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BLOCKADE OF ADRENORECEPTORS INHIBITS THE SPLENIC RESPONSE TO STROKE

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Abstract

Recent studies have highlighted the involvement of the peripheral immune system in delayed cellular degeneration after stroke. In the permanent middle cerebral artery occlusion (MCAO) model of stroke, the spleen decreases in size. This reduction occurs through the release of splenic immune cells. Systemic treatment with human umbilical cord blood cells (HUCBC) 24 hours post-stroke blocks the reduction in spleen size while significantly reducing infarct volume. Splenectomy two weeks prior to MCAO also reduces infarct volume, further demonstrating the detrimental role of this organ in stroke-induced neurodegeneration. Activation of the sympathetic nervous system after MCAO results in elevated catecholamine levels both at the level of the spleen, through direct splenic innervation, and throughout the systemic circulation upon release from the adrenal medulla. These catecholamines bind to splenic α and β adrenoreceptors. This study examines whether catecholamines regulate the splenic response to stroke. Male Sprague-Dawley rats either underwent splenic denervation two weeks prior to MCAO or received injections of carvedilol, a pan adrenergic receptor blocker, prazosin, an $\alpha 1$ receptor blocker, or propranolol, a β receptor blocker. Denervation was confirmed by reduced splenic expression of tyrosine hydroxylase. Denervation prior to MCAO did not alter infarct volume or spleen size. Propranolol treatment also had no effects on these outcomes. Treatment with either prazosin or carvedilol prevented the reduction in spleen size, yet only carvedilol significantly reduced infarct volume ($p < 0.05$). These results demonstrate that circulating blood borne catecholamines regulate the splenic response to stroke through the activation of both α and β adrenergic receptors.

INTRODUCTION

Infiltrating immune cells have long been known to contribute to secondary cellular death associated with stroke. Recent studies have shown that the spleen plays a role in the stroke-induced immune response. Transfusion of human umbilical cord blood cells (HUCBC) after

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stroke has provided insight into the role of the spleen in stroke-induced neurodegeneration. This treatment is effective up to 48 hours after permanent middle cerebral artery occlusion (MCAO) in rats (Newcomb et al., 2006). The HUCBC migrate to the injured hemisphere of the brain and to the spleen when transfused 24 hours after MCAO (Vendrame et al., 2004). Moreover, the spleen shrinks in response to ischemic injury, and this reduction in size is associated with an increase in circulating regulatory T cells and macrophages (Offner et al., 2006a, Offner et al., 2006b, Vendrame et al., 2006).

Administration of HUCBC after stroke not only blocks the reduction in spleen size, but alters splenic cytokine expression from a pro-inflammatory (TNF- α , IL-1 β) to an anti-inflammatory (IL-10) profile (Vendrame et al., 2006). A subsequent study then showed that splenectomy two weeks prior to MCAO resulted in a >80% decrease in infarction, and this effect was accompanied by a significant reduction in the number of infiltrating neutrophils and activated microglia/macrophages in the injured brain (Ajmo et al., 2008). Taken together, these results suggest that the spleen acts as a reservoir of immune cells that are released in response to cerebral ischemia.

Sympathetic innervation accounts for 98% of the nerve fibers innervating the spleen (Klein et al., 1982) through the splenic nerve, with norepinephrine as the primary neurotransmitter. The neural circuitry regulating the immune response through these splenic fibers has been identified with neuronal tracing studies using the trans-synaptic retrograde tracer pseudorabies virus (Cano et al., 2001). Central pathways labeled with this technique include the parvocellular paraventricular nucleus (pPVN), lateral hypothalamus (LH), and medial preoptic (MPO) nucleus of the hypothalamus. Most of these fibers innervate the white pulp, specifically modulating lymphocytes, eosinophils, mast cells, and macrophages (Felten et al., 1985). Sympathetic nervous system (SNS) activation leads to splenic contraction through α 1 receptors on smooth muscle cells. Further, increased cortisol production (Feibel et al., 1977) leads to activation of the hypothalamic-pituitary-adrenal axis (Fassbender et al., 1994) and SNS hyperactivation (Feibel et al., 1977). There is also a loss of parasympathetic tone (Korpelainen et al., 1994).

