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Comparison of stimulated tissue factor expression by brain microvascular endothelial cells from normotensive (WKY) and hypertensive (SHR) rats

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The amounts of tissue factor (TF) expressed by brain microvascular endothelial cells (BMECs) from normotensive Wistar–Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) were compared after stimulating the cells with different doses of lipopolysaccharide (LPS), thrombin, phorbol myristic acid (PMA), Ca^{2+}-ionophore (A23187), or tumor necrosis factor (TNF) and interleukin-1 (IL-1). Treatment of cultured BMECs from WKY and SHR with all of these factors dose-dependently increased their total amount of TF; no substantive differences in the levels of enhanced TF expression were observed between WKY and SHR BMECs. We conclude that stimulated endothelium from rats with hypertension, a major stroke risk factor, is not hyperresponsive with respect to TF expression when compared to normotensive controls.

We have previously reported that rats with risk factors for stroke such as hypertension, advanced age and diabetes, develop more stroke events than control rats when the animals are challenged with a single dose of lipopolysaccharide (LPS)[10]. Differences in response to a provocative dose of LPS in vivo between rats with and without risk factors for stroke may depend on the degree of prothrombotic transformation which ultimately occurs in local endothelium[4–6]. This local transformation could be promoted by the risk factor indirectly through accumulation of perivascular macrophages that release cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1). These cytokines render endothelium procoagulant[4,5,7] and increase cellular adhesion[26]. Another possibility is that the sensitivity to different endothelial cell (EC) activators of hemostasis such as IL-1, TNF-α and LPS has been enhanced by the risk factor acting directly on the endothelium. It would be consistent with the latter possibility if the response of brain EC from hypertensive rats to LPS, IL-1 and TNF-α were to be an enhanced expression of tissue factor (TF) compared to that of brain ECs from normotensive rats.

TF is a 50 kDa phospholipid–protein complex that triggers blood coagulation in association with factor VII via the extrinsic pathway[22]. It is known to be constitutively synthesized by a variety of cells including fibroblasts[15,16], macrophages[2,23], smooth muscle cells[15,16] and EC[6,14,16,21]. In addition, numerous studies have shown that LPS[6,14,21], TNF-α[3,7,24], and IL-1[3,25], increase TF synthesis and expression in a variety of cells in culture.

In the present paper, we tested the hypothesis that the differences in stroke events between normotensive and hypertensive rats observed after LPS treatment in vivo[11] may in part be due to differences (as a result of the hypertension) in the reactivity of their brain ECs to prothrombotic mediators such as LPS, IL-1 and TNF-α. Therefore, we have compared the effects of these factors on TF expression by brain microvascular EC

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(BMEC) isolated from WKY (normotensive) and SHR (hypertensive).

BMEC were isolated following the procedures described by Williams et al.[27] with some modifications[9]. For each preparation, five (16–22-week-old) WKY and SHR rats were utilized and isolated BMECs were plated in 24-well tissue culture plates precoated with Matrigel (Collaborative Research, Bedford, MA) and incubated at 37°C in a humidified, 5% CO_2 atmosphere; media was changed after 24 h and then every 3 days. The viability of the cells was tested at the end of the preparation by a Trypan blue exclusion technique[1]. Cultures were characterized by staining with antibodies to the endothelial cell-specific marker, von Willebrand (FVIII-related) antigen and the astrocyte-specific marker glial fibrillary acidic protein (GFAP), and examined by indirect immunofluorescence microscopy as previously described[18]. Cell numbers in cultures were quantitated by a fluorometric assay as previously described[17]. The Hoechst 33258 (Hoefer Scientific Instruments; San Francisco, CA) was used as a fluorescent probe, and fluorescence was measured in a Perkin Elmer LS-5 fluorescence spectrophotometer set to 365 nM excitation and 458 nM emission. The measured amount of total DNA was then converted to cell numbers by assuming 6 pg DNA/cell[13]. It was noted that Matrigel, when fresh, contains DNA but the DNA becomes hydrolyzed and disappears during the initial 24–48 h of plating.

Experiments were performed 10–18 days after the initial preparation. After this time, the media was removed, and 1 ml of either the same media (control) or LPS (0.01, 1 or 100 μg/ml) (*E. coli* O111:B4; Sigma), thrombin (1 or 10 U/ml), A23187 (10 μM), PMA (100 nM), or TNF (5 U/ml) and IL-1 (20 U/ml) was introduced. Incubation was carried out for 30 min, 1 h, 4 h, or 8 h at 37°C in a humidified atmosphere in 5% CO_2. After the experiment, the cells were gently washed twice with Tris/saline buffer, and the plates were frozen at −80°C.

