Vascular Factors Are Critical in Selective Neuronal Loss in an Animal Model of Impaired Oxidative Metabolism

NOEL Y. CALINGASAN, DVM, PhD, PAUL L. HUANG, PhD, HONG S. CHUN, PhD, ATTILA FABIAN, MS, AND GARY E. GIBSON, PhD

Abstract. Thiamine deficiency (TD) models the cellular and molecular mechanisms by which chronic oxidative deficits lead to death of select neurons in brain. Region- and cell-specific oxidative stress and vascular changes accompany the TD-induced neurodegeneration. The current studies analyzed the role of oxidative stress in initiating these events by testing the role of intercellular adhesion molecule-1 (ICAM-1) and endothelial nitric oxide synthase (eNOS) in the selective neuronal loss that begins in the submedial thalamic nucleus of mice. Oxidative stress to microvessels is known to induce eNOS and ICAM-1. TD increased ICAM-1 immunoreactivity in microvessels within the submedial nucleus and adjacent regions 1 day prior to the onset of neuronal loss. On subsequent days, the pattern of ICAM-1 induction overlapped that of neuronal loss, and of induction of the oxidative stress marker heme oxygenase-1 (HO-1). The intensity and extent of ICAM-1 and HO-1 induction progressively spread in parallel with the neuronal death in the thalamus. Targeted disruption of ICAM-1 or eNOS gene, but not the neuronal NOS gene, attenuated the TD-induced neurodegeneration and HO-1 induction. TD induced ICAM-1 in eNOS knockout mice, but did not induce eNOS in mice lacking ICAM-1. These results demonstrate that in TD, an ICAM-1-dependent pathway of eNOS induction leads to oxidative stress-mediated death of metabolically compromised neurons. Thus, TD provides a useful model to help elucidate the role of ICAM-1 and eNOS in the selective neuronal death in diseases in which oxidative stress is implicated.

Key Words: Intercellular adhesion molecule-1; Endothelial nitric oxide synthase; Metabolism; Neurodegeneration; Oxidative stress; Thiamine.

INTRODUCTION

Experimental thiamine deficiency (TD) models the cellular and molecular mechanisms by which chronic oxidative deficits lead to death of select neurons in brain. Consistent temporal and spatial patterns of neuropathological changes make TD a unique and powerful model to investigate the mechanisms of region-selective neurodegeneration associated with impaired oxidative metabolism. TD is characterized by cholinergic insufficiency (1, 2), memory deficits (3), and reduced activities of thiamine-dependent enzymes in brain (4, 5). TD compromises oxidative metabolism as measured by reduction of the activities of the mitochondrial enzyme α-ketoglutarate dehydrogenase complex (KGDHC), as well as transketolase throughout the brain before the onset of pathologic changes (6). These generalized biochemical deficits appear to predispose the brain cells to various insults such as oxidative stress (7). During TD, indicators of oxidative stress including heme oxygenase-1 (HO-1; 8), superoxide dismutase (9), ferritin, and reactive iron (10) accumulate in microglia, while the nitration product of peroxynitrite, nitrotyrosine (10) and the lipid peroxidation product, 4-hydroxynonenal (8) increase in neurons of vulnerable regions, particularly the thalamus. TD increases the concentration of reactive oxygen species in the thalamus (11). Although cell- and region-specific oxidative stress accompanies neuronal damage, the mechanism responsible for the selective vulnerability in TD remains unclear. Our recent studies demonstrate that HO-1 induction during TD correlates with the neuronal dropout in the submedial nucleus, the initial site of neuronal damage in the thalamus (8). However, the initiating trigger for the early neuronal death has not yet been discovered.

The blood-brain barrier (BBB) is an important site of numerous TD-induced alterations in regions where neurons are destined to die. Selective increases in IgG permeability occur in regions that exhibit neuronal death at relatively late stages of TD (12, 13). IgG extravasation is preceded by neuronal loss and HO-1 induction (8). In microvessels of vulnerable regions, TD increases endothelial nitric oxide synthase (eNOS) and ferritin immunoreactivities, as well as nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase reactivity (10). Furthermore, TD induces an inflammatory response in vulnerable regions, as shown by the proliferation of perivascular, activated microglia (10, 14) and granulocytes (15). Inflammatory processes influence the levels of vascular factors including nitric oxide (NO) and intercellular adhesion molecule-1 (ICAM-1; 16).

