Hypertonic Mannitol Loading of NF-κB Transcription Factor Decoys in Human Brain Microvascular Endothelial Cells Blocks Upregulation of ICAM-1

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Background and Purpose—An acute inflammatory response exacerbates tissue injury during acute ischemic stroke. The transcription factor nuclear factor (NF)-κB plays a key role in endothelial cell activation and the inflammatory response. Targeted genetic disruption of NF-κB activation in cerebral endothelial cells may be protective in stroke. We determined whether a NF-κB transcription factor decoy (TFD) could block intercellular adhesion molecule (ICAM)-1 upregulation, an indicator of endothelial cell activation.

Methods—We modeled ischemia-reperfusion in vitro by exposing cultured human brain microvascular endothelial cells (HBMEC) to tumor necrosis factor (TNF)-α and conditions of hypoxia-reoxygenation (H/R). Mannitol was used to load phosphothiorated oligonucleotides containing 3 copies of the κB binding sequences (TFDs) into cultured HBMEC. An NF-κB TFD, a mutated NF-κB TFD, and a scrambled TFD were studied for their effect on ICAM-1 mRNA levels and surface ICAM-1 by ELISA.

Results—Hyperosmolar loading with mannitol permitted rapid transfection of TFD into endothelial cell nuclei. The NF-κB TFD but not the mutated or scrambled TFD competed with a κB sequence for binding to nuclear extracts from HBMEC exposed to TNF-α. The NF-κB TFD blocked the TNF-α–induced and H/R-induced increase in ICAM-1 mRNA levels and the upregulation of surface ICAM-1.

Conclusions—Mannitol delivers phosphothiorated oligonucleotides into cultured HBMEC. An NF-κB decoy blocks both TNF-α–induced and H/R-induced ICAM-1 upregulation in HBMEC. Targeted genetic disruption of endothelial NF-κB activation may be of benefit in acute ischemic stroke. (Stroke. 2000;31:1179-1186.)

Key Words: endothelium ■ intercellular adhesion molecule-1 ■ nuclear factor kappa-B ■ transcription, genetic

An acute inflammatory response with influx of neutrophils exacerbates tissue injury during acute ischemic stroke.1,2 This acute inflammatory response is more intense with temporary than with permanent middle cerebral artery occlusions.3,4 Inhibiting neutrophils or neutrophil-endothelial cell interactions reduce the infarct size after temporary but not permanent occlusions of the middle cerebral artery.5,6 This suggests that inflammatory responses may be a potential hazard of thrombolytic therapy and that inhibiting these inflammatory responses may be useful adjunctive therapy to thrombolytics. Therapeutic benefit has been achieved by combining anti–intercellular adhesion molecule (ICAM)-1 antibody treatment with tissue-type plasminogen activator.7,8

During ischemia-reperfusion, a set of inflammatory endothelial genes including ICAM-1, E-selectin, and IL-8 are transcribed, resulting in an “activated” endothelial cell phenotype.9 The activated endothelial cell upregulates the surface expression of cell adhesion molecules and secretes cytokines, which act to sequester neutrophils in the ischemic zone. Ideally, to achieve an optimal “anti-inflammatory” therapeutic effect, multiple endothelial adhesion molecules and multiple inflammatory mediators must be targeted. Rather than targeting multiple individual inflammatory mediators, a more parsimonious strategy would be to target the “upstream” common pathway involved in the upregulated expression of multiple inflammatory genes.

The transcription factor nuclear factor (NF)-κB binds to the promoter of a large number of genes involved in the inflammatory response such as ICAM-1, E-selectin, vascular cell adhesion molecule (VCAM), IL-1, tumor necrosis factor (TNF)-α, IL-8, IL-6, COX 2, and intercellular nitric oxide synthase (iNOS).10 NF-κB is activated by a variety of stimuli that include cytokines such as TNF-α and IL-1 and oxidative...
stress. In the resting state, NF-κB is bound by an inhibitor, IκB, in the cytoplasm. After phosphorylation by IKK, IκB is degraded by the proteasome. This degradation of IκB releases NF-κB, which travels to the nucleus where it binds to cis-elements in the promoters of genes involved in the inflammatory cascade.

