Colonic bacterial translocation as a possible factor in stress-worsening experimental stroke outcome

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ING STROKE patients, infections impede neurological recovery and increase morbidity as well as mortality. Thus it is recognized that early mortality is due to direct complications from large strokes. However, infections are the leading cause of death in the postacute phase of stroke, regardless of hospitalization (29). In this context, chronic and acute infectious diseases and subsequent inflammatory status have been implicated as risk factors of stroke and in the pathophysiology of cerebral ischemia (22). Furthermore, experimental evidence demonstrates that animals spontaneously develop diseases due to bacterial infections several days after stroke (33), contributing to the prognosis of the disease. In this context, it has been shown that antibiotic treatment reduces mortality rate and neurological deficit in a mouse model of stroke (28).

On the other hand, epidemiological evidence demonstrates comorbidity between psychological stress and depressive symptoms and stroke risk (20, 41), but only a few experimental studies have examined the possible mechanisms by which prior stress may affect stroke outcome. Interestingly, in the experimental field, some studies have shown an exacerbated stress outcome in animal models of social stress (13, 38). In addition, contributions of our group indicate that a double-faced mechanism is involved in this exacerbation, involving both proexcitotoxic [decreased glutamate uptake concomitant to changes in excitatory amino acid transporter (EAATs) expression] and proinflammatory (increased expression and activity of inflammatory enzymes and cytokine release) effects (5, 6, 25).

Apart from that, there is growing evidence from experimental studies supporting the ability of psychosocial stress, such as immobilization, to induce physiological abnormalities in the gut, such as increased permeability (4, 16, 35). Recent findings show that exposure to acute immobilization stress induces a strong colonic inflammatory response and also alters colonic epithelial barrier, leading to colonic epithelial barrier dysfunction and allowing the passage of intestinal bacteria to internal organs (32).

The aim of the present study was to investigate further the mechanisms by which prior stress worsens stroke outcome. Specifically, we assessed the effect of repeated short exposure to immobilization stress before experimental brain ischemia on colonic inflammatory and functional parameters, as well as on bacterial translocation. Understanding the implication of bacterial translocation after stroke is of great potential clinical relevance, given the high incidence of infections after severe stroke and their main role in mortality and morbidity in stroke patients.

stress; stroke; bacterial translocation; intestinal barrier; stroke outcome

INSTRUCTIONS

MATERIALS AND METHODS

Animals. Adult male Fischer rats weighing 225–250 g were used. All experimental were approved by the Animal Welfare Committee of the Universidad Complutense according to European legislation (2003/65/EC). Rats were housed under standard conditions of temperature and humidity and a 12:12-h light-dark cycle (lights on at 06:00).

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with free access to food and water. All animals were maintained under constant conditions for 7 days before stress.

**Immobilization stress.** Rats were exposed to stress at 1000 in a room adjacent to the animal homeorem. The protocol of stress used was a subacute model consisting of 1 h immobilization for seven consecutive days (S7). Immobilization was performed using plastic rodent restrainers (Decapi-cone type, Braintree, MA) that allowed for a close fit to rats.

**Permanent focal ischemia.** Permanent occlusion was made in the left common carotid artery (CCA) and in the ipsilateral distal middle cerebral artery (MCA), as described previously (12). Briefly, for permanent CCA occlusion, a silk ligature was used, whereas the MCA was occluded by applying an electrocoagulator tip (Select-Sutter Medizintechnik, Freiburg, Germany) while suspending the vessel on a wire hook. After heat was transferred through the wire, the MCA was cauterized and severed.

**Experimental groups.** Several groups (n = 6–12 in each group) were used as follows: 1) a control group (animals were handled for a few seconds one time at 1100); 2) a subacute stress group killed 24 h after the last immobilization exposure (S7); 3) a permanent middle cerebral artery occlusion (MCAO) group killed 24 h after operation (MCAO); 4) group 3 with prior exposure to stress, operated 24 h after the last immobilization exposure, and killed 24 h after operation (S7 + MCAO); 5) group 4 with antibiotic (ab) decontamination (see below) from first day of stress to the moment of death; and 6) other three groups carried out with control, stressed, and MCAO animals receiving antibiotics.

