Effects of plasma from hibernating ground squirrels on monocyte-endothelial cell adhesive interactions

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Yasuma, Yoshihide, Richard M. McCarron, Maria Spatz, and John M. Hallenbeck. Effects of plasma from hibernating ground squirrels on monocyte-endothelial cell adhesive interactions. Am. J. Physiol. 273 (Regulatory Integrative Comp. Physiol. 42): R1861–R1869, 1997.—Adhesion and subsequent penetration of leukocytes into central nervous system ischemic tissue proceeds via a coordinated inflammatory mechanism involving adhesion molecules at the blood-endothelium interface. Mammalian hibernation is a state of natural tolerance to severely reduced blood flow-oxygen delivery (i.e., ischemia). Hibernating thirteen-lined ground squirrels were investigated in an attempt to identify factors responsible for regulating this tolerance. Since leukocyte adhesion is closely associated with entrance into hibernation, the role of leukocyte adhesion to endothelium in this phenomenon was examined. Intercellular adhesion molecule-1 (ICAM-1) is expressed by endothelium and regulates interactions with circulating leukocytes that may result in margination or extravasation. ICAM-1 expression by rat cerebral microvascular endothelial cells (EC) cultured with plasma from hibernating (HP) or nonhibernating (NHP) thirteen-lined ground squirrels was dose dependently increased by HP and, to a lesser extent, by NHP. Treatment of EC with HP coincidentally induced significantly greater increases in monocyte adhesion to EC (37.2%) than were observed with NHP (23.9%). Study of the effects of HP and NHP on monocyte adhesion to EC may identify mechanisms responsible for ischemic tolerance in hibernators and could lead to the development of novel therapeutic approaches to the treatment of stroke.

adhesion; endothelial cells; hibernation; intercellular adhesion molecule-1; stroke

A PHENOMENON EXISTS IN NATURE that conveys an apparent tolerance to ischemia-like conditions; this phenomenon is hibernation. Hibernation represents a unique circannual physiological adaptation that allows animals to conserve energy and survive extended periods of food deprivation. During hibernation, organs are able to survive long periods of time under very severe circumstances without any ultimate loss of function. Mammalian hibernation involves a controlled suppression of interactive physiological responses that preserve homeostatic balance. These include energy metabolism, biophysical changes, and circadian rhythms. Entrance into hibernation is characterized by dramatic reductions in body temperature, as well as metabolic, heart, and respiratory rates (1, 14, 29). During bouts of hibernation, ground squirrel cerebral blood flow is reduced to ischemic levels (1, 14). With respect to the severely reduced blood flow and oxygen availability, hibernation presents a natural case of adaptation to conditions that provoke brain damage in animals that do not normally hibernate (i.e., euthermic animals).

The absence of neurological changes in animals aroused from hibernation indicates a tolerance to these stroke-like conditions. Although the capacity of hibernating animals to tolerate stroke-like conditions of low oxygen is certainly related to the capacity to lower metabolic demands, the induction of leukocytopenia in hibernating animals may also contribute to the absence of injury in these animals. Hibernating mammals have been investigated in attempts to identify and isolate factors responsible for regulating this tolerance. This research has identified a variety of factors including hormones (7, 21) and opioids (10, 23, 45, 47) with a demonstrated capacity to regulate hibernation.

Of particular interest to the research presented here are the hematological changes that accompany the physiological responses during the hibernating process. Entrance into hibernation is associated with a rapid and profound leukopenia (≥90% fall in circulating leukocytes), whereas arousal is associated with restoration of leukocytes to normal levels (14, 29, 35, 39). Since circulating leukocytes are intimately involved with pathogenic mechanisms associated with central nervous system (CNS) ischemia and reperfusion injury (3, 11, 15, 26), ischemic tolerance observed in hibernating animals may, to a certain extent, be a consequence of their severely leukocytopenic state. It should be pointed out that this is not necessarily a brain endothelium-specific event. In fact, leukocyte adhesion occurring by leukocyte-endothelium interactions is very likely to occur in non-CNS areas; it is quite possible that cells in hibernating animals are sequestered in a definitive manner. In fact, we have preliminary evidence that indicates that the majority of red cells and many of the leukocytes in hibernating squirrels are sequestered in the spleen and, to a lesser extent, in the liver (N. Azzam, personal communication).

