Delta$_2$-Specific Opioid Receptor Agonist and Hibernating Woodchuck Plasma Fraction Provide Ischemic Neuroprotection

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Abstract

Objectives: The authors present evidence that the δ opioid receptor agonist Deltorphin-$D_{variant}$ (Delt-D_{var}) and hibernating woodchuck plasma (HWP), but not summer-active woodchuck plasma (SAWP), can provide significant neuroprotection from focal ischemia in mice by a mechanism that relies in part on reducing nitric oxide (NO) release in ischemic tissue.

Methods: Cerebral ischemia was produced in wild-type and NO synthase–deficient ($NOS^{-/-}$) mice by transient, 1-hour middle cerebral artery occlusion (MCAO). Behavioral deficits were determined at 22 hours and infarct volume was assessed at 24 hours after MCAO. Mice were treated with saline or Delt-D_{var} at 2.0 and 4.0 mg/kg, or 200 μL of HWP or SAWP. $NOS^{-/-}$ mice were treated with either saline or Delt-D_{var} at 4.0 mg/kg. NO release was determined using an N9 microglial cell line pretreated with δ- or μ-specific opioids and HWP or SAWP prior to activation with lipopolysaccharide and interferon-γ. Nitrate in the medium was measured as an indicator of NO production.

Results: Infusion of Delt-D_{var} or HWP (but not SAWP) decreased infarct volume and improved behavioral deficits following 1 hour of MCAO and 24 hours of reperfusion. In $NOS^{-/-}$ mice, endothelial $NOS^{+/+}$ is required to provide Delt-D_{var}–induced neuroprotection. Delt-D_{var} and HWP dose-dependently decreased NO release in cell culture, while SAWP and other δ- and μ-specific opioids did not.

Conclusions: Delt-D_{var} and HWP, but not SAWP, are effective neuroprotectant agents in a mouse model of transient MCAO. In cell culture, the mechanism of this ischemic neuroprotection may rely in part on their ability to block NO release.

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Definitive specific therapy for ischemic stroke remains to be established, despite numerous studies undertaken to elucidate its complex pathophysiology.[1–3] In the presence of diminished cerebral blood flow and reduced availability of oxygen and glucose, cellular homeostasis in the brain is lost as cascades of inflammatory events lead to progressive neuronal damage and ultimately cell death. Experimental therapies for stroke over the past 15 years have generally been directed at select events in the ischemic process.[4–8] Given the complexity of the pathophysiologic derangements and numerous mediators implicated in the ischemic process (i.e., cytokines, endothelial cells, leukocyte adhesion molecules, nitric oxide (NO), and cyclooxygenase production), it may well be that no one factor is dominant and that progression of ischemic damage is caused by loss of homeostatic control.[9] Mammalian hibernation presents a unique

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example of a natural ability in animals to preserve cellular homeostasis without any accompanying brain and organ system damage during profound hypothermia, bradycardia, and respiratory depression, with its associated hypoxia and aglycemia.[10] Thus, intervention designed to preserve cellular homeostasis may be a new approach for the treatment of ischemic brain injury.

We have previously demonstrated that both a delta opioid–like hibernation induction trigger (HIT) derived from the plasma of deeply hibernating woodchucks and a nonspecific delta opioid, [D-Ala^2,D-leu^5]enkephalin (DADLE, which mimics the activity of the HIT molecule in inducing natural hibernation), can markedly extend the survival of ischemic rabbit heart, dog lung, and dog multiorgan systems.[11–19] We have also shown that the HIT molecule and delta$_2$ opioid agonists, which are highly specific for the delta$_2$ receptor subtype such as Deltorphin-A (Delt-A) and Deltorphin-D$_{variant}$ (Delt-D$_{var}$), can provide extended myocardial ischemic protection (decreased left ventricular infarct volume) in rats[20] and pigs[21] when infused 24 hours to 45 minutes prior to ischemia. More recently, we have demonstrated that Delt-D$_{var}$ can also markedly improve survival of severely hemorrhaged rats when infused 24 hours prior to hemorrhage without concomitant fluid resuscitation.[22] However, the HIT containing woodchuck plasma and these delta$_2$-specific opioids originally isolated from skin secretions of two South American frog species have to the best of our knowledge never before been tested for their ability to prevent cerebral ischemia.[23,24] Thus, we tested the hypothesis that hibernating woodchuck plasma (HWP) and the delta-specific opioid Delt-D$_{var}$ can provide ischemic protection in wild-type C57BL/6 mice undergoing 1 hour of middle cerebral artery occlusion (MCAO). To investigate the role of NO production on the inflammatory ischemic process, we utilized 1) a murine N9 microglial cell line treated with HWP and summer-active woodchuck plasma (SAWP), opioids, lipopolysaccharide (LPS), and interferon-γ (IFN-γ)[25] and 2) mice deficient in inducible nitric oxide synthase (iNOS$^{-/-}$), neuronal nitric oxide synthase (nNOS$^{-/-}$), or endothelial nitric oxide synthase (eNOS$^{-/-}$) that had been infused with opioids following 1 hour of MCAO.