ICAM-1 (CD54) is an inducible cell-surface glycoprotein expressed in endothelial cells, leukocytes, tissue...
macrophages, and dendritic cells (17). ICAM-1 promotes adhesion of leukocytes to vascular endothelium, allowing extravasation of these cells (18). In cell culture, ICAM-1 induction occurs in response to inflammatory factors (19) and oxidative stress (20). In brain, ICAM-1 is induced in endothelial, microglial, and mononuclear cells in inflammatory and necrotic lesions, as well as in multiple sclerosis plaques (21) and in senile plaques of Alzheimer brains (22). Whether ICAM-1 contributes to neuronal death following TD, or in these disorders, has not been tested.

Nitric oxide (NO) is a biological messenger and neurotransmitter, as well as a potential mediator of neurotoxicity. Excessive NO is capable of altering the BBB permeability. TD induces nitric oxide synthase (NOS) in susceptible brain regions (10). Inducible NOS (iNOS) increases in macrophages/microglia in the TD mouse thalamus, but not in spared regions. However, iNOS is not a required component of the cascade in TD pathology since deletion of the iNOS gene does not mitgicate the TD-induced brain damage nor HO-1 induction (8). Although TD induces eNOS in microvessels within areas of neuronal damage (10), whether eNOS is deleterious to neurons during TD is unknown.

The current studies tested the role of ICAM-1 and eNOS in the selective neuronal death induced by TD. Using mice lacking the gene encoding ICAM-1 or eNOS, these studies demonstrate that both ICAM-1 and eNOS are critical factors in selective neuronal loss following TD.

**MATERIALS AND METHODS**

**Induction of Thiamine Deficiency**

In Experiment One (time-course studies of ICAM-1 induction in TD), TD was induced in separate groups of 3-month-old C57BL/6 mice for 7 (n = 4), 8 (n = 6), 9 (n = 7), 10 (n = 6), or 11 (n = 6) days. As described previously (8, 10, 12), mice received a thiamine-deficient diet (ICN Nutrition Biomedicals, Cleveland, OH) ad libitum and daily intraperitoneal injection of the thiamine antagonist, pyrithiamine hydrobromide (0.1 ml of saline/10 g body weight; Sigma Chemical Co., St. Louis, MO). Control animals (n = 5) received a normal thiamine-containing diet ad libitum and intraperitoneal saline injections daily (0.1 ml/10 g body weight).

In Experiment Two (effect of gene-targeted deletion of ICAM-1 and eNOS on TD-induced lesions), TD was induced in 3-month-old male ICAM-1 knockout (n = 9; Jackson Laboratories, Bar Harbor, Maine), eNOS knockout (n = 7), and nNOS knockout (n = 5) mice, and wild-type mice (n = 5 for ICAM-1 knockout, 5 for both eNOS and nNOS knockouts) for 11 days, as described above. All knockout mice have the same genetic background (i.e. C57BL/6). Hence, for all these studies, C57BL/6 mice were used as wild-type controls. Controls were treated as in Experiment One (n = 9 for ICAM-1 knockout and 5 for corresponding wild-type; 7 for eNOS knockout, and 5 each for nNOS knockout and wild-type). The Institutional Animal Care and Use Committee of the Weill Medical College of Cornell University approved all animal procedures employed in these studies.

**Tissue Preparation**

Mice were euthanized with a lethal dose of sodium pentobarbital (6 mg/100 g intraperitoneal; Fort Dodge Laboratories, Fort Dodge, IA) at different time points of TD. Prior to perfusion, tissue samples from tails of knockout mice were collected for genotyping. Mice were perfused transcardially with 0.9% NaCl solution followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.4). Brains were removed, postfixed for 2 h in the same fixative, and then placed in 30% sucrose solution overnight at 4°C for cryoprotection. Coronal sections (35-μm-thick) were cut on a sliding microscope.

**Immunocytochemistry**

The staining protocol employed a modified avidin-biotin-peroxidase immunocytochemistry procedure (23) as described previously (8, 10, 12). Briefly, sections were pretreated with 3% H2O2 in 0.1 M sodium phosphate buffered saline (PBS) for 30 min. The sections were incubated sequentially in (a) 1% bovine serum albumin (BSA) and 0.2% Triton X-100 in PBS for 30 min, (b) ICAM-1 (1:100; Chemicon, Temecula, CA), eNOS (1:1,000, Transduction Laboratories, Lexington, KY), myeloperoxidase (1:1,000, Chemicon), or HO-1 (1:4,000, StressGen, British Columbia, Canada) antibody in 0.1 M PBS/0.5% BSA for 18 h, (c) biotinylated anti-hamster or anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:200 in PBS/0.5% BSA for 1 h, and (d) avidin-biotin-peroxidase complex (Vector) diluted 1:200 in PBS for 1 h. All incubations and rinses were performed at room temperature with continuous agitation on an orbit shaker. Immunoreactivity was visualized by incubating the sections in 0.05% 3,3′-diaminobenzidine tetrahydrochloride dihydro (DAB; Sigma) containing 0.003% H2O2 in PB.

