 0.05, ANOVA) from media alone.

4. Discussion

Hypertension, as well as other stroke risk factors, such as atherosclerosis and diabetes, are associated with increased subendothelial accumulation of monocytes and macrophages [3,27]. A possible mechanism by

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### Table 3

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Treatment</th>
<th>Incubation time (h)</th>
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<tbody>
<tr>
<td>SHR EC</td>
<td>Media</td>
<td>5.18 ± 2.27</td>
</tr>
<tr>
<td></td>
<td>Media</td>
<td>6.02 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>IL-1</td>
<td>7.11 ± 1.21</td>
</tr>
<tr>
<td></td>
<td>TNF+IL-1</td>
<td>6.60 ± 0.28</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Media</td>
<td>6.40 ± 1.61</td>
</tr>
<tr>
<td></td>
<td>Media</td>
<td>12.33 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>IL-1</td>
<td>11.44 ± 1.35</td>
</tr>
<tr>
<td></td>
<td>TNF+IL-1</td>
<td>10.58 ± 1.28</td>
</tr>
</tbody>
</table>

* Values for vWF are presented as ng/10^5 cells (mean ± S.D.) obtained from three separate wells of a representative experiment out of two experiments with similar results.

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### Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.23 ± 0.25</td>
</tr>
<tr>
<td>Mo:EC</td>
<td>7.15 ± 0.78</td>
</tr>
<tr>
<td>5:1</td>
<td>8.34 ± 1.72</td>
</tr>
<tr>
<td>Activated Mo:EC</td>
<td>10.04 ± 0.75 *</td>
</tr>
<tr>
<td>5:1</td>
<td>12.29 ± 1.10 *</td>
</tr>
<tr>
<td>PMA (100 ng/ml)</td>
<td>29.73 ± 1.82 *</td>
</tr>
<tr>
<td>Monocyte sup.</td>
<td>6.89 ± 0.98</td>
</tr>
<tr>
<td>Activated monocyte sup.</td>
<td>7.47 ± 0.51</td>
</tr>
</tbody>
</table>

* Values for vWF are presented as ng/mg cells (mean ± S.D.) obtained from three separate wells.

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*Monocytes (Mo) were obtained as described in section 2 and were added at indicated ratios at initiation of experiment.

* Activated Mo were incubated with LPS (100 ng/ml) for 2 h prior to addition to EC at indicated ratios.

* Supernatants (sup) from resting and LPS-activated Mo were present at a concentration of 25% (v/v) and were added at start of experiment.
which these factors contribute to the increased incidence of stroke may ultimately involve their capacity to convert endothelium from an anticoagulant to a procoagulant surface. In addition to LPS, TNFα and IL-1 are capable of "preparing" tissues for a local Schwartzman-like reaction [21] thus rendering the endothelium procoagulant [22]. It has been shown in rats that stroke risk factors including hypertension prepared brainstem tissue (i.e. Schwartzman-like reaction) for the development of localized ischemia and hemorrhage after i.v. or i.c.v. injection of LPS [12]. In addition, hypertensive rats injected with LPS produced more TNFα than similarly treated normotensive rats [31].

It is known that vWF is synthesized by only two cell types, megakaryocytes, platelets and EC [16,34]. Megakaryocytes and platelets do not constitutively secrete vWF; they store vWF in organelles known as α-granules and acutely release it in response to platelet activation. On the other hand, the secretion of vWF by EC, which are known to be the major source of vWF, occurs by both constitutive and regulated pathways [19,28,35].

It is shown here that cerebrovascular EC cultures derived from SHR and WKY rats constitutively secreted vWF into the medium. Treatment with various effectors, such as thrombin and PMA, that are widely known to induce release of vWF from cultured EC, also had no effect on vWF secretion by either SHR or WKY EC cultures (Table 1) [18,19,23]. Treatment of HUVEC cultures with these same factors induced the release of vWF in a time-dependent manner (Table 2). One possible reason for this discrepancy may involve the the species and/or anatomical region from which the EC were derived. Although vWF has been detected in cultured EC derived from different species as well as vascular beds, some of these studies do not specifically address induced as opposed to constitutive secretion of vWF [16,17,30]. EC derived from large vessels, such as umbilical veins, possess rod-shaped Weibel–Palade bodies which release vWF by the regulated pathway [19,28,35]. Weibel–Palade bodies are absent in cultured EC derived from human, rat and mouse cerebral microvessels [4,7,33] which implies that these microvessels might release vWF only by the constitutive pathway. However, it was recently shown by assessment of stained preparations that primary cultures of human brain microvessel EC, devoid of Weibel–Palade bodies, could be stimulated to release vWF [7]. Previous experiments demonstrating some vWF release in brain microvascular EC raise the possibility that the amount of vWF released is near the detection threshold [6].

Various aspects of the acute phase response in man, which include elevations in plasma levels of vWF, have been attributed to the cytokines TNFα and IL-1 [24,25]. Although these cytokines have been shown to enhance vWF release by EC, they had little to no effect on vWF release by the HUVEC or SHR and WKY EC cultures used here [6,29]. Similar negative findings have been also been reported by others [11,23,38].

In some experimental conditions where these cytokines had no direct effects on vWF release, they modulated vWF secretion stimulated by other factors [23]. The ability of additional monocyte factors or monocytes themselves to affect vWF release by cultured EC was tested (Table 4). The data demonstrated that in vitro activated peripheral blood monocytes and, to a lesser extent supernatants from these cells, significantly elevated the levels of vWF released by cultured HUVEC; others have also shown a similar monocyte requirement for stimulated release of vWF [11,13]. No stimulated release was seen using resting monocytes. Similar monocyte co-culture experiments using SHR or WKY EC did not demonstrate any significant changes in constitutive vWF secretion (results not shown).

The evidence presented here suggests that LPS-induced elevations in plasma vWF in SHR and WKY rats is not derived from cerebrovascular EC. Perhaps, the elevated vWF levels observed in vivo are from EC in other areas of the vasculature which may have been exposed to LPS which leaked out of the ventricle and into the CSF and bloodstream. It is also possible that certain factors may induce release of vWF by EC vivo but are not active on cultured EC (i.e. in vivo activity may be mediated by as yet unknown intermediates which are not present in the in vitro system) [28].

The results presented here (Fig. 3) also show that LPS (i.v.) decreased FVIII:c levels in plasma. During clotting, the large amount of thrombin that is produced, inactivates FVIII:c and stimulates vWF release from endothelium and platelets, which leads to increases in the vWF/FVIII:c ratio [9]. These results may therefore indicate an increase in thrombin activity [13,23,29].

Although no differences were observed between hypertensive and normotensive rats in the levels of vWF released by cerebrovascular EC in vitro, the in vivo experiments provide evidence supporting the hypothesis that the stroke risk factor hypertension may generate a hypercoagulant-hypertrombotic milieu by enhancing interactions with vascular endothelium. The possible influence of a variety of other factors such as the presence of considerably greater numbers of monocytes in the circulation of SHR as opposed to WKY rats [31,32] may be an important factor. Also, in addition to storing vWF in Weibel–Palade bodies, EC store P selectin [2,20]. Its expression can be induced within minutes by factors which also affect vWF and it may mediate adhesion of leukocytes to activated endothelium [10,14]. Future experiments examining the complex interactions involving vascular endothelium and circulating blood cells and soluble factors may help to clarify the existence of the proposed effects of stroke.
risk factors and suggest modes of prophylactic intervention.

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