There is currently an abundance of evidence indicating the participation of leukocytes in development of pathological consequences of CNS ischemia. In ischemic injury to the brain, various mediators including free radicals, excitotoxins, cytokines, and prostaglandins have been implicated in pathogenesis. A significant contribution to the overall damage comes from activated cells and results in an inflammatory response orchestrated by factors such as cytokines and chemokines. The consequent adhesion of leukocytes at the blood-endothelium interface and their subsequent penetration into ischemic tissue proceeds via a coordinated inflammatory mechanism involving cytokines, chemokines, and adhesion molecules. Increased leukocyte interactions with endothelium results in further activation of both cell types and is manifested by pathological alterations in both the blood vessel wall and the
surrounding endothelial tissue (3, 11, 15, 26). The generation of endothelial adhesion molecules and their roles in the subsequent pathogenic responses to ischemia have been widely studied (for review, see Ref. 31). Similar data have been obtained in in vitro models of ischemia-reperfusion-induced endothelial injury (34, 44). The active involvement of endothelium in these inflammatory events is underscored by the transformation of the endothelial facade to a prothrombotic and proinflammatory surface (18). These changes are indicated by the release of a plethora of endothelial-derived factors such as Factor VIII/von Willebrand factor, platelet-activating factor (PAF), interleukin 1 (IL-1) thromboxane A2, endothelin, nitric oxide, and superoxide anions (17, 19). The ultimate consequences of this endothelial activation are the attachment-accumulation, activation, and subsequent extravasation of circulating leukocytes at the localized sites of inflammation. The expression of adhesion molecules is a prerequisite for the development of this inflammatory process. The pathogenic role of adhesion molecules in disorders such as ischemic stroke has recently received widespread attention (for review, see Ref. 31).

It is interesting that in addition to previous work demonstrating the association of leukocytopenia with hibernating states, onset of hibernation has been induced by the passive transfer of plasma from hibernating animals (8, 40). The ability of extracts from hibernating but not active ground squirrels to cause hypometabolic and hypothermic effects in a homeotherm such as the rat further indicates the capacity of crude extracts from hibernating ground squirrels to induce physiological changes (i.e., torpor) that resemble, albeit to a lesser extent, onset of a hibernating state (25).

This study utilized thirteen-lined ground squirrels, a mammalian species that hibernates. The in vitro effects of plasma from hibernating as well as active animals on monocyte (Mo) adhesion to endothelial cells (EC) were examined. We hypothesized that factors that regulate leukocyte adhesion may be upregulated in plasma of hibernating animals. The results indicate that treatment of EC cultures or Mo with plasma of hibernating, as well as active, ground squirrels affects both the expression of adhesion molecules and adhesive interactions.

MATERIALS AND METHODS

Animals. Thirteen-lined ground squirrels (Spermophilus tridecemlineatus) were obtained from TLS Research (Bartlett, IL). Female Wistar-Kyoto (WKY) rats obtained from Taconic Farm (Germantown, NY) were used at 12–24 wk of age to isolate cerebromicrovascular EC and peripheral blood Mo. All animal studies and procedures were performed in accordance with a protocol approved by the National Institute of Neurological Disorders and Stroke Animal Care and Use Committee and the National Institutes of Health (NIH) “Guide for the Care and Use of Laboratory Animals” [DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205].

Animal preparation for induction of hibernation and sampling. Ground squirrels were housed individually in facilities with an ambient room temperature of 21°C and a 12:12-h light-dark cycle. They were fed standard rodent chow and water ad libitum and were weighed biweekly to monitor the seasonal state of their circannual cycle (weight gain is known to precede spontaneous entry into the hibernating state). All experimental animals were studied during both hibernating and active phases under either normal or hibernating conditions (i.e., in cages bedded with wood shavings within a cold chamber [hibernaculum] that was kept at 5°C, at 60% humidity, and in constant darkness, except for a photographic red safelight (3–5 lx)). These environmental conditions approximated those to which these animals are normally exposed in the winter season. Hibernation generally began from 1 to 7 days after the animals were placed in the hibernaculum. The chamber could be entered only through a darkened anteroom; noise within the chamber was kept to a minimum. Blood samples (citrated) were withdrawn via external jugular venous puncture during hibernation, and the plasma was used fresh or was stored at −70°C.

EC cultures. The isolation and cultivation of microvascular endothelium from WKY rats were performed with some modification as previously described (38). All EC cultures routinely exhibited a characteristic cobblestone appearance and were used at the time they attained confluence on 96-well flat-bottom microtiter plates. Cultures contained <95% EC as identified by immunocytochemistry and fluorescence-activated cell sorter (FACS) analysis using EC-specific anti-Factor VIII-related antigen (Accurate Chemical and Scientific, Westbury, NY). Where indicated, EC were cultured at 37°C in media alone or with media containing plasma from hibernating [hibernating plasma (HP)] or active [nonhibernating plasma (NHP)] squirrels, or indicated concentrations of recombinant human tumor necrosis factor-α (TNF-α; Endogen, Boston, MA) or lipopolysaccharide from Escherichia coli 0111:B4 (LPS) (Sigma, St. Louis, MO). All cytokines contained <10 pg/ml endotoxin as determined by RE-toxate assay kit (Sigma). In some experiments, EC were also cultured with 1–10 U/ml thrombin (Sigma) or 10–100 µM histamine (Sigma).