**Bacterial translocation.** Rats were anesthetized (at the time indicated above) with pentobarbital sodium (320 mg/kg ip). After death, cardiac blood, mesenteric lymph nodes (MLN), liver, spleen, and lungs were removed under sterile conditions. Samples were planted in agar plates and analyzed as previously described (32).

**Assessment of colonic permeability.** In the time corresponding to death (see experimental groups for details), rats were anesthetized with isoflurane. A catheter (OD 1 mm) was inserted rectally at 4 cm from the anus. We slowly perfused 1.5 ml of 51Cr-EDTA (Perkin-Elmer, Madrid, Spain) in 0.5 ml of 0.9% NaCl in the colon (0.25 ml/h). After perfusion (2 h), rats were killed using pentobarbital sodium, and blood was collected by cardiac puncture. The 51Cr-bound radioactivity was counted using a gamma counter to measure radioactivity of the samples. The permeability was expressed as the ratio between blood and total 51Cr instilled and reported as a percentage, as previously described (32).

**Myeloperoxidase activity.** Colonic samples were homogenized (glass/glass) and centrifuged. Tissue levels of myeloperoxidase (MPO) activity were determined on supernatants using hydrogen peroxide as substrate for the enzyme. One unit of MPO activity was defined as that converting 1 μmol of hydrogen peroxide to water in 1 min at 40°C (2).

**Assessment of colonic damage.** The colon was opened longitudinally and scored for macroscopic visible damage on a 0–4 scale according to criteria previously described (11): 0, normal mucosa; 1, hyperemia and edema, no ulcers; 2, as before + small linear ulcers or petechiae; 3, as before + wide ulcers and necrosis and/or adhesions; 4, as before + megacolon and/or stenosis and/or perforation.

**Western blotting.** Colonic protein levels of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) were analyzed by Western blot. Samples containing 40 μg of protein were loaded, and the proteins were size-separated in 7–10% SDS-PAGE (90 V). Proteins were blotted on a polyvinylidene difluoride membrane (Hybond-P; Amersham Biosciences) and incubated with specific primary antibodies against COX-2 (1:1,000 dilution; Santa Cruz Biotechnologies) or iNOS (1:500; Santa Cruz Biotechnologies). Proteins recognized by the antibody were revealed by the ECL-kit (Amersham Biosciences) following the manufacturer’s instructions. Finally, β-actin levels were used as loading controls for total protein expression. Autoradiographs were quantified by densitometry using an image analyzer (Image J 1.39a; National Institutes of Health), and several time expositions were analyzed to ensure the linearity of the band intensities.

**Antibiotic decontamination.** We followed a described protocol that results in intestinal decontamination (1). Rats were given drinking water ad libitum containing streptomycin sulfate (2 mg/ml) and penicillin G (1,500 U/ml) from the first day of stress (at 0800) until the moment of death to reduce the indigenous gastrointestinal microflora.

**Infarct size.** Infarct volume was measured as previously described (24). In short, brains were removed, and a series of 2 mm of coronal slices was obtained and stained in 1% 2,3,5-triphenyl-tetrazolium chloride in 0.1 M phosphate buffer. Infarct volumes were measured by sampling stained sections with a digital camera (Nikon Coolpix 990), and the image of each section was analyzed by an image analyzer (Image J 1.39a; National Institutes of Health). The contralateral hemisphere perimeter was overlapped on the ipsilateral hemisphere to exclude edema, and infarct borders in both the cortex and subcortex (corpus caudatum excluded) were delineated. The area of infarct was determined by counting pixels contained within the outlined regions of interest and expressed in square millimeters. Infarct volumes (in mm³) were integrated from the infarct areas over the extent of the infarct calculated as an orthogonal projection.

**Plasma corticosterone levels.** Plasma was obtained from blood samples by centrifuging the sample at 1,500 g for 10 min immediately after stress taken at 1100. Determinations in all experimental and control groups were carried out at this time to avoid circadian variability. Samples were stored at −80°C before assay by using a commercially available kit by RIA of 125I-labeled rat corticosterone (DPC, Los Angeles, CA). A gamma counter was used to measure radioactivity of the samples.