**Western Blot Analysis**

Control (n = 2) and 11-day TD (n = 2) C57BL/6 mice were killed under halothane anesthesia and perfused transcardially with 150 mM NaCl. The whole thalami were carefully dissected from the brains and homogenized in 20 mM Tris-HCl buffer (pH 7.6) containing 2 mM EGTA, 14 mM 2-mercaptoethanol and protease inhibitor cocktail (Sigma; 100 μl/10 ml). Protein content was determined using the Micro BCA kit (Pierce, Rockford, IL). Proteins were electrophoretically resolved on 10% polyacrylamide gels (1.5 mm slabs) and electroblotted to nitrocellulose membranes. The membranes were blocked overnight in 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl [Tris buffered saline (TBS)] containing 5% nonfat dry milk. Immunoreactivity was detected by employing the avidin-biotin-peroxidase technique (Vector) and DAB for ICAM-1, or the nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate detection system (Sigma) for eNOS.

**Neuron-specific Nuclear Protein (NeuN) Immunocytochemistry**

As in our previous studies (8), quantification of neuronal loss was performed using sections that were immunostained with an
antibody against an excellent neuronal marker, neuron-specific nuclear protein (NeuN; 24). NeuN immunostaining eliminates the problem of distinguishing between small interneurons and glial cells, which is encountered in routine hematoxylin and eosin or cresyl violet staining. Sections were pretreated with 0.05 M potassium phosphate buffered saline (KPBS) containing 1% NaOH and 3% H2O2 for 30 min. After rinsing in KPBS 3 times for 10 min each, sections were treated with 0.4% Triton X-100 and 1% BSA in KPBS for 30 min. The sections were incubated in NeuN antiserum (Chemicon; 1:1,000 in KPBS/1% BSA/0.4% Triton) for 18 h. After rinsing in KPBS containing 0.25% BSA and 0.02% Triton X-100, sections were incubated in biotinylated anti-mouse IgG (1:200 in KPBS/0.25% BSA/0.02% Triton) for 1 h, followed by avidin-biotin-peroxidase complex (1:200 in KPBS) for 1 h. Sections were rinsed in 0.05 M KPBS, and the reaction was developed in 0.05% DAB and 0.003% H2O2 in KPBS. All steps were performed at room temperature with continuous agitation.

**Blood-brain Barrier (BBB) Disruption**

The integrity of the BBB was assessed on the same sections that were stained with NeuN antibody. Since the monoclonal NeuN antibody requires the use of anti-mouse IgG as secondary antibody, leakage of IgG, a measure of BBB breakdown (12, 25) was immunohistochemically detectable in the same sections stained for NeuN. The pattern of IgG immunostaining in NeuN-labeled sections was identical to that of semiadjacent sections that were stained specifically for mouse IgG following the standard immunohistochemical detection of BBB disturbances (25). IgG immunoreactivity did not interfere with NeuN staining of neurons.

**Quantitative Analysis of Neuronal Loss**

By the criteria of Coggeshall and Lekan (26), areal density nuclear profile counts were determined as indicators of neuronal cell numbers. This strategy was used for several reasons. First, estimates from nuclear profile counts deviate less from true numbers compared with total profile counts, which include cytoplasmic edges, since the diameter of the neuronal nuclei in the submedial thalamic nucleus is small (average wide diameter ~10 μm; average narrow diameter ~8 μm) in relation to section thickness (35 μm).

Secondly, TD did not alter the size of individual neuronal nuclei in the submedial nucleus. For example, the mean (±SEM) widest diameter (μm) of neuronal nuclei in control ICAM-1 /−/− mice was 10.76 ± 0.1 (n = 105 nuclei), while that of TD ICAM-1 /−/− mice was 10.42 ± 0.1 (n = 101 nuclei). The mean (±SEM) narrowest diameter (μm) of nuclei in control ICAM-1 /−/− mice was 8.46 ± 0.1 (n = 105 nuclei), while that of TD ICAM-1 /−/− mice was 8.1 ± 0.1 (n = 101 nuclei).