Mo. Peripheral blood was obtained from WKY rats by cardiac puncture and collected into heparin-coated tubes. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation (Hibayse 1119; Histopaque 1077; Sigma) for 30 min at 700 g, 24°C. Mononuclear cells (separated from plasma and granulocytes) were collected, washed, and further purified by centrifugation on hypersonic Nycodenz density gradients (Accurate Chemical) for 15 min at 600 g, 24°C. Mo preparations were extensively washed in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 2% EDTA to remove contaminating platelets. The Mo-enriched fractions were counted (>95% viable as determined by trypan blue exclusion), and their purity was assessed as >90% by their ability to phagocytize latex beads and by FACS analysis for ED-1 and ED-2 antibody-treated cells as described in flow cytometry.

Enzyme-linked immunosorbent assay. The relative degree of intercellular adhesion molecule-1 (ICAM-1) expression by EC cultures was quantitated by enzyme-linked immunosorbent assay with antibodies [murine immunoglobulin (IgG) to rat ICAM-1 (Seikagaku America, Rockville, MD) essentially as previously described (42). All cultures in 96-well microtiter plates (2 · 10 graded 50 µl of 1 ml−1 well−1) were incubated at 37°C in 5% CO2. Nonspecific binding sites were blocked using heated-aggregated rabbit IgG (Sigma), and cultures were sequentially incubated with anti-ICAM-1 antibody (1:1,000); biotinylated anti-mouse IgG (1:2,000); and 0.33 µg/ml avidin-horseradish peroxidase. All incubations were performed at 37°C for 1 h, and plates were washed three times with PBS.
containing 2% BSA after incubation with each of the above additions. The reaction was developed by addition of phosphate-citrate buffer/0.004% O-phenylenediamine, 0.012% H$_2$O$_2$ (25°C, 30 min), and stopped by addition of 4 N HCl. The optical density (OD) was immediately read at 490 nm (reference 630 nm). Background values (cells incubated with normal mouse serum in lieu of primary antibody) for each condition were subtracted from test values (identical cultures incubated with anti-ICAM-1).

Mo adhesion. Mo used in adhesion experiments were labeled with sodium $[^{51}C]r$chromate (0.1 mCi/ml) for 30 min at 37°C, washed twice with warm Hank's balanced salt solution (HBSS) containing 4% fetal calf serum (FCS) and added (2–5 $\times$ 10$^4$ 50 ml$^{-1}$-well$^{-1}$) to EC monolayers cultured in microtiter plates, which were subsequently incubated at 37°C for 30 min. After incubation, nonadherent cells were removed, EC monolayers were washed, and adherent cells were lysed with 2% (vol/vol) Triton-X (0.2 ml/well). Radioactivity in all fractions was counted on a LKB Compu gamma 1282 gamma counter. The number of adherent cells was evaluated by the measured ratio of counts/min to cell counts established for each experiment using known numbers of Mo. These numbers were verified with calculations for percentage of adherent cells calculated by dividing Triton-X counts/min by the total counts/min added. All experiments were performed in quadruplicate. In some experiments, Mo adhesion was calculated by measuring myeloperoxidase activity as previously described (4); no significant variations in the percent adhesion were observed in these experiments compared with those using radioactivity data. Also, >90% of cells adhering to EC monolayers stained positively for ED-1, indicating that adhesion by contaminating lymphocytes was minimal under the conditions described here.

Antibody blocking experiments. In some experiments, Mo were preincubated (20 min at 37°C) in serum-free media with saturating concentrations (25 µg/ml) of indicated monoclonal antibodies (MAb) or in serum-free medium alone. Control experiments using isotype-matched antibodies showed no effects (i.e., no significant differences compared with untreated Mo). In experiments using anti-ICAM-1, EC monolayers were preincubated with antibody (30 min, 37°C) and washed three times with warm HBSS containing 4% FCS. The inhibitory effects of antibody treatment on adhesion were calculated by the formula 100% $\times$ [%adhesion (no MAb) – %adhesion (+MAb)]/%adhesion (no MAb).

Flow cytometry. Adhesion molecule expression on EC and peripheral blood Mo freshly prepared from WKY rats was quantitated by FACScan analysis (flow cytometry) using a FACS IV analyzer (Becton Dickinson FACS System, Mountain View, CA). Cells were stained with antibodies to rat ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), very late after activation antigen-4 (VLA-4), E-selectin, CD11a, CD11b, or CD18 and analyzed as previously described (32). The data are presented as percent cell population positively stained and mean fluorescence intensity (MFI) representing semiquantitative data (in channel units) on the mean number of fluorescent reagent-binding molecules expressed by the cells.

The purity of peripheral blood monocyte preparations was also assessed as >90% by FACScan analysis using ED-1 and ED-2 antibodies (MFI = 153.4 and 22.5, respectively).