**Protein assay.** Proteins were measured using bicinchoninic acid.

**Materials and statistical analysis.** Reagents and drugs were from Sigma (Madrid, Spain) or as indicated in the text. Results are expressed as means ± SD of the indicated number of experiments; statistical analysis involved one-way ANOVA (or the Kruskal-Wallis test when the data were not normally distributed) followed by individual comparisons of means (Student-Newman-Keuls’s or Dunn’s method when the data were not normally distributed). Comparisons between the groups of rats under two different factors (presence or not of stress and presence or not of antibiotic) were performed with two-way ANOVA with the post hoc Newman-Keuls’s test (integroup analysis). P < 0.05 was considered statistically significant.

**RESULTS**

**Effect of stress and stroke on bacterial translocation.** A conjunction of stress and ischemia (S7 + MCAO) induced translocation of different bacteria species (colony-forming units (CFUs)) to MLNs, spleen, liver, and lung in 100% of animals (Fig. 1). Liver showed the highest number of CFUs per gram of tissue, followed by spleen, lung, and MLNs. All bacterial species detected were aerobic or anaerobic gram-positive (*Enterococcus faecalis, Staphylococcus aureus, Peptostreptococcus sp., and Propionibacterium acnes*) with the exception of *Bacteroides fragilis*, which is anaerobic gram-negative. Neither stress nor MCAO alone resulted in the presence of bacteria in any of the organs studied. The groups with antibiotic decontamination did not show bacterial translocation (data not shown).

**Effect of stress and stroke on colonic IgA levels.** To assess possible mechanisms of this bacterial translocation, levels of one of the main colonic defense mechanisms, IgA, were assessed. Secretory IgA represents a first-line defense mechanism against...
Damage compared with control, S7, or MCAO animals (Fig. 3).

In this study, the combination of immobilization and stroke activity in the colon correlates with bacterial translocation (32). Also, we have shown previously that MPO infiltration and has been widely used as a marker of acute inflammation (37). The tissue activity of this enzyme correlates linearly with neutrophil infiltration and has been widely used as a marker of acute inflammation (37). The tissue activity of this enzyme correlates linearly with neutrophil infiltration and has been widely used as a marker of acute inflammation (37).

Effect of stress and stroke on colonic MPO activity and colonic macroscopic damage. A common finding observed in several models of stress-induced intestinal inflammation is an increased MPO activity in tissue homogenates (4). The tissue activity of this enzyme correlates linearly with neutrophil infiltration and has been widely used as a marker of acute inflammation (37). The tissue activity of this enzyme correlates linearly with neutrophil infiltration and has been widely used as a marker of acute inflammation (37).

Effect of stress and stroke on colonic permeability to $^{51}$Cr-EDTA. The percentage of $^{51}$Cr detected in the blood of rats instilled with $^{51}$Cr-EDTA remained the same for control, S7 + MCAO, and S7 + MCAO(ab) groups (0.396 ± 0.018, 0.376 ± 0.020, and 0.381 ± 0.037, respectively).

Effect of stress and stroke on colonic COX-2 and iNOS expression. To clarify the possible molecular pathways involved in the bacterial translocation detected, the expression of two major inflammatory enzymes, COX-2 and iNOS (both previously related with colonic inflammation and bacterial translocation (11, 32), was assessed in colonic homogenates. The S7 + MCAO group exhibited significantly higher values of COX-2 and iNOS expression than in the other groups, and both stressed and MCAO groups showed higher COX-2 and iNOS expression relative to the control group (Fig. 4, A and B).

Effect of antibiotic decontamination on infarct volume. As we described previously, stress before ischemia increases infarct volume (Fig. 5). The intestinal decontamination did not modify the infarct volume in prior stressed animals [S7 + MCAO(ab)] when compared with the same experimental group without antibiotic administration (S7 + MCAO) nor the MCAO infarct volume in nonstressed animals.