In addition, TD did not alter the overall size of the submedial nucleus. To avoid over- or under-estimation of NeuN-positive nuclei because of TD-induced changes in the overall size of the submedial thalamic nucleus and adjacent regions due to tissue and cell edema, we compared 2-dimensional distances between known landmarks encompassing the submedial thalamic nuclei. The distance between the left and right mammillothalamic tracts, as well as the distance between the mammillothalamic tract and the rhomboid nucleus were measured. The area inclusive of the submedial nucleus and adjacent regions was then estimated. The analysis revealed no differences between control and TD groups. For example, in ICAM-1 /−/− mice, TD did not alter the size of the submedial thalamic nucleus area (3.26 ± 0.25 mm2; mean ± SEM) compared with controls (3.28 ± 0.14 mm2) (p > 0.47). Similarly, no statistical difference was found between C57BL/6 (wild type) control (3.14 ± 0.21) and TD (3.4 ± 0.25) (p > 0.19).

NeuN-immunostained sections were used for quantifying neuronal loss. As described in our previous studies (8), neuronal counts were obtained for an area covering the submedial nucleus (previously called gelatinous nucleus) where TD-induced neuronal loss is first detected within the thalamus in mice (8), as in rats (27). The mouse brain atlas of Franklin and Paxinos (28) was used as a guide, sections through 3 rostrocaudal levels (175 μm apart) of the submedial thalamic nucleus were analyzed under the 10× objective. Total neuronal cell counts were made within a 0.46 mm2 area for each side of the brain. This area encompasses the submedial nucleus and part of adjacent subnuclei. Care was taken to position a calibrated eyepiece grid by using the mammillothalamic tract as a landmark (Fig. 2). Results are presented as means of 3 total counts from different rostrocaudal levels.

**Quantitative Analysis of HO-1 Induction**

For evaluation of HO-1 induction, HO-1-stained sections adjacent to those used for NeuN immunoreactivity were used. HO-1 induction was measured by counting the intensely stained glial cells throughout the brain sections. For each animal, 3 sections (175 μm apart) were examined. Only the intensely stained cells that were distinctly above the background level of staining were counted.

**Genotyping of ICAM-1, eNOS, and nNOS Knockout Mice**

At the conclusion of Experiment Two, the genotype of the knockout mice was confirmed. Genomic DNA was isolated from tails by phenol extraction and ethanol precipitation. For ICAM-1 knockout mice, polymerase chain reaction (PCR) amplification of genomic DNA extracted from the tails was performed using GeneAmp PCR Reagent Kit (Perkin Elmer, Branchburg, NJ). The PCR primers used were oIMR017, 5′-CTGAGCCAGCTGGAGGTCTCG; oIMR018, 5′-GAGCGG-CAGAGCAAAGAAGC; and oIMR019, 5′-AGGACAGCA-GGGGAGGGGATT (Kitagawa et al, 1998; Gene Link, Thornwood, NY). The cycle conditions were: initial denaturation at 94°C for 3 min followed by 94°C for 20 s, 64°C for 30 s, 72°C for 35 s, and 72°C for 2 min for 35 cycles. The genotype was also confirmed by using ICAM-1 antibody to immunostain 11-day TD ICAM-1 knockout brain sections in parallel with 11-day TD wild-type control sections, which are known to display robust ICAM-1 staining of microvessels in the thalamus (see Results section).

Genotyping of eNOS- and nNOS-knockout mice was performed as described previously (29, 30). Genomic DNA was isolated from the tails as described above. PCR analysis for nNOS used the following primers: PHL nNOS B1 primer: 5′ CCTTTTGAGAGTAAAGGAAGGGCG 3′; PHL nNOS B2...
changes in the TD model makes it useful for testing the
coordination and loss of righting reflex after 10 to 11
terations below). All animals showed motor in-
pathological changes in brain (see results of the gene de-
weight loss can predict the severity of TD-induced

primer: 5’ GGGCGATCTACGGCTTCCCAAGGCTGCCAC 3’.
For eNOS, the primers used were PLH eNOS E1: 5’
GGATCCCTGGAAAAGGCGGTGAGG 3’.
The cycle conditions were initial denaturation at 94°C for 4 min followed by
94°C for 30 s, 60°C for 30 s, 72°C for 1 min for 35 cycles. For
Southern blot, 10 μg of genomic DNA was digested with Spe
I, resolved by electrophoresis through 1% agarose gels, and
transferred to nitrocellulose. The blots were hybridized with the
32P-labeled pKS probe in 50% formamide, 6

transectionally progressing lesion experiments below). All animals showed motor in-
and loss of righting reflex after 10 to 11
days of TD.