The combined effects of this autonomic dysregulation would be increased adrenergic (from adrenals) or noradrenergic pro-inflammatory influences in the spleen and dampening of anti-inflammatory processes. We hypothesize that the splenic response is regulated by sympathetic neurotransmission via the splenic nerve and/or by increased circulating catecholamines released from the adrenal medulla. In the present study, we quantified cerebral infarct volume and spleen weight after 1) mechanical denervation of the splenic nerve or 2) in the presence of adrenoceptor blockade using the α , β adrenergic antagonist carvedilol, the α 1 receptor antagonist prazosin, or the β blocker propranolol. Denervation had no effect on the reduction of spleen size or neurodegeneration occurring at the cerebral injury site. Blockade of α and β adrenergic receptors significantly inhibited the reduction in spleen size and reduced infarct volume, while α 1 receptor antagonism only blocked the shrinkage of the spleen. Our findings indicate that activation of both α and β adrenergic receptors mediate the splenic response to stroke, and therefore are potential targets for therapeutic treatment against ischemic injury.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 300 to 350 g were housed in a climate controlled room with water and laboratory chow available *ad libitum*. Animals were cared for according to the NIH guidelines for the Care and Use of Animals and overseen by the University of South Florida's Institutional Animal Care and Use Committee. A total of 44 animals were used in this study. Experimental groups were as follows: sham-MCAO (n=8),

sham-splenectomy (n=3), sham-MCAO+sham-splenectomy (n=5), sham-MCAO +denervation (n=3), MCAO-only (n=8), denervation-MCAO (n=9), and splenectomy-MCAO (n=8).

Splenectomy and Denervation

Rats were anesthetized with 3 to 4% isoflurane in 100% oxygen at a flow rate of 2 L/min. Splenectomy or denervation was performed by making a 2 cm dorsal midline skin incision at the caudal terminus at the level of the 13th rib. With blunt forceps, the spleen (with accompanying blood vessels and pancreatic tissue) was exteriorized through the incision. For splenectomy, the blood vessels were ligated, the spleen removed and stored at -80°C . For the denervation experiments, the nerves leading into the spleen were cut after which the spleens were placed back into resting position beneath the abdominal wall. The abdominal wall and incision were then closed with sutures. Rats were allowed to recover 2 weeks prior to the MCAO surgery.

Laser Doppler Blood Flow Measurement

Prior to MCAO surgery, anesthesia was induced with oxygen containing 5% isoflurane in an induction chamber, and the head and neck shaved. Before Doppler insertion, rats were treated prophylactically with Ketoprofen (10 mg/kg IM), atropine (0.25 mg/kg SC), and Baytril (20 mg/kg IM), which were approved according to IACUC guidelines. Ketoprofen injections were continued 3 days post-MCAO to minimize pain and discomfort. Each rat was placed on the operating table dorsal-side-up and anesthesia was supplied through a nose cone (3 to 4% isoflurane in 100% oxygen, flow rate 2 L/min). Using a scalpel, an incision was made just lateral to the midline of the dorsal plates of the skull on the side that was ipsilateral to the MCAO. Once the incision was made, the skin was spread open and the membrane covering the skull pushed aside with a cotton-tipped applicator. Using a microdrill, a small hole was drilled into the skull at 1 mm posterior and 4 mm lateral to bregma. A hollow, stainless steel guide screw was screwed into the hole in the skull and a fiber optic cable (500 μm) was inserted through the screw guide and secured with super glue. Blood perfusion in the brain was then detected using the Moor Instruments LTD laser Doppler with MoorLAB proprietary Windows-based software on a standard laptop. Once surgery was complete, the screw guide was removed and the scalp incision closed with surgical sutures. Rats that did not show $\geq 60\%$ reduction in perfusion during MCAO were excluded from the study.

Permanent Middle Cerebral Artery Occlusion Model

Permanent focal ischemia was achieved during MCAO by using the intraluminal suture technique (Longa et al., 1989). After the Doppler probe was set, an incision was made in the neck and the right common carotid, external carotid, internal carotid, and pterygopalatine arteries were isolated by blunt dissection. The external carotid artery was ligated, cut, and a 4cm long 4-0 monofilament was advanced through the internal carotid artery into the middle cerebral artery. The filament was then permanently anchored at the internal/external carotid junction to produce permanent occlusion. The incision was then sutured and the animal was given a 1ml injection (s.c.) of saline.

Drug Administration

Carvedilol (1.5 mg/kg twice daily, s.c.; Tocris Cookson Ltd., Ellisville, MO), prazosin (1 mg/kg daily, s.c.; Tocris Cookson Ltd., Ellisville, MO), propranolol (30mg/kg daily, s.c.; Sigma, St. Louis MO) or vehicle (100% ethanol for carvedilol and prazosin; sterile water for propranolol; s.c.) were injected at 24 hours preoperative, 0 hours, and 24 hours postoperative. Doses were selected based on safety and efficacy as reported in the literature (Savitz et al., 2000, Prass et al., 2003, Maximo Cardoso et al., 2006)