Effect of stress and stroke on plasma corticosterone levels. To validate our stress protocol and also to detect a possible interference in the stress response following pharmacological treatment, the plasma corticosterone levels were analyzed. Both stress and MCAO and their combination (S7 + MCAO) increased plasma corticosterone levels (173.13 ± 18.9, 284.33 ± 23.1, and 401.56 ± 37.4 ng/ml, respectively; $P < 0.05$). Values obtained in control animals (105.22 ± 10.7 ng/ml) matched the kit manufacturer’s expected values in adult male rats at the time of blood extraction (~1100). There were no significant differences between groups with and groups without antibiotic decontamination (data not shown), indicating that our pharmacological treatment had no effect on the stress response or on the hypothalamic-pituitary-adrenal axis (HPA) axis activation produced by immobilization stress and MCAO.

DISCUSSION

The results of the present study demonstrate that immobilization stress before brain ischemia induces subtle colonic inflammation and dysfunction, leading to the presence of bacteria of intestinal origin in different organs. These findings...
also support the previously demonstrated notion that subacute stress worsens stroke outcome (e.g., stress increases infarct size and decreases behavioral and neurological scores).

In previous works (5, 6, 25), we have demonstrated that acute stress (i.e., 6 h immobilization) worsens experimental stroke outcome by increasing the interleukin (IL)-1β levels in the postsischemic cortex. However, subacute immobilization stress (i.e., 1 h/day during 7 days) induces an increase in tumor necrosis factor-α but not in IL-1β levels in the cortex. This form of subacute stress also causes an increase in the excitotoxic response (increasing synaptic glutamate levels and decreasing the expression of receptors that uptake glutamate). Thus different stress protocols can increase the inflammatory response in the brain following experimental ischemia via different mechanisms.

In the present study, we add an alternative component to the excitotoxic and inflammatory mechanisms discussed above. Here, we have demonstrated that subacute immobilization stress before experimental cerebral ischemia leads to bacterial translocation. It has been described that infections are a leading cause of death in patients with acute central nervous system (CNS) injury, such as stroke. Most infections after CNS injury may be attributed to exposure of these patients to invasive medical procedures and hospitalization, dysphagia, and aspiration, etc. (29). However, these factors do not sufficiently explain why stroke patients have such a high risk of infection. Thus it has become clear that CNS injury is an independent risk factor that, through specific mechanisms, significantly increases susceptibility to infection (15). Furthermore, experimental studies have demonstrated that stroke increases the risk of suffering severe bacterial infections (mostly pneumonia and sepsis) (33).

Interestingly, in a previous study, our group demonstrated that acute immobilization stress produces bacterial translocation to different organs, such as MLNs, spleen, and liver (32). In the present study, the analysis of bacteria species reveals that all bacteria detected after a stress + MCAO protocol are gram-positive, with the exception of B. fragilis, which is gram-negative. These bacteria are members of normal intestinal microbiota, thus suggesting that bacterial translocation might be a likely source of bacterial infections in stress-induced stroke worsening.

The intestinal tract is an internal source of bacteria. A severe brain trauma can initiate a cascade of changes in the gut such as an increase in intestinal/colonic permeability (15) and subsequent luminal bacterial translocation (39). Second, it has been hypothesized that brain ischemia, as a stressful situation (both physiological and psychological), can lead to significant structural and functional anomalies in the intestinal barrier (18): dysfunction leading to bacterial translocation and increasing blood endotoxin levels, which in turn can provoke a generalized exaggerated inflammatory response in brain (30). Third, previous studies show that short-term immobilization produces a significant increase in colonic permeability to 51Cr-EDTA and disruption of epithelial tight junctions (TJs) (32).

The gastrointestinal tract is a well-known target of physiological modifications that occur during stressful life events. Stress is known to increase intestinal permeability, leading to an excessive uptake of luminal antigens and bacterial products that may initiate or modulate an inflammatory response (10, 36). In addition, stress-related factors, such as corticotropin-releasing hormone (CRH) and glucocorticoids (GCs), have been shown to increase intestinal permeability (26, 35). Interestingly, our results show that stressed rats subjected to MCAO (the only experimental group where we detected the presence of bacteria out of the colon) exhibit the highest value of corticosterone (the main GC in rodents). Thus the increase in CRH and GC levels can be an explanation for the bacterial translocation detected.