The highly replicable progression of neuropathological
changes in the TD model makes it useful for testing the
effect of various genotypes. Time course studies in
C57BL/6 mice revealed no apparent macroscopic brain
lesions on days 8 or 9. After 10 to 11 days of TD, pin-
point hemorrhages occurred in the thalamus, mammillary
body, inferior colliculus, dorsal lateral and medial genic-
ulate nuclei, superior and inferior olives, and some peri-
ventricular regions. Brain ventricles displayed edematous
enlargement at day 11. The current studies focused main-
ly on the thalamus because of the identification of the
initial site of neuronal loss in specific thalamic subnuclei,
which progressively spreads to other nuclei until the
whole thalamus is affected (8). The consistent pattern of
neuronal loss makes it possible to study the temporal re-
lationships between neuronal loss and the region-select-
ive neuropathological changes.

Experiment One: Time-course Studies of ICAM-1
Induction in TD

Pattern of Neuronal Loss in the Thalamus of C57BL/6
Mice during TD: As in previous studies (8), the earliest
signs of definite neuronal loss in the submedial nucleus of
the thalamus of C57BL/6 mice occurred on day 9 of
TD (Fig. 2). The submedial nucleus, formerly known as
gelatinous nucleus, is a small bilateral thalamic structure
positioned near the midline dorsomedial to the mammil-
lothalamic tract (28). Bilateral, well-defined areas of neu-
ronal death were apparent in the submedial nucleus, and
partly the neighboring ventromedial and ventrolateral nu-
clei. Ten days of TD produced more severe neuronal
death, and by day 11, almost all thalamic nuclei were
affected.

ICAM-1 Induction Preceded Early Neuronal Loss in
C57BL/6 Mice during TD: As in past reports (31),
ICAM-1 immunoreactivity was virtually undetectable in
most endothelial cells in normal mouse brain. However,
very low levels of ICAM-1 immunoreactivity occurred
in vascular endothelium in patchy areas of control and 7-
day TD brains (Fig. 2). Microvascular ICAM-1 immu-
noreactivity increased in the thalamus, particularly in the
submedial and ventromedial nuclei after 8 days of TD, 1
day prior to the onset of neuronal loss. Nine days of TD
further enhanced ICAM-1 expression. The pattern of mi-
crovascular ICAM-1 induction roughly overlapped with
neuronal loss. By day 10, ICAM-1 immunoreactivity in
vascular walls was remarkably increased in larger areas
of the thalamus, including those where severe neuronal
dead was not yet apparent. Thus, subsequent ICAM-1
induction occurred prior to neuronal damage. As in our
previous report (8), focal increases in IgG immunoreac-
tivity (indicating selective increases in BBB permeabili-
ty) also occurred at this stage. These areas of IgG ex-
travasation did not overlap with the region of initial
neuronal loss. The intensity and extent of ICAM-1 in-
duction progressively increased in microvessels, irrespec-
tive of their size, during the course of TD pathology.
Fig. 2. NeuN (left) and ICAM-1 (right) immunoreactivity in adjacent sections of the thalamus of C57BL/6 mice in control, and days 8, 9, and 10 of TD showing the patterns of early neuronal loss and ICAM-1 induction in the submedial thalamic nucleus during TD. ICAM-1 is induced in microvessels (arrows) on day 8, prior to neuronal loss (asterisks), which occurs in the submedial nucleus on day 9. Note the progression of ICAM-1 induction in microvessels on day 10, even in areas where neurons are still intact. Quantification of neuronal loss was performed in the boxed areas. Scale bar = 250 μm.

Immunoblotting analysis of brain homogenate from dissected thalami verified the identity of the protein recognized in microvessels by immunocytochemistry. Eleven days of TD increased the expression of the ~95 kDa protein that is consistent with ICAM-1 compared with control (Fig. 3). Thus, the protein that increased in region-specific microvessels with TD was an ICAM-1 immunoreactive species of appropriate size.