METHODS

Study Design

This was an animal investigation of neuroprotection from focal ischemia in mice by a mechanism that relies in part on reducing NO release in ischemic tissue. All protocols were reviewed and approved by the Lexington Veteran's Administration Medical Center Institutional Animal Care and Use Committee.

Animal Subjects

Male C57BL/6 mice (25–30 g, Harlan Labs, Indianapolis, IN) and male knockout mice on a C57BL/6 background (25–35 g, The Jackson Laboratories, Bar Harbor, ME) deficient in iNOS$^{-/-}$, nNOS$^{-/-}$, and eNOS$^{-/-}$, were given free access to food and water.

MCA Occlusion

Mice were anesthetized with chloral hydrate (350 mg/kg) and xylazine (4 mg/kg), and rectal temperatures were maintained at 37 ± 0.5°C. The left femoral artery was cannulated with a PE-10 catheter for measurement of arterial blood pressure. Arterial blood samples (50 μL) were analyzed for pH, arterial oxygen pressure (pO_2), and partial pressure of carbon dioxide (pCO_2) using a blood gas/pH analyzer (Corning 178, CIBA Corning Diagnostics, Medford, MA) before occlusion, 10 minutes after occlusion, and 10 minutes after reperfusion. The left common carotid artery was exposed, and the occipital, superior thyroidal, maxillary, and lingual branches of the external carotid artery were coagulated; the pterygopalatine artery was ligated as described elsewhere.[26–28] The base of the external carotid artery was secured with a microsurgical clip, the distal end of the external carotid artery was then ligated with a 6-0 nylon suture, and the external carotid artery was cut. A blunted 5-0 blue monofilament nylon was placed in the end of the external carotid artery to prevent both slipping of the internal suture and bleeding. The suture remained in place for 1 hour, after which reperfusion of the brain and restoration of blood flow were accomplished by retreating the suture from the middle cerebral artery. Delt-D$_{var}$ sequence (Tyr-D-Ala-Phe-Ala-Asp-Val-Ala-Ser-Thr-Ile-Gly-Asp-Phe-Phe-His-Ser-Ile-NH$_2$) was administered intravenously (IV) by tail vein injection over a 1-minute period at concentrations of 2 or 4 mg/kg in 100 μL of phosphate-buffered saline (PBS). In a second series of experiments, 200 μL of HIT-containing HWP or 200 μL of SAWP was injected IV. Controls received 200 μL of PBS IV.

Study Protocol

Measurement of Infarct Volume. Twenty-four hours after MCAO, mice were euthanized with a halothane overdose. The brain was immediately removed and placed into a mouse brain matrix (ASI Instruments Inc., Warren, MI), and coronal sections (2 mm thick) were stained with 2% 2,3,5-triphenyl tetrazolium chloride (TTC). Infarct size was determined according to the formula (Contralateral volume – Ipsilateral undamaged volume) × 100/Contralateral volume. Quantitation of contralateral and ipsilateral volumes was determined by image analysis using a Scion Image (NIH Image Version 1.59, NIH, Bethesda, MD) modified by Scion Corp. (Frederick, MD) and Adobe Photoshop 2.0.1 (Adobe Systems Inc., San Jose, CA).

Behavioral Assessment. Mice were assessed by a blinded observer (JY) for neurologic deficits at 22 hours following ischemia using the following numerical ranking scale criteria: 0 = no observable deficits (normal); 1 = failure to extend right forepaw upon lifting the tail (mild); 2 = circling to the contralateral side (moderate); and 3 = leaning to the contralateral side at rest or no spontaneous motor activity (severe). This protocol has been used previously to determine the behavioral changes in mice following cerebral ischemia.[28]

Measurement of Cerebral Blood Flow. Regional cerebral blood flow was monitored by laser Doppler flowmetry (Laser-flow BPM, Vasamedics Inc., St. Paul, MN) in anesthetized animals under resting conditions 30 minutes prior to ischemia, 10 minutes after MCAO, and 20 minutes after reperfusion. Two flexible fiberoptic probe tips were placed 2 mm posterior and 6 mm lateral to the bregma on the ipsilateral hemisphere of the brain. After surgery, the probes were removed and the mice were returned to their cages with access to food and water.