In previous studies, we have found that NF-κB is involved in the TNF-α–induced and hypoxia-reoxygenation (H/R)-induced upregulation of the ICAM-1 gene in human brain microvascular endothelial cells (HBMEC). Blocking NF-κB activation with pyrrolidine dithiocarbamate or the proteasome inhibitor n-Tosyl-Phe-chloromethyl ketone blocked the upregulation of the ICAM-1 gene. Transcription factor “decoys” (TFD) are double-stranded oligodeoxyribonucleotides that compete with endogenous cis DNA sequence elements in the regulatory regions of gene promoters for the binding of transcription factors. TFDs that can bind or “trap” NF-κB and prevent the transcription of genes involved in the inflammatory cascade could be of potential therapeutic value in acute ischemic stroke and other neurological disorders characterized by cerebral endothelial cell activation. We undertook this study to (1) determine if we could deliver TFDs into cultured HBMEC and (2) determine if a NF-κB TFD could inhibit the cytokine and H/R mediated upregulation of ICAM-1. In the course of our experiments, we found that hypertonic loading with mannitol achieved rapid introduction of TFDs into HBMEC, which are refractory to transfection with DNA alone.

**Methods**

**Human Brain Microvascular Endothelial Cells**

HBMEC were isolated from temporal lobectomy specimens from patients undergoing ablative surgery for partial complex seizures and were maintained in culture as previously described. Low-passage HBMEC were stably transfected with the plasmid construct pSV3NEO (ATCC 37150), which is encoded for the SV-40 large T-antigen and a bacterial gene, which confers resistance to neomycin. G418-resistant clones were obtained, and one of these, designated IT-1, displayed a substantially extended lifespan in culture. IT-1 cells are not immortalized but maintain vigorous growth for 20 to 30 passages in culture when grown in the presence of 200 μg G418/mL. They exhibit the following endothelial cell phenotypes: cobblestone morphology at high cell density (Figure 1A), tube formation when grown on matrigel (Figure 1B), and expression of von Willebrand factor (Figure 1C and D). These cells do not express smooth muscle actin, which suggests that they are not contaminated with vascular smooth muscle cells.
Individual primary isolates of HBMEC show unpredictable rates of senescence and usually cease cell growth by passage 5 to 8. They also vary greatly in their response to proinflammatory mediators. IT-1 cells, although not an immortal cell line, were used for most of the experiments described in this study because of their stability of phenotype, vigorous growth characteristics, and predictable response to proinflammatory mediators such as cytokines and H/R. Although IT-1 cells can be maintained in culture without noticeable senescence for prolonged periods, reference cultures were frozen at low passage and used between passages 3 and 6.

Decoys
Upper-strand and reverse-complement phosphorothioated oligonucleotides (38 mer) were synthesized, HPLC-purified, and annealed by Oligos, Etc, Inc. For fluorescence studies, TFDs were 5′-end labeled with fluorescein (FITC). Three oligonucleotide sequences were synthesized and used. In addition to the NF-κB TFD and its mutant analogue were as described by Goldring et al. The double-stranded NF-κB TFD contained 3 copies of a consensus, high-affinity NF-κB binding site found in the κ-light chain enhancer in B cells. In the mutant NF-κB TFD, the 3 guanine residues at the 5′ terminus of the NF-κB consensus sequences were changed to pyrimidines, TCT. The scrambled NF-κB TFD sequence was designed with the use of Transcription Element Search Software. It had the same base composition as the NF-κB TFD but contained no sequence predicted to bind a transcription factor in the Transcription Element Search Software database. The 38-mer (38-mer) NF-κB TFD sequence was 5′-GGGGACCTTCCGTGAGCAGGGCAGGTTCGGTCTGT-3′ and 5′-GTCTACTTTCCGCTGGACATCTTCCAGGGGAGTCTTCC-3′, with the consensus sequences underlined. The 38-mer scrambled NF-κB sequence (upper strand) was 5′-GGGGACCTTCCGTGAGCAGGGCAGGTTCGGTCTGT-3′.