Blood Pressure Measurement and Quantification

Blood pressures were collected non-invasively in the morning at 24 hours preoperative, at 0 hours (the day of surgery), and at 24 hours postoperative. All readings were taken prior to daily injections. Groups were as follows: sham-MCAO (n=3), MCAO+vehicle (n=6), MCAO+prazosin (n=5), and MCAO+carvedilol (n=5). The MCAO+propranolol group was omitted because this compound showed no efficacy in either inhibiting the MCAO-induced reduction in spleen weight or decreasing infarct volume. Readings were collected from the ventral artery at the base (widest portion) of the tail using a Critikon #1 neonatal inflatable cuff (GE Healthcare, Milwaukee, WI) attached to an Advisor Multiparameter Vital Sign Monitor (Smiths Medical/Surgivet Division, Waukesha, WI). Each rat was gently restrained in a plastic cone and placed in a dark tunnel with only the tail exposed. The tail was warmed during collection by applying warm water with a gauze pad. Three consecutive attempts (3 min maximum each) were made to collect readings each day. If it was not possible to obtain any readings, the rat was returned to its cage and a final attempt was made following the measurement of all other rats (24 hours preoperative = 2, 0 hours = 3). Readings were obtained for all animals. For data analysis, group means were subjected to one-way ANOVA with repeated measures.

Brain Extraction and Sectioning

Animals were euthanatized at 48 hours post MCAO and perfused with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The brains were harvested, post-fixed in paraformaldehyde and saturated with increasing sucrose concentrations (20%, 30%) in phosphate buffered saline (PBS, pH 7.4). Brains were frozen and sectioned coronally at 30 μ m thickness using a cryostat. Sections were either thaw-mounted onto glass slides or placed in Walter's Anti-freeze cryopreservative and stored at -20°C .

Fluoro-Jade Histochemistry

Six sections were chosen from each rat brain at specific intervals (from 1.7 mm anterior to bregma through -3.3 mm posterior to bregma) that included striatal and hippocampal regions of the infarct. Fluoro-Jade (Histochem, Jefferson, AR) staining was performed to label degenerating neurons. This method was adapted from that originally developed by Schmued et al. (Schmued et al., 1997) and has been subsequently detailed (Duckworth et al., 2005). Tissues were thaw-mounted and dried onto glass slides. Slides were then placed in 100% ethanol for 3 min followed by 70% ethanol and deionized water for 1 min each. Sections were then oxidized using a 0.06% KMnO_4 solution for 15 min followed by three rinses of double distilled water for 1 minute each. Sections were then stained in a 0.001% solution of Fluoro-Jade in 0.1% acetic acid for 30 min. Slides were again rinsed, allowed to dry at 45°C for 20 min, cleared with xylene and coverslipped with DPX mounting medium (Electron Microscopy Sciences, Ft. Washington, PA).

Infarct Volume Quantification

Infarct volumes were calculated separately from both the Fluoro-Jade and thionin labeled sections. Images of six 30 μ m thick brain sections were taken at intervals from $+1.7$ to -3.3 mm from bregma to determine the volume of infarction. Stained tissues were digitally photographed with an Olympus IX71 microscope controlled by DP manager software (Olympus America Inc, Melville, NY) at a magnification of 1.25x. Each image was edited with Jasc Paintshop Pro to sharpen and enhance contrast to the same specifications. Total volume of neurodegeneration was measured using the NIH Image J software. The volumes of the contralateral hemispheres were also measured and used to control for possible edema in the ipsilateral hemispheres by dividing each individual infarct by the contralateral measurement.

Tyrosine Hydroxylase Immunohistochemistry

Thaw-mounted sections were equilibrated to room temperature and rinsed with PBS. Sections were incubated for 20 min at room temperature in a 3% H₂O₂/ddH₂O solution to reduce endogenous peroxidase activity. After additional washes in PBS, samples were placed in a permeabilization buffer containing 10% goat serum, 3% 1M lysine, and 0.3% Triton X-100 in PBS for 1 hr at room temperature. Next, they were incubated overnight at 4°C in a humidified chamber with an anti-tyrosine hydroxylase (TH) rabbit polyclonal antibody (1:1,000 dilution; Pel Freez, Rogers, AR) in primary/secondary antibody solution (PBS, 2% goat serum and 0.3% Triton X-100). Sections were then washed with PBS and incubated for 1 hr at room temperature with a biotinylated goat anti-rabbit (1:300 dilution, Vector Laboratories, Burlingame, CA) secondary antibody in primary/secondary antibody solution. After additional PBS washes, sections were incubated for 1 hr in avidin-biotin horseradish peroxidase macromolecular complex (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA). The ABC solution was then removed, sections were washed with PBS and incubated briefly in metal-enhanced 3',3'-diamino benzidine (DAB) in 0.03% H₂O₂ (ImmunoPure[®] Metal Enhanced DAB Substrate Kit, Pierce, Rockford, IL). After a final series of washes in PBS, slides were dried, dehydrated, cleared with xylene and coverslipped with DPX mounting medium (Electron Microscopy Sciences, Ft. Washington, PA).