Cell Culture and Nitrite Assay. The mouse N9 microglial cell line of neuronal origin was cultured in Dulbecco's modified essential medium containing 10% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, 4.5 g/L glucose, penicillin (50 U/ml), and streptomycin (50 μg/mL). Cells were plated in 96-well plates at a density of 2×10^4 cells per well. After 24 hours, cells were activated by incubation in medium containing 1 μg/mL *Escherichia coli* LPS and 100 U/mL recombinant murine IFN-γ. Deeply HWP and SAWP (Northeastern Wildlife, South Plymouth, NY) and various concentrations of Delt-D_{var}, [D-Pen2,5]enkephalin (DPDPE), and Dermorphin-H (Derm-H; Bachem Inc., King of Prussia, PA) were dissolved in water or dimethyl sulfoxide and were added to N9 cells activated with LPS and IFN-γ. Nitrate concentrations in the medium were measured as an indicator of NO production by the Griess reaction.[25] Standards and supernatant were analyzed in triplicate. Ten minutes after addition of Griess reagent, the plates were read using an enzyme-linked immunosorbent assay plate reader at 550 nm.

Data Analysis

Results are expressed as means ± SEM. Differences were analyzed using one-way analysis of variance (ANOVA). Two-group comparisons were evaluated by Student's t-test, with Bonferroni's correction for multiple comparisons.

RESULTS

Prior to opioid treatment there was no significant differences in mean arterial pressure (MAP), pCO_2, pO_2, pH, or rectal temperature between PBS-treated controls and Delt-D_{var}–treated mice. C57BL/6 control (n = 5) mice were given tail vein injections of 100 μL of PBS. C57BL/6 experimental mice were given either 2 mg/kg Delt-D_{var} (n = 5) dissolved in 100 μL of PBS or 4 mg/kg Delt-D_{var} (n = 6) dissolved in 100 μL of PBS following 1 hour of MCAO. In both control and Delt-D_{var}–treated mice, induction of ischemia resulted in a 75% reduction in cerebral blood flow that was sustained for 1 hour. MCAO produced a unilateral infarction in the striatum and in large areas of the cerebral cortex as visualized by TTC staining of coronal sections. Immediately postischemia there was no significant differences in MAP, pCO_2, pO_2, pH, or rectal temperature between controls and test mice (Table 1). However, at 22 hours postischemia, there was a significant ($p = 0.05$) decrease in neurologic deficits of 2 mg/kg Delt-D_{var}–treated animals (0.89 ± 0.2) versus control animals (1.69 ± 0.2). We also saw a significant ($p = 0.05$) decrease in infarct volume (29%) in test animals (52 ± 8 mm^3) compared to control animals (73.2 ± 6.8 mm^3) at 24 hours postischemia. Injection of 4 mg/kg Delt-D_{var} reduced infarct volume by 68% ($p = 0.006$) from 73.2 (± 6.8) mm^3 in control mice to 23.74 (± 4.8) mm^3 in test mice, and behavioral deficits decreased from 1.69 (± 0.2) in controls to 0.44 (± 0.11) in test mice ($p < 0.05$).

C57BL/6 wild-type and C57BL/6 ($NOS^{-/-}$) mice were subjected to 1-hour MCAO followed by tail vein injection of either 100 μL of PBS in control mice or 4 mg/kg Delt-D_{var} dissolved in 100 μL of PBS in the experimental mice. Infarct volume in PBS-injected $iNOS^{-/-}$ mice ($n = 5$) was 40.91 (± 7.3) mm^3 versus 75.0 (± 6.8) mm^3 in wild-type mice ($n = 5$). Infarct volume in $nNOS^{-/-}$ mice ($n = 5$) was 50.0 (± 8.2) mm^3 while infarct volume in $eNOS^{-/-}$ mice ($n = 5$) was 95.45 (± 7.7) mm^3, which was 27% higher than in wild-type controls. Infarct volume in C57BL/6 wild-type mice ($n = 5$) injected with 4 mg/kg Delt-D_{var} was reduced by 56% to 41.67 (± 13.89) mm^3 versus 75.0 (± 6.8) mm^3 in PBS-injected controls ($n = 5$). Infarct volume of $iNOS^{-/-}$ mice ($n = 5$) was 45.83 (± 8.3) mm^3 and that of $nNOS^{-/-}$ mice ($n = 5$) was 42.0 (± 10.0) mm^3 while infarct volume in $eNOS^{-/-}$ mice ($n = 5$) was 76.39 (± 15.3) mm^3. Immediately postischemia there were no significant differences in MAP, pCO_2, pO_2, pH, or rectal temperature between control and $NOS^{-/-}$ mice.