Accumulation of Myeloperoxidase (MPO)-positive Cells during TD: ICAM-1 binds to leukocytes and facilitates adhesion and transendothelial migration of leukocytes (18). To determine whether leukocytes accumulate
in vulnerable regions where ICAM-1 is induced, the thalamus was examined for the presence of MPO-immunoreactive cells. MPO is a marker for inflammatory cells, particularly neutrophils (32). MPO-positive cells were detected in the thalamus following ICAM-induction. The appearance of MPO-immunoreactive cells began on day 10 of TD. By day 11, numerous MPO-immunoreactive cells were scattered throughout the thalamus (Fig. 4).

**Experiment Two: Effect of Gene-targeted Deletion of ICAM-1 and eNOS on TD-induced Lesions**

**Confirmation of Genotype:** ICAM-1 knockout mice did not exhibit ICAM-1 gene expression, as assessed by DNA on PCR analysis or by immunocytochemistry. PCR analysis with the primers oMR018 and oMR019 amplified a 150-bp band in ICAM-1 knockout samples, while the primers oMR017 and oMR018 amplified a 178-bp DNA fragment in wild-type controls (Fig. 5). These bands are of the expected size for the ICAM-1 knockouts and wild-type controls. Immunocytochemical staining of the TD ICAM-1 knockout sections revealed some cross-reactivity with the secondary antibody in the parenchyma, but no intense ICAM-1 staining in microvessels, which was evident in TD wild-type sections (Fig. 5).

PCR analysis revealed 900 and 404 bp bands, which are the expected sizes for eNOS and nNOS wild-type mice, respectively, but not in knockout mice (Fig. 6, top). Southern blot analysis showed no evidence of eNOS gene expression in eNOS mutant mice (Fig. 6, bottom). The immunocytochemical data showing lack of eNOS protein expression in TD sections (not shown) corroborated the results of the genotyping analysis.

**Neuronal Loss, HO-1 Induction and Blood-brain Barrier Breakdown in ICAM-1 Knockout Mice:** To test whether targeted disruption of the murine ICAM-1 gene would attenuate TD-induced neuronal loss, ICAM-1 knockout mice and wild-type controls were made thiamine-deficient. ICAM-1 knockout mice showed less severe neuronal loss compared with wild-type mice (Fig. 7). While TD in wild-type mice reduced the number of neurons in the submedial nucleus region by 87% compared with controls, only a 56% reduction occurred in ICAM-1 knockout mice. Thus, ICAM-1 deficiency significantly attenuated the severity of TD-induced neurodegeneration (p < 0.01, ICAM-1 knockout vs wild-type).

Light HO-1 immunoreactivity occurred throughout the brain in saline-treated controls and wild-type mice. Eleven days of TD produced intense HO-1 immunoreactivity in microglia within the thalamus of wild-type mice. In contrast, deletion of ICAM-1 gene significantly reduced the number of HO-1 immunopositive microglia in the thalamus (p < 0.05) (Fig. 8). Based on the extent and severity of IgG extravasation, BBB breakdown was attenuated in ICAM-1 knockout mice compared with wild-type controls (data not shown).

**Neuronal Loss, HO-1 Induction and Blood-brain Barrier Breakdown in eNOS and nNOS Knockout Mice:** Our previous studies reveal that TD enhances the expression of eNOS (Fig. 3) and nicotinamide adenine dinucleotide...
Phosphate (NADPH) diaphorase reactivity in microvessels within the thalamus (10). Thus, the current experiments tested whether a deficiency of nitric oxide from eNOS or nNOS alters the severity of TD-induced neuronal loss. Thiamine-deficient eNOS, but not nNOS knockout mice, showed less severe neuronal loss compared with wild-type controls (Fig. 7). TD produced an 85% reduction of neurons in wild-type mice and 55% and 88% reductions of neurons in eNOS and nNOS knockout mice, respectively. Our previous report shows that the neuronal loss in iNOS knockout mice was comparable to wild-type controls (8). Thus, the lack of eNOS significantly reduced neuronal damage produced by TD (p < 0.05, eNOS knockout vs wild-type).

TD led to the accumulation of HO-1 immunoreactive microglia in the thalamus of eNOS and nNOS knockout mice as well as wild-type controls. However, mice lacking eNOS displayed only half the number of HO-1 positive microglia compared with nNOS knockouts or wild-type mice (Fig. 8). The TD-induced IgG accumulation in the thalamus of eNOS knockout mice was less severe than nNOS knockout mice and wild-type controls (data not shown).