Tissue factor activity was estimated by a two-stage amidolytic assay using purified factors X and VII (Sigma) as previously described[15,25]. Briefly, BMEC were submitted 3 times to a freeze and thaw cycle and were pipetted (100 μl) into plastic tubes and placed in a water bath at 37°C. TF activity was assessed by incubation (37°C, 5 min) with 200 μl mixture of factor X (300 nM) and factor VII (0.5 nM) in 20 mM Tris (pH 7.4), 135 mM NaCl, and 0.5% BSA followed by addition of 100 μl of 25 mM $CaCl_2$. Factor Xa amidolytic activity was assessed by monitoring the hydrolysis (increase in absorbance at 405 nm) of the synthetic peptide Bz-Ile-Glu-Arg-*p*-nitronilide (S-2222, KabiVitrum). Under these conditions, factor Xa formation was linear and limited only by the amount of TF present. TF concentration was determined from a standard curve created by serial dilutions of rabbit brain TF (Sigma).

A one-stage clotting (plasma recalcification) assay using either pooled normal plasma (PNP) or factor VII-deficient plasma (George King Bio-Medical, Overland Park, KS) was performed as previously described[4,14]. Similar assays were performed using rabbit brain TF (Sigma) and rat brain TF (saline extract of rat brain homogenate[12]) incubated with indicated concentrations of phospholipase-C (PHL-C; Sigma) or Con-A (30 min, 37°C), which inhibit TF procoagulant activity[2,8,14]. The time to form a clot was monitored with a Fibrometer; one unit of TF was defined as the amount of TF required to generate a thromboplastin time equal to 40 s.

The final BMEC preparation consisted of small groups of cells in the form of microvessel fragments and some single EC. In recent experiments, we have demonstrated by means of total DNA per well quantification, that approximately 10^4 cells were seeded per well and that our plating method permits homogeneous plating with no significant difference in the amount of DNA between wells. Viability of the cells was consistently more than 80% as assayed on an intermittent basis by the Trypan blue exclusion method. Analysis of BMEC cultures by indirect immunofluorescence microscopy revealed that $> 95\%$ of cultured cells were routinely positive for FVIII-related antigen and $< 2\%$ stained positively for GFAP.

The one-stage clotting times of factor VII-deficient plasma and PNP in the presence of different serially diluted concentrations of rat TF were compared. Clotting times generated by TF (1 and 0.1 μg/ml) mixed with factor VII-deficient plasma were much longer (10 s and 135 s, respectively) than those mixed with PNP (40 s and 65 s, respectively). The same dependence on factor VII was found with the amidolytic assay, where almost no TF activity was observed when rat TF was tested with only factor X ($O.D._{405\ nm} = 0.090$) as opposed to factors X and VII ($O.D._{405\ nm} = 0.725$). Moreover, PHL-C (10–100 μg/ml) and Con A (40–200 μg/ml) dose-dependently inhibited both rabbit and rat TF procoagulant activity in the one-stage clotting assay (results not shown).

When BMEC from WKY and SHR were incubated for 8 h with different doses of LPS, both WKY and SHR BMEC dose-dependently increased their total amount of TF (Fig. 1). This effect reached significance at 1 μg/ml and 100 μg/ml ($P < 0.01$). However, there were no significant differences in the amount of TF expressed by WKY and SHR BMECs. Treatment with

100 μg/ml LPS for 1 h and 4 h also resulted in lesser though significant levels of TF release from both SHR and WKY EC; no reproducibly significant differences were observed between SHR or WKY BMECs.

The ability of factors other than LPS to enhance expression was also examined. As shown in Table I, treatment with TNF + IL-1 or thrombin caused significant TF expression by both SHR and WKY BMECs ($P < 0.05$). In additional experiments not reported here, treatment with A23187, PMA, and higher concentrations of thrombin (i.e. 10 U/ml) also resulted in enhanced TF expression above background in all BMEC cultures. In the data shown in Table I, as well as the data obtained from additional experiments described above, no significant differences were observed between SHR and WKY BMEC cultures.

We show that BMEC from normotensive and hypertensive rats express dose-dependent increments of TF when the cells are stimulated with LPS (Fig. 1), thrombin and the combination of TNF + IL-1 (Table I) as well as PMA and A23187 (results not shown). In all experiments, no significant differences in levels of TF expression were observed between the two groups. Validation of the techniques used to measure TF procoagulant activity from rat vessels consisted of a greatly reduced procoagulant effect in factor VII-deficient plasma, and inactivation of rat TF preparation by PHL-C[2,14] and Con-A[8].