Hibernating woodchuck plasma was also tested for its potential to provide ischemic neuroprotection similar to that of Delt-D_{var}. C57BL/6 mice ($n = 5$) per group were subjected to 1-hour MCAO, followed by an injection in the tail vein with 200 μL of PBS, 200 μL of SAWP, or 200 μL of HWP. Following 24 hours of reperfusion, infarct volume in controls was 78.39 (± 7.0) mm^3 while HWP-treated mice had a 32% decreased infarct volume (53 ± 6.0 mm^3, $p < 0.05$) versus control. SAWP-treated mice had an infarct volume of 75 (± 8.0) mm^3, which was not significantly different from controls.

Four different opioid peptides were tested for their potential inhibitor effects on NO production in LPS- and IFN-γ–activated N9 microglial cells, using the Griess assay. The opioids were 1) Delt-D_{var}, 2) DPDPE (a $delta_1$-specific opioid), 3) DADLE, and 4) Derm-H (a μ-specific receptor agonist). One millimolar Delt-D_{var} inhibited NO production by 95% ($p < 0.001$), when compared to control cells, while DADLE (2.5 mM), Derm-H (1 mM), and DPDPE (1 mM) had little or no effect on NO release (Table 2A). Delt-D_{var} also inhibited NO production in a concentration-dependent manner. At 0.5 mM there was a 43% decrease ($p < 0.001$) in NO production compared to control-activated cells, and at 0.2 mM there was a 16% decrease in NO production compared to control-activated cells (Table 2B).

Hibemating woodchuck plasma and SAWP were tested for their potential inhibitor effects on NO production by activated N9 microglial cells. HWP at 50% decreased NO production by 77% ($p < 0.001$) compared to control-activated cells, while 50% SAWP caused a 25% increase in NO production compared to control-activated cells. HWP also inhibited NO production in a

Table 1
Physiologic Parameters before, during, and after Middle Cerebral Artery Occlusion in Control and Opioid-treated Wild-type Mice