**Relationship Between ICAM-1 and eNOS Induction during TD:** To determine whether ICAM-1 and eNOS initiate separate pathways during TD, sections from 11-day TD eNOS knockout mice were immunostained for ICAM-1, and sections from TD ICAM-1 knockout mice were immunostained for eNOS. TD induced ICAM-1 in thalamic microvessels in eNOS knockout mice comparable to that found in C57BL/6 mice (Fig. 9). However, TD did not enhance eNOS immunoreactivity in ICAM-1 knockout mice (not shown).

**DISCUSSION**

The current studies show that vascular factors are critical in a cascade of events that lead to oxidative stress and death of metabolically compromised neurons in specific brain regions following TD. TD induced ICAM-1 in microvessels in the submedial nucleus beginning 1 day prior to the onset of neuronal cell death. ICAM-1 induction led to MPO-positive leukocyte accumulation that was associated with neuronal injury. During late stages
Fig. 7. Neuronal loss in the submedial thalamus of ICAM-1, eNOS and nNOS knockout mice and wild-type controls on day 11 of TD. Values represent the mean ± SEM. ICAM-1 −/− TD (n = 9); ICAM-1 −/− control (n = 9); wild-type for ICAM-1 TD (n = 5); wild type for ICAM-1 control (n = 5); eNOS −/− TD (n = 7); eNOS −/− control (n = 7); nNOS −/− TD (n = 5); nNOS −/− Control (n = 5); wild type for NOS −/− TD (n = 5); wild type for NOS −/− control (n = 5). *p < 0.01 vs wild-type (top); *p < 0.05 vs wild-type (bottom).

Fig. 8. Microglial heme oxygenase-1 induction in 11-day TD thalamus of ICAM-1, eNOS and nNOS knockout mice and respective wild-type controls. Values represent the average number of HO-1 labeled microglia in 3 rostrocaudal levels of the thalamus (mean ± SEM). n values are the same as Fig. 7. P < 0.05 vs wild-type.

of TD, eNOS was induced in microvessels within the thalamus. Studies utilizing mice that lack ICAM-1 or eNOS gene demonstrate directly the deleterious consequences of ICAM-1 or eNOS induction in TD. Absolute inactivation of ICAM-1 or eNOS afforded significant neuroprotection in the thalamus during TD. This finding contrasts with our previous studies in which neither deletion of iNOS gene nor pharmacological NOS inhibition attenuates TD-induced cell loss (8). ICAM-1 induction in TD is not mediated by nitric oxide from eNOS. ICAM-1 induction occurred first, and a deficiency of eNOS in knockout mice did not block ICAM-1 induction following TD. Furthermore, TD failed to induce eNOS in ICAM-1 knockout mice. Thus, an ICAM-1-dependent pathway of eNOS induction in susceptible regions is critical in the pathogenesis of TD:

TD → ICAM-1 → eNOS → neuronal death

Whether TD causes neurons to produce a signal that induces ICAM-1 cannot be assessed from the current experiments. These results further demonstrate the utility of genetic knockout mice in the study of the role of vascular factors in models of neurodegeneration as in focal cerebral ischemia (33) and ischemia-reperfusion injury (34).

The early events in the submedial nucleus demonstrate that neurons respond to oxidative stress by dying, whereas other cells appear to upregulate antioxidant processes and survive. In the current study, only loss of neurons was monitored. Neuronal changes undoubtedly precede this cell death. Our unpublished studies measuring DNA fragmentation or using modified Tunel assay (ApopTag kit; Oncor, Gaithersburg, MD) did not reveal any evidence of apoptosis either before or during the onset of ICAM-1 induction. On day 9 of TD, when most neurons in the submedial nucleus have degenerated, ICAM-1 immunoreactive microvessels and HO-1 containing microglia were still apparent within the area of neuronal loss, and exhibited signs of an antioxidant response. These observations are consistent with the demonstration that in vitro TD reduces KGDHC activity, causes accumulation of the lipid peroxidation product 4-hydroxynonenal, and
induces apoptosis exclusively in cultured primary neurons, but not in other brain cell types (35). During late stages of TD in mice, 4-hydroxynonenal and nitrotyrosine immunoreactivities accumulate in neurons, while iron increases in microglial cells within the thalamus (8, 10). Excess iron may damage neurons by increasing the formation of hydroxyl radicals (36). Neurons have a low iron-storage capacity compared with macrophages and microglia, which can survive a heavy iron load. Taken together, the in vivo and in vitro studies clearly demonstrate that the cell-specific responses to impairment of oxidative metabolism from TD reflect inherent properties of the different brain cell types.