Cell Loading
TFDs were introduced into HBMEC by a variation of the hypertonic Cell Loading solution (0.2 mL), cell monolayers were rinsed with PBS and rocking the plate to facilitate absorption of the PBS. The filter paper remained in contact with the monolayer for 10 seconds and then washed once with Ca/Mg-free PBS. After removal of PBS from the well by pipetting, residual PBS was removed by placing a 1-cm diameter 35 mm). Generally, cells from 3 wells provided sufficient RNA or nuclear extract for Northern blot and EMSA assays.

Hypoxia-Reoxygenation
IT-1 cells were grown in 6-well plates, and cells from 3 wells were labeled with fluorescein for RNA extraction. It is difficult to lower oxygen in the media in 96-well plates, and for this reason, gelatin-coated 48-well plates were used for the ELISA experiments. TFDs were loaded into cells with mannitol as above. The medium was then replaced with media that had been deoxygenated by bubbling 100% nitrogen for 45 minutes, and the cells were placed in the chamber for 15 hours. Reoxygenation was accomplished by removing the cells from the chamber and placing in a CO2 incubator at 37°C. Four hours later, RNA was extracted from the cells. In the experiments in which surface ICAM-1 was measured by ELISA, the cells were either fixed with paraformaldehyde immediately at the end of the hypoxia period or reoxygenated for 24 hours.

Electrophoretic Mobility Shift Assays
Nuclear extracts were obtained from IT-1 cells as described by Ledebur and Parks, and protein concentrations were measured with a BCA protein assay (Pierce). Parallel cultures of IT-1 cells were untreated or treated with TNF-α (100 U/mL; Genzyme Diagnostics) for 30 minutes. For the EMSA, a 22-mer, double-stranded DNA target probe containing a high-affinity NF-κB binding site was obtained from Stratagene and end-labeled with 32P-ATP and T4 kinase with the use of standard protocols. Binding reactions were initiated by mixing 17 μL of binding buffer (Stratagene GelShift Assay kit), 5μL of protein extract (usually 5 μg protein), and 1 μL of unlabeled competitor oligonucleotide (100-fold molar excess relative to the radiolabeled target probe). In reactions without protein or unlabeled competitor, 5 μL or 1 μL, respectively, of 25% glycerol was added to the reaction. After preincubation at room temperature for 5 minutes, reactions were placed on ice and 1 μL of radioactive target probe (100 000 dpm, 100 pg DNA) was added. Volume was adjusted to 25 μL with water, and reaction mixtures were incubated for 25 minutes on ice. Reactions were analyzed by electrophoresis in 4%, 0.1, 7-cm polyacrylamide gels in 1× Tris-glycine-EDTA buffer at 70 V (room temperature). Bands corresponding to complexes between NF-κB and the radiolabeled target oligonucleotide were detected by autoradiography. Relative band density was determined by scanning with a Shimadzu CS-9301 PC dual wavelength flying spot densitometer.

ICAM-1 ELISA
IT-1 cells were grown to confluence in 96-well plates (Costar) coated with 0.5% gelatin. To test the ability of various TFDs to influence TNF-α activation of NF-κB, oligonucleotides were hypertonically loaded into some wells as described above. After a 30-minute recovery period, loaded cells were treated with TNF-α (100 U/mL) for 4 or 24 hours. Control wells were untreated or received TNF-α alone. After cytokine treatment, cells were fixed for 15 minutes with 2% paraformaldehyde followed by blocking for 1 hour with 2% BSA in PBS. Monoclonal antibody to ICAM-1 (Becton Dickinson) diluted 1:7500 was added for 45 minutes at room temperature. In control wells, the first antibody was omitted. After 3 washes with PBS, peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) was added at a 1:1000 dilution for 45 minutes. After 3 washes with PBS, the substrate (TMB) was added and the reaction stopped in 10 to 20 minutes with 3N sulfuric acid. Optical densities were read in a plate reader at 450 nm. For the H/R experiments, after the cells were loaded with TFDs with mannitol, they were exposed to severe hypoxia for 15 hours and some cells exposed to a further 24-hour period of reoxygenation. After fixing with paraformaldehyde, the ELISA was performed as above. After adding the substrate, the solution in the well was transferred to the wells of a 96-well plate, and optical densities were read.