On the other hand, previous studies have shown that experimental ischemia induces long-lasting depression of the cell-mediated immunity associated with spontaneous bacteremia and pneumonia (33). In addition, these studies also show that pharmacological inhibition of the activation of either sympathetic nervous system (SNS) or HPA prevented the stroke-induced lymphocyte apoptosis, lymphopenia, and monocytic deactivation. However, poststroke lymphocyte dysfunction and bacterial infections were only prevented by SNS inhibitors (33). Thus, considering the well-known actions of psychological stress on SNS activation, we cannot discard the SNS as a factor involved in the bacterial translocation detected here.

In addition, in a previous study, we demonstrated that a decrease in the levels of colonic IgA is related to bacterial translocation after acute immobilization stress (32). Secretory IgA represents a first line of defense against pathogens in the mucosal surfaces by means of, among other mechanisms, agglutinating bacteria and preventing them from binding to intestinal epithelial cells. Therefore, a decrease in the amount of IgA in the colonic lumen could contribute, together with an increased colonic permeability related to TJ disruption, to bacterial translocation and increased antigen uptake (4). Interestingly, now we show that the group with stress before MCAO (S7 + MCAO; the experimental group with presence of bacteria) presents a reduction in IgA levels when compared with the rest of the groups. If the stress-induced decrease in IgA levels in the colonic mucosa results from a true decrease of IgA synthesis by lamina propria plasma cells, or if there is a lack of transportation to the colonic lumen, deserves further investigation (42).

### Table 1. Macroscopic colonic aspect

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>S7</th>
<th>MCAO</th>
<th>S7 + MCAO</th>
</tr>
</thead>
<tbody>
<tr>
<td>No lesions (score = 0)</td>
<td>10/10</td>
<td>9/10</td>
<td>8/10</td>
<td>2/10</td>
</tr>
<tr>
<td>Edema, hyperemia (score = 1)</td>
<td>0/10</td>
<td>1/10</td>
<td>2/10</td>
<td>8/10</td>
</tr>
<tr>
<td>Perforation or stenosis (score = 4)</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Mean score ± SE</td>
<td>0 ± 0.1</td>
<td>0.1 ± 0.05</td>
<td>0.2 ± 0.14</td>
<td>0.8 ± 0.14*</td>
</tr>
</tbody>
</table>

S7, 7 consecutive days of stress; MCAO, middle cerebral artery occlusion. See methods for details; n = 6 experiments. *P < 0.05 vs. other groups.
MPO is an enzyme found in neutrophils, and its activity in the colon is linearly related to neutrophil infiltration (37). These cells are important sources of oxygen radicals and nitrogen species, which accumulate after several days, leading to delayed tissue damage (17). An increase in MPO activity has been related with colonic dysfunction and bacterial translocation (32). The present study shows that animals exposed to stress before MCAO (S7/MCAO) exhibit an increase in colonic MPO activity when compared with the rest of the treatment groups. Similarly, an increase in the expression of COX-2 and iNOS has been also correlated with bacterial translocation (32). In fact, iNOS-derived nitric oxide (NO) has been shown to increase intestinal epithelial permeability both in vitro and in vivo (32, 36). This effect seems to be related to NO-induced cytoskeleton rearrangement and subsequent TJ dysfunction (4, 32). Furthermore, acute stress induces mast cell degranulation in the lamina propria as well as release of prostaglandins (4), and this process is downstream of COX-2 signaling. Interestingly, our results show an increase in the expression of these two proinflammatory enzymes, and also we have detected that stress exposure followed by ischemia (S7 + MCAO) induced an increase in macroscopic damage (related to inflammation) compared with the rest of experimental groups.

One of the most used experimental approaches to assess the intestinal permeability in vivo is the lumen-to-blood ratio after instillation of $^{51}$Cr-EDTA or other molecules such as small carbohydrates (4, 32). However, rats subjected to stress and experimental ischemia did not show differences in the $^{51}$Cr-EDTA permeability. A possible explanation is the timing of the experiment. The permeability assay was performed 24 h after MCAO (the time point at which infarct volume and the rest of the parameters were analyzed), but it is plausible that the colonic dysfunction occurs in any instant after MCAO and is not resolved until later than 24 h. Thus, although we cannot affirm that we detect a colonic dysfunction, the results obtained in other parameters (iNOS and COX-2 expression, IgA levels, MPO activity, etc.) and the fact that in standard conditions the liver and the spleen are always germ-free organs suggest that the colonic dysfunction and the subsequent bacterial translocation are a reasonable explanation for the presence of bacteria.