Endothelial cells are a critical site of inflammation and free radical production leading to permeability changes of the BBB in vulnerable regions during TD. The present findings complement the demonstration that in vitro TD enhances the permeability and decreases oxidative metabolism of cultured endothelial cells (37). Metabolic disturbances lead to endothelial release of a variety of harmful substances including adhesion molecules, NO, and histamine (38). Histamine release and granulocyte number increase within the thalamus during TD (15). The data suggest that the endothelial cell response to impaired oxidative metabolism varies between anatomic sites. Studies comparing the response of endothelial cells derived from vulnerable and invulnerable regions to TD will be required to directly resolve the issue. Nevertheless, the data are consistent with the idea that selective changes in endothelial cells lead to selective neuronal death.

ICAM-1 induction following TD may promote neurodegeneration by several mechanisms. ICAM-1 mediates inflammation-induced rolling, adhesion, and accumulation of polymorphonuclear leukocytes to the endothelial surface, and their subsequent migration across the endothelial cells (18). The precise mechanisms by which leukocytes migrate across the endothelium are not known. Recent immuno-ultrastructural evidence shows ICAM-1 expression in endothelial cell vesiculotubular structures in injured BBB, suggesting that these vesiculotubular structures are a possible route for transendothelial migration across the BBB (39). Interestingly, our previous studies reveal numerous vesicular profiles in the endothelial cytoplasm of TD thalamus where the BBB is disrupted (12). The transmigration of leukocytes, especially granulocytes, may contribute to neurodegeneration in several ways. First, leukocyte adherence to capillary endothelium can occlude microvessels (40, 41) resulting in microcirculatory failure. The rat thalamus is supplied by arterioles that have an abrupt origin and small diameters, rendering these vessels more prone to hemorrhagic stroke (42, 43). Well-circumscribed lesions in the rodent thalamus in late stages of TD resemble those in ischemia, a disease model where infarct size is attenuated by ICAM-1 deficiency (33). Another possible mechanism for leukocyte-mediated pathology is that leukocytes can release free radicals and damage neurons (44). Myeloperoxidase secreted by leukocytes converts hydrogen peroxide to hypochlorous acid, which destroys tissue by forming aldehydes and oxidizing biomolecules (45). Finally, diapedesis of leukocytes, particularly neutrophils into the perivascular space, can further aggravate the inflammatory response and subsequently contribute to neuronal injury and demise. Neutrophil recruitment into the brain parenchyma leads to extravasation of plasma proteins (46 for review). The current results do not distinguish between these possibilities. The attenuation of TD-induced neuronal damage and the impairment of the inflammatory response in TD mice lacking the ICAM-1 gene suggest that ICAM-1 induction is a critical initial event that leads to neuronal death.

Although eNOS mediates basal vasodilation (30), excessive endothelium-derived NO damages neurons. Results of the current studies with eNOS knockout mice suggest that the beneficial effects of vasodilation are less important than the toxic effects during TD. The finding that targeted disruption of the eNOS gene attenuated the neuronal damage from TD confirms the hypothesis that
NO derived from endothelial cells contributes to TD-induced neurodegeneration. A sustained NO production is capable of altering the BBB permeability allowing extravasation of blood proteins that damage neurons. In models of focal ischemia (47) and meningitis (48), pharmacological inhibition of NOS mitigates the increased BBB permeability. Although the direct toxicity of NO is modest, excessive endothelial NO may react with superoxide, a free radical that has been suggested to accumulate in the thalamus (9), to form peroxynitrite (ONOO\(^{-}\)). Peroxynitrite in turn can diffuse from its vascular origin and produce hydroxyl radicals that are harmful to neurons (49). Peroxynitrite can also injure neurons by modifying tyrosine in proteins, producing nitrotyrosine. TD increases nitrotyrosine immunoreactivity in axons and axon terminals in the thalamus (10). Tyrosine nitration can affect structural proteins with significant pathological consequences (50).

The current results are consistent with the hypothesis that a generalized impairment of oxidative metabolism from TD leads to an ICAM-1 dependent cascade of inflammatory and oxidative stress responses in vulnerable regions. Deleterious compounds or cytokines are released from activated leukocytes and inflammatory cells including microglia. ICAM-1 and eNOS induction compromises the integrity of the BBB allowing entry of extraneuronal proteins and iron, thereby exacerbating the oxidative damage to metabolically compromised neurons that eventually die. Thus, the TD model may help elucidate the role of vascular ICAM-1 and eNOS in the selective neuronal death in diseases in which oxidative stress is implicated.

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