Antibodies. The following antibodies, which are specific for the indicated rat adhesion molecules, were obtained from Seikagaku America: 1A29, ICAM-1 (CD54); TA-2, VLA-4 (CD49d); WT.1, LFA-1a chain (CD11a); WT.5, Mac-1a chain (CD11b); and WT.7, LFA-1b chain (CD18). Monoclonal anti-rat ED-1 and ED-2, murine IgG1 antibodies specific for Mo/macrophage and macrophages, respectively, and anti-E-selectin (CD62E) were obtained from Chemicon International (Temecula, CA).

Statistics. All data are presented as means ± SD. Statistical differences were evaluated by analysis of variance followed by Fisher's protected least-significance difference test between particular experimental groups.

RESULTS

General observations. The use of outbred animals in these studies and the assessment of a factor(s) that may be closely associated with diet, duration of hibernation, and seasonal variations, resulted in differing levels of responses.

Characterization of Mo binding to EC. Time-course studies demonstrated that Mo (5 $\times$ 10$^4$/well) binding to EC monolayers increased rapidly within the first 15 min of incubation and reached a maximum by 30 min incubation at 37°C (Fig. 1A). Incubation for greater...
than 30 min did not result in significant increases in the number of adherent Mo. To determine Mo concentrations necessary for optimal binding to EC monolayers, various numbers of Mo were used to assess relative binding capacity. Addition of 0.1–1.0 × 10^5 Mo/well resulted in levels of Mo adhesion directly proportional to numbers of Mo added (Fig. 1B). This linear binding (r² > 0.982) was used to establish the maximum allowable numbers of Mo that could be added to each well. Addition of >1 × 10^5 Mo/well did not result in proportional increases in the percent of adherent cells. Therefore, unless otherwise noted, ~2–5 × 10^5 Mo/well were routinely used in experiments described here.

Mo adhesion to treated EC. The treatment of microvascular EC cultures with HP as well as NHP (final concentration = 1:4) reproducibly induced significant increases in Mo adhesion to EC (Fig. 2). Exposure of EC to NHP caused a 23.9% increase in the number of Mo binding/well (10,900 ± 316 vs. 8,800 ± 53) that was similar to that observed by EC treatment with optimum concentrations (50 U/ml) of TNF-α (25.6% increase). Treatment of EC with HP caused an even greater increase in the number of adherent Mo/well (12,073 ± 345). This increase in Mo adhesion to HP-treated EC (37.2%) was significantly greater (P < 0.001) than that observed with NHP-treated EC. Treatment of EC with NHP collected in summer (henceforth referred to as NHP-summer) increased Mo adhesion to EC to a lesser extent than NHP collected in winter (10.9%; P < 0.05). Similar results were also observed in experiments using other Mo preparations and EC cultures. As shown in Fig. 2, maximum increases in adhesion were observed at highest concentrations of plasma. Increasing dilutions of plasma resulted in concomitant decreases in the level of adhesion observed in treated EC. In all cases, less adhesion-enhancing activity was observed with diluted (1:10) plasma.

Both HP and NHP treatment increased Mo adhesion in a time-dependent manner. Although treatment of EC with 10 U/ml thrombin or 100 µM histamine increased adhesion (28.4 and 12.3%, respectively) as early as 15 min after treatment, no reproducibly significant upregulation of adhesion was observed on EC treated with HP or NHP for <2 h (results not shown). Significant upregulation of adhesion by plasma-treated EC was seen as early as 4 h after initiation of treatment and attained maximum levels 16–24 h after treatment (Fig. 3). Incubation of EC with plasma for >24 h did not result in further increases in the level of Mo adhesion.

Aside from significant differences observed in the overall level of adhesion, both HP and NHP exhibited similar kinetics regarding their ability to upregulate adhesion. Maximum adhesion to both HP- and NHP-treated EC was also observed at 30 min, similar to that observed on untreated EC (Fig. 1A).

Adhesion molecule expression by EC and Mo. Relatively low levels of ICAM-1 were constitutively expressed on all EC cultures. Treatment with various concentrations of LPS (1–100 ng/ml) or TNF-α (1–100 U/ml) dose dependently increased ICAM-1 expression...
EFFECT OF HIBERNATING PLASMA ON MO-EC INTERACTIONS