Parameter	Preischemia	Ischemia	Postischemia
Cerebral blood flow (% of baseline)			
Control	100 ± 7	19 ± 3	98 ± 6
Delt-D_{var} (2 mg/kg)	100 ± 5	21 ± 5	97 ± 8
Delt-D_{var} (4 mg/kg)	100 ± 4	21 ± 5	97 ± 6
Wild type	100 ± 5	20 ± 4	96 ± 5
Wild type (4 mg/kg Delt-D_{var})	100 ± 6	18 ± 2	97 ± 8
iNOS	100 ± 5	21 ± 4	94 ± 7
iNOS (4 mg/kg Delt-D_{var})	100 ± 3	19 ± 5	99 ± 4
nNOS	100 ± 7	17 ± 2	97 ± 6
nNOS (4 mg/kg Delt-D_{var})	100 ± 5	18 ± 3	96 ± 7
eNOS	100 ± 6	16 ± 4	98 ± 8
eNOS (4 mg/kg Delt-D_{var})	100 ± 4	20 ± 5	95 ± 5
PBS	100 ± 6	18 ± 5	99 ± 7
HWP	100 ± 5	16 ± 4	95 ± 5
SAWP	100 ± 7	17 ± 3	98 ± 6
pH			
Control	7.36 ± 0.05	7.33 ± 0.04	7.33 ± 0.03
Delt-D_{var} (2 mg/kg)	7.36 ± 0.04	7.35 ± 0.05	7.35 ± 0.05
Delt-D_{var} (4 mg/kg)	7.35 ± 0.03	7.34 ± 0.05	7.33 ± 0.04
Wild type	7.34 ± 0.04	7.32 ± 0.06	7.34 ± 0.03
Wild type (4 mg/kg Delt-D_{var})	7.36 ± 0.05	7.33 ± 0.04	7.33 ± 0.05
iNOS	7.35 ± 0.03	7.34 ± 0.05	7.34 ± 0.04
iNOS (4 mg/kg Delt-D_{var})	7.34 ± 0.04	7.33 ± 0.03	7.34 ± 0.05
nNOS	7.33 ± 0.04	7.35 ± 0.05	7.35 ± 0.05
nNOS (4 mg/kg Delt-D_{var})	7.35 ± 0.05	7.36 ± 0.06	7.34 ± 0.04
eNOS	7.36 ± 0.04	7.34 ± 0.04	7.33 ± 0.03
eNOS (4 mg/kg Delt-D_{var})	7.34 ± 0.05	7.33 ± 0.05	7.33 ± 0.03
PBS	7.35 ± 0.03	7.34 ± 0.03	7.35 ± 0.04
HWP	7.36 ± 0.03	7.35 ± 0.05	7.36 ± 0.04
SAWP	7.36 ± 0.04	7.35 ± 0.05	7.33 ± 0.05
MABP (mm Hg)			
Control	89.1 ± 10.1	89.8 ± 9.5	89.6 ± 10.2
Delt-D_{var} (2 mg/kg)	88.9 ± 9.8	89.2 ± 9.7	88.7 ± 10.1
Delt-D_{var} (4 mg/kg)	87.2 ± 9.5	90.1 ± 9.4	86.6 ± 10.3
Wild type	89.5 ± 9.3	86.4 ± 10.3	84.7 ± 10.2
Wild type (4 mg/kg Delt-D_{var})	88.8 ± 9.7	87.7 ± 9.5	87.2 ± 9.4
iNOS	86.4 ± 10.4	90.3 ± 9.2	89.8 ± 9.6
iNOS (4 mg/kg Delt-D_{var})	88.3 ± 9.6	91.5 ± 9.9	87.4 ± 9.9
nNOS	87.8 ± 9.9	86.2 ± 10.5	90.5 ± 9.8
nNOS (4 mg/kg Delt-D_{var})	90.6 ± 9.5	89.8 ± 9.4	90.8 ± 10.6
eNOS	86.8 ± 9.7	88.9 ± 10.2	88.7 ± 9.7
eNOS (4 mg/kg Delt-D_{var})	85.9 ± 10.2	87.6 ± 9.5	86.1 ± 9.2
PBS	88.4 ± 9.9	85.4 ± 10.6	88.3 ± 9.5
HWP	86.3 ± 9.2	89.6 ± 9.3	86.7 ± 10.4
SAWP	88.2 ± 10.3	87.3 ± 9.5	84.9 ± 9.7
pCO_2 (mm Hg)			
Control	48.8 ± 4.7	48.2 ± 4.7	48.5 ± 4.6
Delt-D_{var} (2 mg/kg)	48.6 ± 5.1	49.1 ± 4.8	48.9 ± 4.9
Delt-D_{var} (4 mg/kg)	46.5 ± 4.8	48.4 ± 4.6	50.1 ± 5.3
Wild type	49.9 ± 4.7	45.5 ± 5.3	46.9 ± 4.6
Wild type (4 mg/kg Delt-D_{var})	45.4 ± 5.1	50.8 ± 4.9	48.6 ± 5.2
iNOS	48.7 ± 4.6	47.2 ± 4.6	49.8 ± 4.8
iNOS (4 mg/kg Delt-D_{var})	47.6 ± 4.9	49.9 ± 6.1	50.7 ± 4.6
nNOS	50.3 ± 4.5	46.4 ± 4.5	47.3 ± 4.7
nNOS (4 mg/kg Delt-D_{var})	47.5 ± 5.3	50.1 ± 4.8	44.5 ± 4.9
eNOS	49.9 ± 4.8	47.4 ± 5.2	51.4 ± 5.5
eNOS (4 mg/kg Delt-D_{var})	50.1 ± 4.6	48.6 ± 4.5	46.8 ± 4.8
PBS	51.4 ± 5.2	49.8 ± 4.7	48.3 ± 4.6
HWP	45.9 ± 4.4	46.3 ± 4.8	50.5 ± 5.3
SAWP	49.3 ± 4.8	51.4 ± 5.0	45.9 ± 4.9
pO_2 (mm Hg)			
Control	93.2 ± 4.3	93.1 ± 4.8	92.6 ± 4.9
Delt-D_{var} (2 mg/kg)	93.5 ± 3.8	92.8 ± 4.4	92.9 ± 3.9
Delt-D_{var} (4 mg/kg)	96.1 ± 4.7	92.4 ± 3.7	89.8 ± 4.2
Wild type	91.5 ± 3.4	91.7 ± 4.4	93.3 ± 3.5
Wild type (4 mg/kg Delt-D_{var})	97.4 ± 4.9	96.1 ± 4.2	93.6 ± 4.4
iNOS	94.7 ± 4.7	91.5 ± 3.8	90.7 ± 3.8
iNOS (4 mg/kg Delt-D_{var})	93.8 ± 3.5	96.3 ± 4.1	93.4 ± 3.4
nNOS	97.3 ± 4.8	93.7 ± 4.4	94.1 ± 3.9
nNOS (4 mg/kg Delt-D_{var})	92.9 ± 4.2	95.3 ± 3.9	91.9 ± 4.2