Tyrosine Hydroxylase Quantification

Digital photographs of spleen sections immunostained with TH were taken at 10x magnification with a Zeiss Axicam Color camera and Zeiss Axioscope 2 microscope controlled by Openlab software (Improvision Ltd, Lexington MA). Images were edited with Jasc Paintshop Pro to sharpen and enhance contrast of the images to the same specifications. Image analyses were performed using NIH Image J software to determine the total area of neurons labeled positive with TH by particle analyses across the tissue sections.

Flow Cytometry for Splenic Cell Profile

Splenocyte Isolation—Rat spleens were harvested and placed in ice cold culture media (RPMI-1640 medium containing 5% fetal bovine serum). They were then washed once with additional media and placed into stomacher bags with approximately 12 ml of culture media. The stomacher bags were secured, placed into the stomacher, paddled for 10 seconds and placed immediately on ice. The homogenized spleen solutions from each bag were pipetted into 50 ml centrifuge tubes and culture media was added to a volume of 40 ml for washing. The solution was centrifuged at 1200 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 3 ml of 0.5% PharmLyse (BD Biosciences) in dH₂O and incubated for 20–30 seconds. The cell suspension was washed with 30–50 ml of RPMI culture media and centrifuged at 1200 rpm for 10 min. The supernatant was decanted and the pellet was resuspended in 10 ml of culture RPMI media. Cell count and viability were determined using the Trypan Blue exclusion method.

Preparation of Spleen Cells for FACS Analysis—1 x 10⁶ spleen cells were placed into 200 µl of blocking solution containing Fc-block[™] (BD Pharmingen) to reduce non-specific antibody binding and incubated for 1 hour. After blocking, cells were labeled with at least two of the following fluorescent conjugated antibodies (BD Pharmingen) assigned as follows: group I - CD3 (PE), CD4 (APC) and CD45 (FITC); and group II - CD8a(PE) and HIS48 (granulocyte) (FITC). Cells were incubated with antibodies in the dark for 1 hour. The cells were then washed in 10ml of PBS/FBS solution and centrifuged for 7 minutes at 400g (no brake). The supernatant was removed and the cells were resuspended in 400 µl of sterile PBS and transferred to flow cytometer tubes (BD Falcon polystyrene 5 ml tubes) for FACS analysis. Control samples matched for each fluorochrome and each antibody were used to set

compensation and negative staining criteria. Dead cells were excluded using propidium iodide staining. FACS analysis was performed on the BD LSR II flow cytometry system. Data was analyzed using BD FACS Diva software.

ELISA Assay for Measurement of Cytokine Levels

For ELISA, 96-well plates were coated with capture antibody (2 ng/ml) and incubated overnight at 4°C. The following morning, plates were washed with Tris buffered saline (TBST; 20 mM Tris pH 7.5, 150 mM NaCl, and 0.05% Tween 20) and blocked for 1 hour at room temperature with 1% BSA in TBST. A standard curve was prepared using recombinant protein standards. Following block, the standards and unknowns were incubated for 1 hr at room temperature, washed with TBST and incubated for 2 hrs at room temperature with biotinylated detection antibody (100 ng/ml). After incubation, plates were washed with phosphate buffered saline (PBS; 3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl, 135 mM NaCl, pH 7.4). Plates were then incubated for 20 min at room temperature with streptavidin conjugated to horseradish peroxidase, washed again, and incubated for 5 minutes in the dark with 100 µl of 3,3',5,5'-tetramethyl-benzidine (TMB) (Sigma-Aldrich, St. Louis, MO). Finally, 50 µl of 1 M H₂SO₄ stop solution was added to quench the reaction, and optical density was measured using a microplate reader set at 450 nm.

Antibodies used were purchased from R&D Systems, Inc. (Minneapolis, MN) and consisted of the following: for capture, tumor necrosis factor- α (MAB510 anti-rat antibody;) and interleukin-10 (MAB519 anti-rat antibody). For detection, tumor necrosis factor- α (#BAF510 biotinylated anti-rat antibody) and interleukin-10 (#BAF519 biotinylated anti-rat IL10 antibody). The IL-1 β ELISA was conducted using a commercial kit generated against rat IL-1 β (#88-6010; eBioscience, San Diego, CA). All antibodies used were included in the kit, and all steps were performed as indicated by the manufacturer's protocol. The same wash buffers and detection methods were used as described for the TNF- α and IL-10 ELISAs.