A: effects of active and hibernating squirrels’ plasma on intracellular adhesion molecule-1 (ICAM-1) expression by EC cultures. Data are means of optical density (OD) ± SD values of quadruplicate EC cultures. EC monolayers were incubated alone, with 100 ng/ml lipopolysaccharide (LPS), 50 U/ml TNF-α or in the presence of 1:2 (solid bars), 1:5 (closely hatched bars), or 1:10 (widely hatched bars) dilution of HP or NHP plasma. EC cultures were incubated with additions for 24 h before assay by enzyme-linked immunosorbent assay (ELISA) as described in MATERIALS AND METHODS. Data are from a representative of 5 experiments. *Significant differences by ANOVA + F-PLSD (P < 0.001) between HP and NHP treatments. B: effects of seasonal variation of squirrel plasma on ICAM-1, expression by EC cultures. Data are means of OD ± SD values of quadruplicate EC cultures. EC monolayers were incubated alone, with 50 U/ml TNF-α, or in the presence of HP, NHP, or NHP/S plasma. EC cultures were treated for 24 h before assay by ELISA as described in MATERIALS AND METHODS. Data are from a representative of 3 experiments. *Significant differences by ANOVA + F-PLSD (P < 0.05) between HP and NHP treatments; **significant differences by ANOVA + F-PLSD (P < 0.005) between NHP/S and NHP/W treatments.

Fig. 4. A: effects of active and hibernating squirrels’ plasma on intracellular adhesion molecule-1 (ICAM-1) expression by EC cultures. Data are means of optical density (OD) ± SD values of quadruplicate EC cultures. EC monolayers were incubated alone, with 100 ng/ml lipopolysaccharide (LPS), 50 U/ml TNF-α or in the presence of 1:2 (solid bars), 1:5 (closely hatched bars), or 1:10 (widely hatched bars) dilution of HP or NHP plasma. EC cultures were incubated with additions for 24 h before assay by enzyme-linked immunosorbent assay (ELISA) as described in MATERIALS AND METHODS. Data are from a representative of 5 experiments. *Significant differences by ANOVA + F-PLSD (P < 0.001) between HP and NHP treatments. B: effects of seasonal variation of squirrel plasma on ICAM-1 expression by EC cultures. Data are means of OD ± SD values of quadruplicate EC cultures. EC monolayers were incubated alone, with 50 U/ml TNF-α, or in the presence of HP, NHP, or NHP/S plasma. EC cultures were treated for 24 h before assay by ELISA as described in MATERIALS AND METHODS. Data are from a representative of 3 experiments. *Significant differences by ANOVA + F-PLSD (P < 0.05) between HP and NHP treatments; **significant differences by ANOVA + F-PLSD (P < 0.005) between NHP/S and NHP/W treatments.

Table 1. Effect of treatment on adhesion molecule expression by EC

<table>
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<tr>
<th>Treatment</th>
<th>ICAM-1</th>
<th>VCAM-1</th>
<th>E-selectin</th>
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<tbody>
<tr>
<td>0</td>
<td>11.4 (57.3)</td>
<td>0</td>
<td>0</td>
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<tr>
<td>TNF-α (6 h)</td>
<td>38.3 (84.6)</td>
<td>33.4 (66.3)*</td>
<td>61.7 (111.8)</td>
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<tr>
<td>TNF-α (20 h)</td>
<td>82.1 (126.8)</td>
<td>68.5 (122.9)*</td>
<td>9.4 (19.2)</td>
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<td>HP (6 h)</td>
<td>27.1 (70.4)</td>
<td>14.1 (43.4)</td>
<td>55.0 (87.6)†</td>
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<td>HP (20 h)</td>
<td>78.4 (133.9)</td>
<td>49.3 (88.6)</td>
<td>12.8 (26.4)‡</td>
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<tr>
<td>NHP (6 h)</td>
<td>18.9 (64.0)</td>
<td>15.6 (48.0)</td>
<td>41.3 (29.7)</td>
</tr>
<tr>
<td>NHP (20 h)</td>
<td>76.4 (118.9)</td>
<td>44.7 (99.6)</td>
<td>8.6 (17.1)</td>
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</tbody>
</table>

Cerebrovascular endothelial cells (EC) were incubated in the presence of 50 U/ml tumor necrosis factor-α (TNF-α), diluted (1:2) plasma from hibernating (HP) or nonhibernating (NHP) animals for 6 h or 20 h, as indicated. Cells were treated with antibodies to the indicated adhesion molecules and examined by FACScan analysis as described in MATERIALS AND METHODS. Data are expressed as % of total cells that stained positively for the indicated adhesion molecule; this value was obtained after subtracting overlap (on y-axis) of control cultures incubated with normal mouse serum and fluorescein isothiocyanate goat anti-mouse IgG. The mean fluorescence intensity, which approximates the number of fluorescent-binding molecules, expressed by the cell is shown in parentheses. ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1. *Significant differences by analysis of variance (ANOVA) + Fisher’s protected least-significant differences test (F-PLSD) (P < 0.01) between TNF-α and HP or NHP treatments. †Significant differences by ANOVA + F-PLSD (P < 0.05) between HP and NHP treatments.