Table 1
Continued

Parameter	Preischemia	Ischemia	Postischemia
eNOS	98.5 ± 3.7	92.6 ± 4.4	95.3 ± 3.6
eNOS (4 mg/kg Delt-D_{var})	94.2 ± 4.1	91.2 ± 4.5	91.7 ± 4.3
PBS	94.5 ± 4.7	90.8 ± 3.7	90.8 ± 4.5
HWP	95.1 ± 4.3	94.6 ± 4.2	92.5 ± 3.8
SAWP	93.2 ± 3.9	90.7 ± 4.5	93.1 ± 4.1

Values are mean ± SD for measurements in five to six mice per group. Measurements were made 10 minutes before occlusion, during ischemia (10 minutes following occlusion), and 10 minutes following reperfusion (except cerebral blood flow which was measured 30 minutes following reperfusion.
Delt-D_{var} = Deltorphin-$D_{variant}$; eNOS = endothelial nitric oxide synthase; HWP = hibernating woodchuck plasma; iNOS = inducible nitric oxide synthase; MABP = mean arterial blood pressure; nNOS = neuronal nitric oxide synthase; SAWP = summer-active woodchuck plasma; PBS = phosphate-buffered saline; pCO_2 = partial pressure of CO_2; pO_2 = oxygen pressure.

Table 2A
Effect of Different Opioids on Nitrite Release in Culture Medium 24 Hours prior to Activation of N9 Microglial Cells with Lipopolysaccharide and Interferon-γ

Test Group	Nitrite (μM), Mean ± SD
Unactivated cells	0.94 ± 0.05
Activated cells	21.23 ± 0.11
Activated cells + Delt-D_{var} (1 mM)	0.94 ± 0.15*
Activated cells + DADLE (2.5 mM)	21.23 ± 0.52
Activated cells + DPDPE (1 mM)	22.64 ± 1.11
Activated cells + Derm-H (1 mM)	18.87 ± 1.28

DADLE = [D-Ala2,D-leu5]enkephalin; Delt-D_{var} = Deltorphin-$D_{variant}$; Derm-H = Dermorphin-H; DPDPE = [D-Pen2,5]enkephalin.
*$p < 0.001$ vs. activated cells.

Table 2B
Effect of Different Concentrations of Opioid Deltorphin-$D_{variant}$ (Delt-D_{var}) on Nitrite Release in Culture Medium 24 Hours prior to Activation of N9 Microglial Cells with Lipopolysaccharide and Interferon-γ

Test Group	Nitrite (μM), Mean ± SD
Unactivated cells	0.90 ± 0.12
Activated cells	21.13 ± 0.51
Activated cells + Delt-D_{var} (0.5 mM)	11.56 ± 0.88*
Activated cells + Delt-D_{var} (0.2 mM)	17.84 ± 0.37
Activated cells + Delt-D_{var} (0.1 mM)	19.44 ± 0.93
Activated cells + Delt-D_{var} (0.05 mM)	18.77 ± 0.34

*$p < 0.001$ vs. activated cells.

concentration-dependent manner, while SAWP showed little to no inhibitory effect at similar concentrations. At a concentration of 25 and 12.5% HWP, there was a 57% ($p < 0.001$) and a 47% ($p < 0.001$) decrease in NO release compared to control-activated cells, respectively, while at 25% SAWP, there was a slight increase in NO production compared to control-activated cells (Table 3).