Northern Blot
Northern blotting was performed as described previously. Briefly, total RNA was extracted from 1 to 3x106 cells with TRIzol reagent (Life Technologies), subjected to electrophoresis in 1.2% horizontal agarose gels, which contained 2.2 mol/L formaldehyde, and then capillary transferred to nylon membranes. A radiolabeled human
ICAM-1 cDNA probe and a human GAPDH were hybridized consecutively to the membranes. Labeled ICAM-1 bands were detected with autoradiography.

Results

The NF-κB decoy was detected within the nuclei of HBMEC within 10 minutes after hypertonic loading with mannitol (Figure 2). Without hypertonic loading, there was no evidence of penetration of TFD into the cell or the nucleus even after prolonged exposure (4 to 24 hours). The hypertonic loading successfully transfected the oligonucleotides into endothelial cell nuclei in both primary cultures of HBMEC and IT cells.

To determine if the TFD specifically competed for NF-κB binding with a NF-κB consensus sequence, an EMSA was performed. In the EMSA, the NF-κB TFD but not the mutated or scrambled TFD competitively inhibited binding to a NF-κB oligonucleotide in nuclear protein extracts from IT-1 cells exposed to TNF-α for 30 minutes (Figure 3).

In Northern blot analysis of ICAM-1 mRNA levels, the NF-κB TFD blocked the TNF-α–induced upregulation of ICAM-1 mRNA in HBMEC down to unstimulated levels. The mutated TFD slightly reduced ICAM-1 mRNA levels, whereas the scrambled decoy had no effect on ICAM-1 levels (Figure 4). When HBMEC cells were exposed to conditions of severe H/R, there was an increase in ICAM-1 mRNA levels. This increase in mRNA levels was inhibited nearly to unstimulated levels (>70%) by the NF-κB decoy but only slightly by the mutated or scrambled decoy (Figure 5).

The NF-κB decoy reduced surface ICAM-1 levels as measured by ELISA. TNF-α–induced cell surface ICAM-1 was completely blocked (to unstimulated levels) by the NF-κB TFD but not by the mutated or scrambled TFD (Figure 6). Similarly, the NF-κB decoy completely

Figure 2. A, IT-1 cells mannitol-loaded with FITC–end-labeled NF-κB TFD oligonucleotide. Intense fluorescence is seen in more than 95% of cell nuclei. B, Hoechst stain (DAPI filter) of same cells demonstrating nuclei.
blocked the H/R-induced upregulation of surface ICAM-1 (Figure 7). A partial effect was seen with the mutated decoy, but no significant effect was seen with the scrambled decoy.

**Discussion**

During acute cerebral ischemia-reperfusion, the cerebral endothelium is exposed to inflammatory cytokines such as TNF-α and IL-1 and oxidative stress. Exposure of brain microvascular endothelial cells to TNF-α and conditions of H/R “models” ischemia-reperfusion to the brain endothelium in vitro. In our experiments, we use ICAM-1 as a “reporter” or “read-out” of endothelial cell activation. Our studies demonstrate that a NF-κB TFD blocks TNF-α–induced and H/R-induced ICAM-1 upregulation in human brain microvascular cells. This inhibition of upregulation was complete because both the TNF-α–induced ICAM mRNA levels and surface ICAM by ELISA were reduced to unstimulated levels. Similarly, the NF-κB decoy blocked the H/R-induced increase in ICAM mRNA levels. This confirms the importance of NF-κB in the TNF-α–induced and H/R-induced upregulation of ICAM-1 in HBMEC.