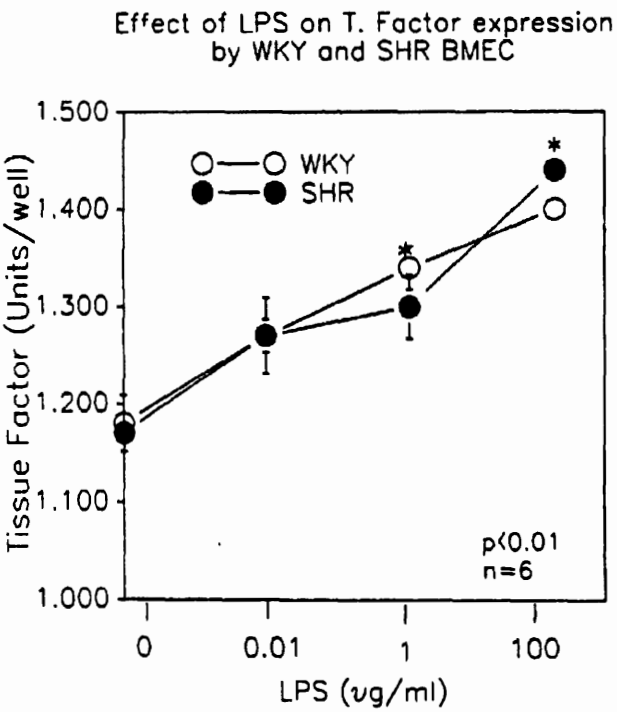

Fig. 1. Comparison of TF activity present on BMECs from WHY and SHR rats after LPS treatment. BMECs from WKY and SHR rats were isolated and plated onto Matrigel as described before. After 14 days, the cells were incubated with 1 ml of different doses of LPS diluted in M199, 2 mM glutamine. Incubation was for 8 h at 37°C in a humidified incubator after which the plates were placed at 37°C in a water bath, and washed 3 times with Tris/saline buffer. After the last wash, the buffer was removed and cells were subjected to 3 freeze and thaw cycles, followed by the amidolytic assay as described in Materials and Methods. The data were interpolated from a standard curve prepared by serial dilutions of Sigma TF. One unit is defined as the amount of TF necessary to form a clot in 40 s in the one-stage clotting assay ($n = 6$; * = $P < 0.01$). The data have been standardized by measuring the amount of DNA in each well and assuming a DNA concentration equal to 6 μg/cell[13].

TABLE I

Effects of TNF + IL-1 or thrombin on TF expression by SHR and WKY BMEC

Treatment [a]	*TF Expression* [b]	
	SHR	*WKY*
1 h		
0	0.056±0.004	0.059±0.005
TNF+IL-1	0.072±0.001	0.074±0.004
Thrombin	0.072±0.007	0.092±0.013
4 h		
0	0.078±0.004	0.084±0.008
TNF+IL-1	0.101±0.009	0.096±0.005
Thrombin	0.211±0.011	0.297±0.017

[a] Cells were treated for indicated time periods with TNF (5 U/ml) + IL-1 (20 U/ml) or thrombin (1 U/ml).

[b] Data for TF expression are presented on $O.D._{405\ nm} \pm S.E.M.$ obtained from amidolytic assay as described in Materials and Methods.

We have postulated that risk factors for stroke could create an environment in which the probability of an *interaction* between perivascular monocytes and macrophages with endothelium via cytokines such as TNF-α and IL-1 is increased[10,11]. This *interaction* could increase the probability of local thrombosis or hemorrhage in focal regions of the brain circulatory system and is viewed as equivalent to the preparatory step in the local Shwartzman reaction paradigm[19]. Such an *interaction* could be intensified by increasing the number of perivascular monocytes and macrophages in extracranial and intracranial vessels and/or rendering these cells hyperresponsive. Conversely, the endothelium lining blood vessels of the brain in animals with risk factors for stroke could be hyperresponsive and express more TF after appropriate stimulation than animals devoid of such risk factors. We have observed that SHR produce more TNF-α in blood and cerebrospinal fluid in response to LPS than their normotensive progenitor, WKY[11]. This suggests that hypertension may be associated with activation of the monocyte/macrophage arm of the postulated interaction. The present study has explored whether the EC of hypertensive animals is similarly activated, and the data suggest that for induction of TF by LPS and other factors used in these experiments, it is not. This finding

does not, however, exclude the possibility that stroke risk factors are associated with other forms of EC activation, such as adhesion receptor expression or that in vivo stimulation with factors utilized here might produce differential TF expression by EC in the two groups of animals.

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