Table 3
Effect of Hibernating Woodchuck Plasma (HWP) and Summer-active Woodchuck Plasma (SAWP) on Nitrite Release in Culture Medium 24 Hours prior to Activation of N9 Microglial Cells with Lipopolysaccharide and Interferon-γ

Test Group	Nitrite (μM), Mean ± SD
Unactivated cells	0.93 ± 0.05
Activated cells	22.08 ± 0.51
Activated cells + 1.25% HWP (v/v)	17.84 ± 0.47
Activated cells + 1.25% SAWP (v/v)	19.97 ± 1.29
Activated cells + 2.5% HWP (v/v)	16.81± 0.73
Activated cells + 2.5% SAWP (v/v)	19.66 ± 1.11
Activated cells + 12.5% HWP (v/v)	12.19 ± 1.18*†
Activated cells + 12.5% SAWP (v/v)	18.63 ± 0.85
Activated cells + 25% HWP (v/v)	10.32 ± 0.62*†
Activated cells + 25% SAWP (v/v)	23.08 ± 4.5
Activated cells + 50.0% HWP (v/v)	1.10 ± 0.07*†
Activated cells + 50.0% SAWP (v/v)	22.64 ± 2.32

*$p < 0.001$ vs. activated cells.
†$p < 0.001$ vs. corresponding SAWP concentration.

DISCUSSION

This study demonstrates that IV infusions of both the delta$_2$-selective opioid agonist Delt-D_{var} and plasma from deeply hibernating (HWP), but not summer-active woodchucks (SAWP), confers ischemic neuroprotection in a mouse MCAO model. There were no significant differences in physiologic parameters and core temperature between controls and test mice (HWP and opioid-treated wild-type mice and NOS-deficient mice), suggesting that the observed neuroprotection in HWP- and opioid-treated mice was not caused by vascular alterations evaluated within our study. In the case of Delt-D_{var}, the neuroprotection is dose-dependent, with the 2 mg/kg opioid dose decreasing infarct volume by 29% and behavioral deficits by 47% compared to controls, while the 4 mg/kg Delt-D_{var} dose decreased infarct volume by 68% and behavioral deficits by 72%. Similarly, the opioid-like HWP decreased infarct volume by 32% and neurologic deficits by 21% compared to controls, while the SAWP decreased infarct volume by only 4%, which was not significantly different from

controls. We have previously demonstrated in isolated and intact ischemic rat heart systems,[20] as well as in an ischemic porcine model,[21] that Delt-D_{var} and HWP can provide myocardial ischemic preconditioning (IPC) and prolonged (24-hour) pharmacologic ischemic preconditioning (PPC), which relies in part on a mechanism(s) requiring activation of ATP-dependent potassium channels.[20,29] We now present evidence that both Delt-D_{var} and HWP ischemic protection may also rely in part on a mechanism(s) that blocks NO release. Injection of PBS into NOS-deficient mice resulted in infarct volumes previously demonstrated in the literature, where $iNOS^{-/-}$ and $nNOS^{-/-}$ mice have reduced brain damage due to the decreased effects of NOS in the brain.[30] In contrast, $eNOS^{-/-}$ mice had an increased infarct volume, indicating a protective effect of $eNOS^{+/+}$ in vascular function in cerebral ischemia. Injection of Delt-D_{var} did not significantly further enhance the decrease in infarct volume in $iNOS^{-/-}$ and $nNOS^{-/-}$ mice, or provide any ischemic protection in $eNOS^{-/-}$ mice. Since there was no enhancement of ischemic protection in $iNOS^{-/-}$ and $nNOS^{-/-}$ mice, our findings give further evidence that the neuroprotective effects of Delt-D_{var} are mediated through a mechanism requiring the presence of $eNOS^{+/+}$.

Utilizing an N9 microglial cell line that was pretreated with either Delt-D_{var} or HWP prior to incubation, we have demonstrated that both Delt-D_{var} and HWP, but not DADLE, DPDPE, or Derm-H, can block LPS- and IFN-γ–induced NO release in this cell line. These findings give further evidence that both the highly purified Delt-D_{var} and the HWP are able to block NO in cells undergoing ischemic stress. We have also previously demonstrated HWP ischemic protection in isolated rat and rabbit heart models.[18,20] In these studies, myocardial ischemic functional parameters were markedly enhanced and left ventricular infarct volume was markedly decreased when HWP was infused into rat heart immediately prior to ischemia or as long as 24 hours prior to ischemia. This IPC and PPC can be blocked by infusion of the universal opioid antagonist naltrexone or the highly specific $delta_2$ opioid receptor antagonist naltriban methane sulfonate, but not by the highly specific $delta_1$ receptor antagonist 7-dehydrobenzylidene naltrexone. Such findings indicate that HWP has specificity for $delta_2$, but not $delta_1$, receptors similar to that of Delt-D_{var}. We have also previously demonstrated that an HWP affinity chromatography albumin fraction that has been further purified by preparative fast protein liquid chromatography resulted in an 88-kDa fraction, which could dose-dependently decrease mouse vas deferens (MVD) contractile activity.[31] The MVD assay is used for monitoring delta opioid receptor activity and potency, thereby indicating the delta opioid nature of the HWP.