(results not shown). Exposure of EC cultures to HP as well as NHP also increased the expression of ICAM-1 (Fig. 4A). Optimal concentrations of both HP and NHP induced significantly more ICAM-1 expression than optimal concentrations of LPS (100 ng/ml) or TNF-α (50 U/ml). ICAM-1 on EC treated with various dilutions of HP (i.e., final concentrations = 1:5–1:20) was significantly more upregulated than EC treated with NHP (Fig. 4A). Interestingly, NHP-summer upregulated the expression of ICAM-1 (0.14 ± 0.07 OD units) to a significantly lesser degree than NHP collected in winter (0.43 ± 0.07 OD units) (Fig. 4B).

Generally speaking, upregulation of ICAM-1 expression on EC by stimulation with inflammatory mediators such as LPS and TNF-α occurred as early as 4 h after treatment and obtained maximal level at 16–24 h and remained elevated for up to 72 h in the presence of continued stimulation. Similar findings were observed with EC monolayers treated with either HP or NHP.

The expression of ICAM-1, as well as VCAM-1 and E-selectin, were also examined by FACScan analysis. Treatment of EC with optimal concentrations of TNF-α, HP, and NHP (1:2 dilutions) upregulated expression of ICAM-1 in a time-dependent manner (Table 1). Although treatment with HP for 6 h usually induced greater levels of ICAM-1 than NHP, no reproducibly significant differences in either the percentage of positively stained cells or in the relative intensity of staining were observed between treatments. Treatment with TNF-α, HP, and NHP also induced expression of VCAM-1 and E-selectin (Table 1). In regard to VCAM-1 expression, treatment of EC with TNF-α resulted in greater levels of expression than seen with EC incubated in the presence of HP or NHP, which induced comparable levels. The expression of E-selectin was maximally induced after 4–6 h of treatment with TNF-α, HP, or NHP. Significantly (P < 0.05) higher levels of E-selectin were observed with HP-treated EC (MFI = 87.6) as opposed to NHP-treated cells (MFI = 29.7). The level of E-selectin was also less downregulated after 20 h treatment with HP.
Peripheral blood monocytes were examined for surface expression of adhesion molecules CD11b, CD18, ICAM-1, and VLA-4 by FACSscan analysis. In a representative experiment, Mo populations expressed comparatively high levels of CD11b (MFI = 83.4) and CD18 (MFI = 110.8) compared with ICAM-1 (MFI = 65.1) and VLA-4 (MFI = 23.7). The mean MFI values for negative isotype control antibodies were <10 (n = 4).

Antibody blocking experiments. The functional roles of specific adhesion molecules were identified by examining the capacity of specific MAbs to block adhesion of Mo to EC. The data shown in Table 2 indicate that adherence of unstimulated Mo to unstimulated EC monolayers was partially inhibited (95.9 and 39.8%) by incubation of Mo with MAB against α-chains of LFA-1 or Mac-1, respectively (Table 2). Incubation with MAB to the common β-chain (CD18) of LFA-1 and Mac-1 inhibited adhesion by more than 50%. Incubation with MAB to rat VLA-4 had no effect on the level of Mo adhesion. The capacity of MAB to ICAM-1 to block Mo adhesion to untreated EC monolayers was equal to the inhibition observed with MAB to its known ligands (CD11a/CD18 and CD11b/CD18), which are present on the Mo.

The effect of the above MAbs on Mo adhesion to cultured EC treated with TNF-α, HP, or NHP is also shown in Table 2. Mo adhesion to all treated EC was less inhibited by MAB CD11b, CD18, and ICAM-1. Also, MAB to VLA-4, which was ineffective on untreated EC, inhibited Mo adhesion to all treated EC. However, treatment with MAB CD11b, VLA-4, or CD18 with or without VLA-4 inhibited adhesion to HP-treated EC to a significantly (P < 0.05) greater degree than was observed with NHP-treated EC. There were no significant differences in the effects of MAB on adhesion to TNF-α or NHP-treated EC. Similar levels of inhibition by MAB treatment were observed in experiments using different EC cultures and Mo preparations.

<table>
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<th>Treatment</th>
<th>EC Incubation Conditions</th>
<th>None</th>
<th>TNF-α</th>
<th>HP</th>
<th>NHP</th>
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<tr>
<td>CD11a</td>
<td>9.5 ± 1.2†</td>
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<td>CD11b</td>
<td>39.8 ± 6.4</td>
<td>19.1 ± 2.2</td>
<td>25.8 ± 2.6†</td>
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<td>CD18</td>
<td>51.2 ± 9.0</td>
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<td>29.9 ± 7.1†</td>
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<tr>
<td>ICAM-1</td>
<td>43.2 ± 7.7</td>
<td>21.5 ± 7.9</td>
<td>17.2 ± 5.5</td>
<td>16.3 ± 4.8</td>
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<td>VLA-4</td>
<td>2.1 ± 4.0</td>
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<td>VLA-4+CD18</td>
<td>48.6 ± 12.3</td>
<td>33.7 ± 5.9</td>
<td>47.4 ± 6.5†</td>
<td>36.9 ± 7.3</td>
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Cerebrovascular EC were incubated in the presence of 50 U/ml TNF-α, diluted (1:2) plasma from HP or NHP animals for 6 h. Monocytes and EC were treated with indicated monoclonal antibodies as described in MATERIALS AND METHODS. Data were obtained from a representative of 3–5 experiments and are presented as percent inhibition of adhesion compared with adhesion in the absence of antibodies (i.e., mean ± SD values for no treatment, 15.3 ± 6.8% TNF-α, 29.4 ± 3.9% HP, 41.4 ± 6.2% and NHP, 35.1 ± 5.0%). *Significant differences by ANOVA + F-PLSD (P < 0.05) between HP and NHP treatments.