Statistical Analyses

All data are expressed as group mean $\pm$ SEM. A value of $p < 0.05$ was considered significant for all analyses. Significance of data was determined by ANOVA followed by Bonferroni's post hoc test.

RESULTS

Splenic Denervation Prior to MCAO Does Not Inhibit Splenic Reaction

To determine whether sympathetic neurotransmission modulates the size reduction of the spleen after stroke, we compared spleen weights of rats that were subjected to splenic denervation prior to MCAO with rats that underwent MCAO-only, sham-MCAO, and sham-MCAO+denervation (Figure 1). MCAO surgery caused significant reductions in spleen weight at 48 hours when compared to sham-operated controls. Spleen weights from denervation-MCAO rats were also significantly reduced compared to sham-MCAO and sham-MCAO +denervation ($p < 0.05$). There were no significant differences between the denervation-MCAO and MCAO-only treatment groups. Lack of tyrosine hydroxylase (TH) immunostaining in the spleens of denervated animals (Figure 2C) verified that the splenic nerve had been transected, in contrast to the abundant TH immunoreactivity present in the spleens of animals that received sham-splenectomy (Figure 2A) or MCAO-only (Figure 2B). Quantification revealed that denervated spleens contained significantly reduced TH staining compared to spleens from sham-splenectomy and MCAO-only rats (Figure 2D; $p < 0.01$).

Splenic Denervation Prior to MCAO does not Effect Infarct Volume

Fluoro-Jade staining was performed to determine whether transection of the splenic nerve altered infarct volume after MCAO. Degenerating neurons were extensively labeled by Fluoro-Jade in the ipsilateral cortex, striatum and hippocampus 48 hours post-MCAO (Figure 3B). Brain sections from rats that underwent splenic denervation two weeks prior to stroke (Figure 3C) displayed a similar pattern of Fluoro-Jade labeling as that observed in rats subjected to MCAO alone. Animals that received sham-MCAO surgeries displayed no Fluoro-Jade labeling (Figure 3A). Splenectomy two weeks prior to stroke reduced Fluoro-Jade staining in the ipsilateral hemisphere as previously published (Ajmo et al.). While quantification of the infarct volumes revealed no significant difference between the denervation-MCAO and MCAO-only groups, splenectomy prior to stroke significantly decreased infarct volume ($p<0.01$) compared to denervation-MCAO and MCAO-only rats (Figure 3D).

To confirm these results, brain sections from the above groups were stained with thionin. In contrast to Fluoro-Jade, absence of staining denotes cell death in thionin-stained sections. MCAO-only (Figure 4B) and denervation-MCAO rats showed a similar pattern of Nissl staining across the majority of the ipsilateral hemisphere including the cortical, striatal and hippocampal regions (Figure 4C). Sham-operated animals showed no reductions in Nissl staining (Figure 4A). Splenectomy prior to stroke preserved the numbers of thionin-labeled cells in the ipsilateral hemisphere as shown previously (Ajmo et al., 2008). While quantification revealed no significant difference between MCAO-only and denervation-MCAO, infarct volumes from splenectomized rats were significantly reduced ($p<0.01$) compared to the MCAO-only and denervation-MCAO groups (Figure 4D).

Adrenoreceptor Antagonism Does Not Alter Blood Pressure

Blood pressures of rats were measured at 24 hours preoperative, 0 hours, and 24 hours postoperative to determine whether alterations occurred after short-term administration of adrenoreceptor antagonists. Measurements were taken each morning prior to the administration of the antagonists. Quantification revealed no significant differences in systolic or diastolic rates across the various treatment groups, demonstrating that the effects of these compounds on spleen size and infarct volume result from mechanisms that do not involve the modulation of blood pressure.

Adrenoreceptor Antagonism Inhibits the Splenic Reaction to Stroke

Adrenergic regulation of spleen weight after MCAO was investigated using α and β receptor antagonists. Spleens from rats treated with either carvedilol, prazosin, or propranolol at 24 hours preoperative, 0 hours, and 24 hours post-MCAO were evaluated against spleens of rats treated with vehicle alone. The spleen weights of rats treated with either carvedilol or prazosin were significantly greater ($p<0.05$) than those from rats treated with either vehicle alone or propranolol (Figure 6).

Adrenoreceptor Antagonism Reduces Infarct Volume

To examine the effects of adrenoreceptor antagonists on stroke-induced neurodegeneration, brain sections were stained with Fluoro-Jade and infarct volumes were measured. Sections from rats subjected to MCAO-only (Figure 7B) showed increased Fluoro-Jade staining when compared to sham-operated controls (Figure 7A). Only carvedilol treatment resulted in reduced Fluoro-Jade staining (Figure 7C) following MCAO. Treatment with either prazosin (Figure 7D) or propranolol (Figure 7E) resulted in similar degrees of Fluoro-Jade staining relative to vehicle controls. Image analysis of the MCAO-induced infarct volumes showed a significant reduction ($p<0.05$) in Fluoro-Jade labeled damage after treatment with carvedilol compared to the MCAO-only group (Figure 7F).