Numerous studies have provided evidence implicating the participation of leukocytes in the pathologic sequence of events leading to central nervous system ischemia.[9,32,33] A coordinated inflammatory response involving cytokines, chemokines, and adhesion molecules is initiated following leukocyte adhesion at the blood–endothelium interface, which ultimately allows for their penetration into ischemic tissue. Increased leukocyte interaction with endothelium results in further activation of both cell types and is manifested by pathologic alterations in both the blood vessel wall and the surrounding tissue, resulting in adhesion molecules.[33] However, the entrance into hibernation is associated with a profound leukopenia and thrombocytopenia (≥90% fall in circulating leukocytes), whereas arousal is associated with restoration of leukocytes to normal levels.[34] The absence of neurologic damage in an aroused circannual hibernator, such as the woodchuck, following extended bouts of hibernation that may last as long as 4 to 6 weeks, is truly remarkable. These animals are able to tolerate reduction in core temperature as low as 4.0 to 6.0°C, aglycemia, a profound bradycardia, and respiratory rates as low as one breath over a 1- to 2-minute period, resulting in severely reduced cerebral blood flow and oxygen availability. We and others postulate that the ability to survive extended stroke-like conditions may in part be related to the sequestration of leukocytes and platelets in the liver and spleen during hibernation bouts. We have indirect evidence that this might occur utilizing a dog multiorgan autoperfusion system consisting of the heart, lungs, liver, kidneys, and a small portion of the duodenum including the pancreas. We were able to extend viability of the autoperfused organ bloc from an average of 14.5 hours in saline infused controls to over 45 hours following infusion of either HWP or DADLE.[12,13] We also noted that even though the hematocrit was maintained at 45% with donor dog blood, white blood cell counts, which were typically greater than 10.0 ($10^3/\mu L$), dropped precipitately within minutes of infusion of either HWP or DADLE to less than 0.03 ($10^3/\mu L$) and remained at these extremely low levels as long as HWP or DADLE was infused at regular 4-hour intervals during the organ bloc preservation.[35,36] However, since the spleen was not included in the organ bloc, we postulate that both HWP and DADLE infusions resulted in liver sequestration of leukocytes and platelets, although at the time this was not the focus of these experiments. Based on these prior observations, it is quite possible that the observed ischemic neuroprotection provided by HWP and Delt-D_{var} infusions in our mouse MCAO model may rely in part to a transient leukocyte sequestration in peripheral organs such as liver and spleen, thus attenuating the resulting inflammatory cascade, which is known to include NO release initiated by leukocyte endothelial cell adhesion.

LIMITATIONS

The most significant limitation of this study is that of linking in vivo the presence of $eNOS^{+/+}$ in cerebral vascular endothelium to the ability to block NO release in vitro in an N9 microglial cell culture. In nonischemic endothelium, constitutive eNOS produces NO as a byproduct of the conversion of L-arginine to L-citrulline, which is used to maintain blood vessel dilatation and blood flow. It is known that eNOS is damaged in myocardial ischemia–reperfusion, decreasing NO generation, and increasing vasoconstriction. This limits blood flow, resulting in platelet activation, formation of thrombi, and leukocyte interaction with endothelium,

which damages it further and negatively affects eNOS.[36] We theorize that both Delt-D_{var} and HWP block platelet and leukocyte aggregation and block the release of inflammatory cytokines in ischemic endothelium, allowing adequate eNOS-generated NO production. In cell culture, blood vessel dilation and flow, as well as platelet and leukocyte aggregation, are not considerations. Here, the ischemic protective effects of Delt-D_{var} and HWP most likely rely on blocking the LPS-IFN-γ–induced inflammatory cytokine cascade, which disrupts cellular integrity allowing for the release of NOS generated NO,[37] or by acting as metabolic inhibitors.[38] While the N9 microglial macrophage cell line is not a neuronal cell line, it is derived from tissue that is autochthonous in the nervous system and has been traditionally used for monitoring NO production by the Griess assay. In future experiments, we will attempt to use human endothelial cell lines such as HUV-EC-C or HAAE-2 to monitor LPS-IFN-γ–induced NO release, since these cells are more representative of the cerebral tissue exposed to ischemic injury.

CONCLUSIONS

Our study provides further evidence that both Delt-D_{var} and HWP provide ischemic protection by mechanisms requiring activation of the $delta_2$ opioid receptor subtype and blocking of NO release.

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