DISCUSSION

The capacity of hibernating mammals to survive conditions of greatly reduced oxygen and substrate delivery without any ultimate loss of function provides an opportunity to identify responsible mechanisms that may have potentially therapeutic applications in disorders associated with such conditions. In addition to the correlation between tolerance of hibernating animals to conditions of low oxygen and lowered metabolic needs, the induction of leukocytopenia in hibernating animals may contribute to the absence of injury. Because leukocytes are known to play important roles in ischemic injury, the leukocytopenia observed in hibernating animals may be involved in this ischemic tolerance. It has been shown that hibernation (with a consequent leukocytopenia) can be induced by transfusion of plasma from hibernating animals (8, 40). It is shown here that treatment of microvascular EC cultures with HP induces significant increases (37.2%) in Mo adhesion to EC. Significantly smaller increases were observed with NHP from winter, cold-adapted animals (23.9%) and NHP from summer animals (10.9%) (Fig. 2). Despite variations between experiments, this hierarchical relationship was consistently observed, suggesting the presence of higher levels of adhesion-promoting capacity of HP. Also, experiments using dilutions of plasma showed dose dependently less adhesion-enhancing activity and no changes in the relative ranking among the plasma samples.

Although treatment of EC with thrombin or histamine increased adhesion within 30 min, no upregulation of adhesion was observed with HP or NHP at this early time point. In general, early primary adhesive events that occur during inflammatory processes involve activation of the local endothelium and rapid expression of P- and E-selectins, resulting in interactions with ligands (i.e., L-selectins, sialyl LewisX) constitutively present on unactivated neutrophils and Mo (6, 22). These transient and reversible interactions lead to the activation of peripheral blood leukocytes resulting in the shedding of L-selectins and the rapid upregulation of functional integrin expression (i.e., conformational alterations in the CD11/CD18 molecules) and adhesive interactions with ICAM-1 (5, 22). However, as shown here, significant and maximum levels of E-selectin expression were observed only after 4–6 h treatment with HP and, to a lesser extent, after NHP (Table 1). Studies addressing the early time course for the appearance of E-selectins (<4 h) did not reproducibly result in increased E-selectin expression and may indicate relatively delayed responses to plasma-derived factors. Alternatively, plasma may contain factors (e.g., soluble L-selectins) that bind E-selectin and actively block their participation in these adhesive interactions. Although immediate adhesive interactions between plasma-activated EC and Mo were not reproducibly observed, the induced and maximal expression of E-selectin on EC after 4–6 h treatment (Table 1) was coincident with early increases seen in adhesive inter-
actions (Fig. 3). Despite this apparent correlation, the role of selectins in the adhesive events observed here remains questionable because adhesive interactions involving other molecules were also observed at this time. This apparent lack of a role for selectins may indicate differences in the orchestration of adhesive interactions that occur during inflammatory responses from those involved in hibernation. It is also possible that under conditions of low blood flow (due to exogenous factors such as hibernation or to endogenous factors such as localized postcapillary venules), perhaps selectin-mediated events are not so critical components of the adhesion cascade resulting in leukocytopenia.

In regard to the time course, and in view of the lack of correlation with early E-selectin expression, the most prominent factor possibly responsible for increased Mo-EC adhesive interactions is ICAM-1. ICAM-1 is a member of the Ig superfamily and is known to be upregulated in response to inflammatory cytokines, phorbol esters, or LPS; ICAM-1 plays an important role in transendothelial migration of leukocytes through its binding to \( \beta_2 \) leukocyte integrins (i.e., LFA-1, Mac-1; see Refs. 28, 37). It is shown here that rat cerebral microvascular EC, on exposure to HP or NHP, exhibit upregulated surface expression of ICAM-1 (Fig. 4A), which was coincident with the upregulation of adhesive interactions between Mo and EC. In most experiments, treatment with undiluted HP or NHP induced similar levels of ICAM-1 expression. However, comparison of effects of diluted plasma indicated the significantly greater potency of HP with regard to upregulating ICAM-1 expression. In addition, the observed lesser potency of summer NHP in induction of ICAM-1 upregulation (Fig. 4B) indicates a possible correlation between ICAM-1 expression and adhesion (i.e., HP > NHP-winter > NHP-summer). This observation supports the implication that ICAM-1 may represent a possible mechanism for the observed reduction in numbers of circulating white blood cells that occurs during onset of hibernation.