Adrenoreceptor Antagonism Does Not Affect the Relative Abundance of Splenic Lymphocyte Populations

To determine whether adrenergic blockade exerts effects on specific immune cell populations within the spleen, flow cytometry was performed on samples collected from rats that were subjected to either sham-MCAO or MCAO and treated with prazosin, propranolol, or carvedilol. Quantification showed no significant differences in the lymphocyte populations present within the splenocyte fractions across treatment groups (Figure 8), indicating that the effects of these compounds were not limited to specific immune cell populations within the spleen.

Adrenoreceptor Antagonism Modulates Cytokine Expression in the Spleen

To determine the effects of adrenergic receptor antagonists on cytokine expression, levels of IL-1 β , TNF- α and IL-10 were quantified from spleens removed 48 hours after MCAO. Cytokine levels were normalized to total spleen weight. IL-10 was undetectable in all spleen samples (data not shown). Of all the adrenergic receptor blockers tested, only prazosin (n=5) significantly decreased TNF- α levels ($p<0.05$) in the spleen relative to those from MCAO- or sham-operated rats (Fig 9A). In contrast, only propranolol treatment (n=5) increased IL-1 β expression ($p<0.05$) in the spleen after MCAO compared to the spleens from MCAO- or sham-operated rats (Fig 9B). Carvedilol treatment (n=12) had no effect on the expression of these cytokines relative to MCAO-only or sham-operated controls.

DISCUSSION

The spleen is a reservoir of peripheral macrophages and other immune cells, and has recently been implicated in an inflammatory response to brain injury (Offner et al., 2006a, Vendrame et al., 2006). Since an increased circulation of macrophages and a reduction in B cells in the spleen have been reported in response to stroke (Offner et al., 2006a), the reduction in spleen size observed in response to ischemic insult may result from immune cell migration from the spleen to the cerebral injury site.

Shortly after ischemic insult, there is an increase of both norepinephrine and epinephrine in the systemic circulation (Meyer et al., 2004). During stroke, the adrenal medulla secretes catecholamines as a result of increased sympathetic tone (Young et al., 1983). Both norepinephrine and epinephrine have been shown to induce significant splenic atrophy (Mignini et al., 2003), and studies have shown that splenic innervation is predominantly sympathetic (Elenkov et al., 2000). During times of physiological stress, the SNS causes contraction of splenic smooth muscle (Stewart and McKenzie, 2002).

Here, the direct effect of sympathetic neurotransmission via the splenic nerve was examined. TH has been shown to be a reliable marker for intact and functioning neurons in the spleen (Lorton et al., 2005). Reduced TH immunoreactivity after transection of the splenic nerve demonstrated that the elimination of sympathetic fibers was successful, yet did not alter the splenic response to stroke as determined by spleen weight. Infarct volumes for the denervated animals showed no variation relative to those subjected to MCAO-only. Therefore, direct sympathetic neurotransmission via the splenic nerves does not appear to contribute to the splenic response to stroke or ischemic injury in the brain.

To further examine the role of sympathetic neurotransmission, animals subjected to MCAO were treated with adrenoreceptor antagonists. Although these compounds are prescribed for antihypertensive therapy, it is well known that these therapies often take several weeks to show appreciable changes in blood pressure (<http://www.healthline.com/galecontent/beta-blockers>). Due to the fact that these compounds

were only administered for 3 days, once daily, it is not surprising that blood pressure was not significantly altered across treatment groups. Further, these data indicate that the effects of these compounds on spleen size and infarction resulted from mechanisms independent of blood pressure regulation.