Finally, our results with antibiotic pretreatment are in agreement with previous studies demonstrating an absence of protection by antibiotic administration in models of stroke (19) and in clinical trials (9). There also are studies showing a neuroprotective action of antibiotic treatment. However, in these studies, the drug used is minocycline, an antibiotic with neuroprotective actions per se (14), or ceftriaxone, a $\beta$-lactam...
antibiotic that offers neuroprotection by increasing glutamate transporter 1 (EAAT-1) expression (23, 34). Here, we used a stringent protocol (streptomycin and penicillin G) only for its intestinal decontamination effects (1). Even more, this decontamination protocol prevented the bacterial translocation presented in groups without antibiotic treatment and did not modify the glutamate transporter expression in the brain cortex (data not shown).

In addition, previous works have shown effective ischemic protection by antibiotics (28), but not 24 h after stroke as we have examined in this study. Thus it seems that there is an antibiotic-induced protection when longer recovery time points are analyzed. These data suggest that long-term prevention of bacterial infections leads to neuroprotection, but the authors do not discard that the antibiotics used may have other, more direct mechanisms of action (e.g., antiexcitotoxic, anti-inflammatory, etc.) apart from their antibacterial effects (14, 23, 28, 34). In this study, we did not detect any neuroprotection, but our time point is shorter to address our hypothesis of bacterial translocation induced by stress. Thus we cannot discard the possibility that the decontamination protocol used could have neuroprotective effects in a long-term analysis (i.e., >72 h after MCAO), given that the detrimental actions of infection occur in the postacute phase of stroke. Moreover, our results also indicate that the measurement of infarct volume at 24 h may not be the best assessment (in the acute phase) of the role of bacterial translocation on stroke outcome, and future studies should assess additional endpoints (e.g., behavioral deficit, neurological tests, etc.).

The analysis of bacteria species reveals that all bacteria detected are gram-positive with the exception of B. fragilis, which is gram-negative. A possible consequence of translocated bacteria is the specific response elicited by the innate immune system involving Toll-like receptor (TLRs) signaling, not only systemically, but also in the brain (27, 31). During inflammation in the brain, TLRs are expressed in microglia, astrocytes, and neurons (21, 40) and activate nuclear factor-kB signaling pathways linked to the transcription of many proinflammatory genes such as COX-2 and iNOS (31). In this context, it has been demonstrated that TLRs are involved in the inflammatory pathology outcome. Indeed, TLR2 and TLR4 propagate stroke-induced CNS injury (3, 8, 21), and we have demonstrated recently that TLR4-deficient mice after subacute immobilization stress have a better behavioral condition compared with mice that express TLR4 normally after subacute immobilization stress have a better behavioral condition compared with mice that express TLR4 normally and that this effect is associated with a decreased inflammatory response (COX-2 and iNOS expression) and lipid peroxidation in brain tissue (7). Thus, since gram-positive and the gram-negative bacteria activate TLR2 and TLR4, respectively, a working hypothesis is that translocated bacteria may be responsible for activation of TLR-dependent inflammatory pathways in the brain.

**Perspectives and Significance**

Infections are the leading cause of death in the postacute phase of stroke, regardless of hospitalization, and thus infectious diseases and subsequent inflammatory status have been implicated in the pathophysiology of cerebral ischemia. The present work is an extension of our earlier work that demonstrates that subacute immobilization stress before MCAO worsens stroke outcome and suggests that bacterial translocation to different organs can be a pathway implicated. This study shows that stress before stroke induces a bacterial translocation from the intestinal flora. Considering that a CNS injury significantly increases susceptibility to infection, the present work indicates that this endogenous bacteria source must be taken into account. The pharmacological modulation of this pathway represents a possible therapeutic approach in stress-related disorders and particularly in CNS disorders such as brain ischemia, given the high incidence of infections after severe stroke and their main role in mortality and morbidity in stroke patients. Clearly, more investigation is required, but the presence of bacteria shown in this study and the results obtained in others support the notion that clinical studies of preventive antibacterial therapy regimens in brain ischemia patients are needed.

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