The occasional similar potency of undiluted HP and NHP in upregulation of ICAM-1 is contrary to the always superior effect of undiluted HP on upregulating Mo adhesion to EC. In addition, the observation that no significant differences between HP and NHP were observed regarding VCAM-1 expression on EC (Table 1) suggests effects on adhesion molecules other than just ICAM-1 and VCAM-1 or possibly direct effects of plasma on Mo.

MAb-blocking experiments were used to assess the functional roles of specific adhesion molecules involved in Mo binding to EC. In addition to providing evidence for LFA-1 or Mac-1 interactions with ICAM-1, these experiments showed that MAb to VLA-4 inhibited adhesion to HP-treated EC and, to a significantly lesser extent, NHP-treated EC (personal observation). This may be due to greater levels of VLA-4 expression by HP-treated Mo, as opposed to NHP-treated Mo (Table 2). The findings indicate that VCAM-1, the ligand for VLA-4, may play a substantive role in hibernation-induced leukocytopenia, even though no significant differences in the level of VCAM-1 expression were observed after treatment of EC with HP or NHP (Table 1) or with dilutions of HP or NHP (data not shown).

Although the expression of adhesion molecules and adhesive interactions are observed in both hibernation and inflammation, their precise roles in these processes underscore the diversity of these biological responses. Induction of hibernation is not explicitly comparable to the development of a systemic inflammatory response. In fact, no apparent immune responses are observed as a consequence of induction of hibernation. Studies such as those demonstrating marked decreases in mitotic activity of cells in hibernating animals further suggest no apparent activation of cells (27). Differences between hibernation and inflammatory responses may involve signals associated with activation of endothelium or leukocytes (e.g., migration inhibitory factor, PAF, IL-8, etc.). These factors, which are involved in the cascade of events driven by inflammatory mediators, may not be expressed in hibernation. Other factors such as soluble adhesion molecules, differential effects on signal transduction, or integrin activation may account for the disparate responses to hibernation compared with inflammation. The reversible interaction of circulating cells with endothelium during leukocytopenia in hibernating ground squirrels without the consequential activation and elaboration of mediators that is observed in inflammatory responses is similar to margination. This process denotes a diverse activation pathway or a controlled activation where circulating leukocytes are not activated or only stimulated to an intermediate state that is not necessarily associated with extravasation.

Studies regarding the induction of leukocytopenia in hibernation may prove useful for the treatment of disorders associated with leukocyte-mediated damage (i.e., reperfusion injury). Depletion of neutrophils induced by a variety of regimens, including treatment with anti-neutrophil serum (30), vinblastine (43), or mechlorethamine (12, 20) demonstrate decreased size of ischemic deficit and edema formation in leukocytic animals. Similar findings have been reported in animals rendered leukocytopenic by whole body irradiation (24, 41). One of the main difficulties with these experiments concerns the inability to completely deplete neutrophils. Since other circulating cell types such as mononuclear cells or platelets may play roles in ischemia-reperfusion injury (2, 13), other approaches to modulate leukocyte-EC interactions used MAbs to adhesion molecules (i.e., CD11b, CD18, and ICAM-1) (9, 31, 46) or pharmacological strategies. Treatment with factors such as transforming growth factor-\( \beta \) (16), adenosine (36), or \( L \)-arginine (33) has also demonstrated probable roles for leukocytes in ischemia-mediated damage. None of these methods are completely effective or free of undesirable side effects, such as activation of cells or detrimental complications as a consequence of affecting cells other than circulating leukocytes.
If the mechanisms that regulate the controlled leukocytopenia and thrombocytopenia characteristic of hibernation could be identified, then applications could be found in stroke therapy. The ischemic protection observed in hibernating animals is most certainly not a brain-specific event. Peripheral organs may likewise be protected because of a similar mechanism. The possibilities and limitations of hibernation factors as a therapeutic approach for the study of ischemia-reperfusion injury in brain as well as other end organs remain to be explored.

Perspectives

Hibernation represents a unique circannual physiological adaptation that allows animals to survive extended periods of severe environmental stress. To date, it has proven difficult to identify the molecular mechanisms that induce and regulate the hibernating state. If hibernating state-specific adhesive interactions between endothelium and circulating leukocytes could be identified, the findings may help to delineate other interactive physiological responses fundamental to the control of hibernation. This research examines the possible mechanisms that regulate the controlled leukocytopenia characteristic of hibernation in thirteennined ground squirrels. The capacity to reversibly marginate leukocytes and platelets without activation could benefit disease processes that encompass interactions between blood-derived factors and tissue. Identification of the physiological mediators that “park” these cells in a quiescent state could have broad therapeutic applications.

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