The administration of the $\alpha 1$ receptor agonist phenylephrine induces contractions of the spleen in a dose dependent manner (Hardy et al., 1994). The contractility of the spleen has been used as a model to identify $\alpha 1$ receptor subtypes and to test the efficacy of $\alpha 1$ receptor antagonists, such as prazosin (Aboud et al., 1993, Oriowo, 1998, Bolognesi et al., 2001). Other groups have suggested that the decrease in spleen size following experimental stroke is due to profound apoptosis of the lymphocyte population in the spleen (Offner et al., 2006b). Our finding that $\alpha 1$ receptor antagonists block the shrinkage of the spleen is not unexpected, and suggests that spleen shrinkage is due to adrenoreceptor stimulation of the smooth muscle capsule surrounding the spleen. Similar to others in the field (Offner et al., 2006a, Offner et al., 2008), we have found that there is no change in the relative populations of the various lymphocytes in the spleen. It is unlikely that a widespread apoptotic event would destroy enough lymphocytes to atrophy the spleen by 40%, yet preserve their relative percentages in the remaining population. The inhibition of spleen reduction with prazosin treatment did not reduce infarct volume, demonstrating that these two events can be dissociated. This finding shows that both α and β receptor blockade is necessary for effective stroke treatment. Leukocytes are released into the blood from the spleen in response to stroke (Offner et al., 2006a) to migrate to the injured brain. The retention of spleen size by treatment with adrenergic blockers demonstrates that the activation of the SNS after stroke is likely linked to this splenic reaction.

Propranolol administration neither provided neuroprotection nor prevented the reduction in spleen weight after MCAO. Propranolol has been shown to be neuroprotective in experimental stroke models (Standefer and Little, 1986, Goyagi et al., 2006). However, each of these studies used transient occlusion models. Other investigators have reported that $\beta 2$ receptor activation enhances neuroprotection after stroke, while administration of propranolol reversed this effect (Semkova et al., 1996, Junker et al., 2002). The $\beta 2$ receptor is the primary adrenoreceptor expressed on innate and adaptive immune cells (Kin and Sanders, 2006), and $\beta 2$ activation causes mainly an inhibitory effect on immune function. This is consistent with the finding that propranolol blocks the post-stroke immune suppression (Prass et al., 2006).

Carvedilol is a β -adrenergic receptor antagonist with $\alpha 1$ -blocking properties and was utilized to inhibit sympathetic activity derived from both the splenic nerve and from blood borne catecholamines released from the adrenal medulla. This drug has been marketed for treatment of congestive heart failure and hypertension, and has been shown experimentally to reduce ischemic injury in the brain (Frishman, 1998, Barone et al., 2007, Cruz et al., 2007). In our stroke model, carvedilol significantly diminished neurodegenerative damage at the site of ischemia and prevented spleen size reduction. These data indicate that simultaneous blockade of both α and β adrenoreceptors is necessary to achieve these outcomes. It is important to note, however, that the differences observed between carvedilol and prazosin with regard to neuroprotection indicate that the effects of adrenergic blockade may extend beyond the spleen specifically, and therefore may reflect another unknown neuroprotective mechanism.

The spleen also produces inflammatory cytokines in response to stroke. Prazosin administration decreased the levels of TNF- α in the spleen. This compound has been shown to exert anti-inflammatory effects (Brosnan et al., 1986), while a related pharmaceutical decreased TNF- α production in blood mononuclear cells (Fukuzawa et al., 2000). However, reduced splenic TNF- α did not translate into reduced MCAO-induced damage in the present study, and propranolol treatment increased levels of IL-1 β in the spleen. Activation of

adrenergic receptors by endogenous catecholamines is linked to decreased splenic natural killer cell activity (Katafuchi et al., 1993). Thus, blocking adrenergic receptors on splenic cells with propranolol may be the mechanism responsible for the increased expression of IL-1 β . Carvedilol treatment, while effective at reducing infarct volume, did not significantly alter cytokine levels, again indicating that these compounds may mediate neuroprotection via mechanisms independent of splenic cytokine signaling.

Interestingly, researchers in the field of liver injury have known for over ten years that the inflammatory response elicited from the spleen plays a major role in ischemia reperfusion injury to liver (Okuaki et al., 1996). Kupffer cells and neutrophils produce reactive oxygen species, TNF- α and nitric oxide (Jaeschke, 2000) in response to ischemia reperfusion, causing damage to the liver as well as kidney, heart, lungs and intestine (Fan et al., 1999). Removal of the spleen reduces leukocyte infiltration and TNF- α release in liver (Jiang et al., 2007). Splenectomy also protects against damage from intestinal ischemia reperfusion and the subsequent inflammation that damages other organs (Savas et al., 2003). We have also found that splenectomy reduces leukocyte infiltration into the damaged brain (Ajmo et al., 2008). Taken together, these data indicate that the spleen plays a role in eliciting an immune/inflammatory response not only in stroke, but after ischemic insult to any tissue. Thus, any treatment regimen aimed at blocking the splenic response to stroke would likely be beneficial in treating other ischemic tissues such as heart, liver, intestine, or kidney.

While the precise mechanisms have yet to be elucidated, data from the present study demonstrate that the splenic response to stroke results, at least in part, from increased catecholamine circulation. These findings are an initial step in the development of stroke therapies that focus not only on the CNS, but also on the peripheral immune cell signaling that plays a pivotal role in ischemic injury progression. Future experiments demonstrating the long-term efficacy of treatment with adrenergic blockers are necessary to determine whether the delayed administration of these compounds is a viable option for treatment in the clinical setting.

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Figure 1. Denervation has no effect on spleen shrinkage 48 hr post-MCAO

Spleens of rats subjected to denervation prior to stroke showed reduced weight when removed 48 hours after MCAO compared to sham-operated controls (* $p < 0.05$). There was no significant difference in spleen weight of animals subjected to denervation-MCAO when compared to MCAO-only.

Figure 2. Tyrosine hydroxylase labeling 48 hr post-MCAO

TH immunoreactivity localized to nerve fibers (arrows) surrounding splenic blood vessels (BV) and was reduced in spleens from animals that underwent denervation (C) in contrast to labeled sections of animals subjected to sham-splenectomy (A). Spleens of rats subjected to MCAO-only (B) showed a similar labeling pattern as observed in sham-operated controls. Quantification (D) revealed a significant decrease in TH immunoreactivity in denervated spleens relative to spleens from animals subjected to sham-splenectomy or MCAO-only (* $p < 0.01$).

Figure 3. Fluoro-Jade stains the infarct of denervated rats 48 hr post-MCAO

Fluoro-Jade staining shows a large area of damage in the ipsilateral hemisphere from brains of denervation-MCAO rats (C). Rats subjected to MCAO-only showed a similar neurodegenerative profile (B). Brain sections from sham-MCAO rats (A) and sham-MCAO +denervation animals showed no Fluoro-Jade labeled areas (not shown). Quantification (D) revealed no significant change in infarct volume between denervation-MCAO and MCAO-only rats, while splenectomy prior to stroke significantly reduced infarct volume (* $p < 0.01$). Scale bar = 2 mm.

Figure 4. Thionin staining is reduced in denervated rats 48 hr post-MCAO

Thionin-stained Nissl Bodies were reduced in tissues from rats subjected to denervation-MCAO (C) and MCAO-only (B). Brain sections from sham-MCAO (A) and sham-MCAO +denervation (not shown) rats showed uniform thionin staining typical of an uninjured brain. Quantification (D) revealed no significant change in infarct volume between denervation-MCAO and MCAO-only rats, while splenectomy prior to stroke significantly reduced infarct volume (* $p < 0.01$). Scale bar = 2 mm.

Figure 5. Short-term treatment with adrenoreceptor antagonists does not alter blood pressure
 Blood pressures of rats were measured at 24 hours preoperative, 0 hours, and 24 hours postoperative to determine whether alterations occurred after short-term administration of adrenoreceptor antagonists. Quantification showed no significant differences in systolic or diastolic rates across treatment groups at any time point examined.

Figure 6. The effects of adrenoreceptor antagonists on spleen weight
Spleen weights from rats treated with vehicle, carvedilol, prazosin or propranolol were recorded and quantified. Results showed that spleen weights from carvedilol- and prazosin-treated rats were significantly greater than those from rats subjected to MCAO-only (* $p < 0.05$).

Figure 7. The effects of adrenoreceptor antagonists on infarct volume

No Fluoro-Jade staining was present in brain sections from sham-MCAO rats (A). Rats that received carvedilol prior to stroke showed reduced Fluoro-Jade staining (C) relative to rats subjected to MCAO-only (B). Treatment with prazosin (D) or propranolol (E) produced a staining profile similar to that observed in MCAO-only rats. Quantification of infarct volume (F) revealed a significant reduction in rats treated with carvedilol compared to prazosin, propranolol, or MCAO-only (* $p < 0.05$). Scale bar = 2 mm.

Figure 8. Adrenoreceptor antagonists do not alter splenic immune cell counts

Flow cytometry was performed on spleen samples collected from rats that were subjected to either sham-MCAO or MCAO with administration of adrenoreceptor antagonists. Quantification showed no significant alterations in the immune cell types present within the splenocyte fractions across treatment groups, indicating that neither prazosin, propranolol, or carvedilol altered the relative abundance of immune cell types present within the spleen.

Figure 9. The effects of adrenoreceptor antagonists on inflammatory cytokine levels

Levels of the inflammatory cytokines IL-1β and TNF-α were quantified from spleens of rats treated with carvedilol, prazosin or propranolol 48 hours after MCAO. Of all the adrenergic receptor blockers tested, only prazosin decreased TNF-α levels in the spleen relative to MCAO-only or sham-operated rats (A) (* $p < 0.05$). In contrast, only propranolol treatment increased IL-1β expression after MCAO compared to the splenic expression from MCAO- or sham-operated rats (B) (* $p < 0.05$). Carvedilol treatment had no effect on the expression of these cytokines.