The regulatory regions that control cytokine-induced expression of the ICAM-1 gene in endothelial cells have been defined by deletional analysis and reveal that a single NF-κB site within a 92-bp region (−227 to −136) confers responses to TNF-α, IL-1β, and lipopolysaccharide. Both the ICAM-1 and IL-8 promoter regions contain an NF-κB site that differs at the extreme 5′ end from the consensus NF-κB sequence, where the conserved guanine residue is replaced by a thymidine residue (GGGRNNYYCC to TGGAAATTCC). NF-κB sites with this base change appear to be able to selectively bind other members of the NF-κB/rel family easily beside the classic p50/p65 heterodimer. Supershift gel shift assays and cotransfection studies indicate that activation of the NF-κB site in the promoters of ICAM-1 and IL-8 depend on p65 homodimers or crel/p65 heterodimers instead of the classic p50/p65 heterodimer. Despite this difference in the 5′ region of the endogenous ICAM-1 NF-κB site, our NF-κB TFD with the κB consensus sequence still completely inhibited the upregulation of ICAM-1.

The NF-κB TFD had relatively specific inhibitory effects on ICAM-1 upregulation. The NF-κB TFD but not the mutant or scrambled decoy competed for binding of activated NF-κB in a gel shift from nuclear protein isolated from HBMEC. With TNF-α stimulation, the mutated decoy had a partial effect in reducing ICAM mRNA levels, whereas the scrambled decoy had none. In our studies of H/R, although the mutated and scrambled decoys slightly reduced mRNA levels, the NF-κB decoy dramatically reduced ICAM-1 mRNA levels. Similarly, the surface expression of ICAM-1 in response to TNF-α was only minimally affected by the mutant and scrambled decoys, whereas the κB decoy reduced ICAM-1 to unstimulated levels. With H/R, the NF-κB decoy...
inhibit NF-κB–dependent gene expression. Figure 5. NF-κB TFDs block accumulation of ICAM-1 mRNA in cells exposed to H/R. ICAM-1 mRNA levels in IT-1 cells were estimated by Northern blot analysis. ICAM-1 mRNA levels were elevated in cells treated with TNF-α (lane 2) and H/R (lane 3) but barely detectable in untreated (UN) control cells (lane 1). Increase in ICAM-1 mRNA induced by H/R was reduced in cells that were mannitol-loaded with the NF-κB TFD (lane 4). Loading with mutated TFD (lane 5) or scrambled TFD (lane 6) resulted in slight reduction again reduced the upregulated surface expression of ICAM-1, whereas the mutated decoy had a partial effect. Our mutated decoy only differs by 3 bp in the consensus sequence, and this may explain why the mutated decoy may have some effect on mRNA levels and on ICAM-1 surface expression. Goldring et al.17 reported that this same mutated decoy also reduced NO production and iNOS levels in macrophages but a reduction less than with the κB decoy. Importantly, our scrambled decoy had no significant effect on either TNF-α–induced or H/R-induced surface ICAM expression.

TFDs have been used successfully in other cell types to inhibit NF-κB–dependent gene expression.17,21–24 Xu and colleagues24 used a hairpin loop oligonucleotide with an NF-κB binding site to block the upregulated expression of iNOS in murine cerebral endothelial cells. Although in many of these studies the TFD was delivered into the cells “naked,” without a carrier or vector, the study by Morishita et al23 required the use of hemagglutinating virus of Japan liposomes to transfect cells.

Figure 5. NF-κB TFDs block accumulation of ICAM-1 mRNA in cells exposed to H/R. ICAM-1 mRNA levels in IT-1 cells were estimated by Northern blot analysis. ICAM-1 mRNA levels were elevated in cells treated with TNF-α (lane 2) and H/R (lane 3) but barely detectable in untreated (UN) control cells (lane 1). Increase in ICAM-1 mRNA induced by H/R was reduced in cells that were mannitol-loaded with the NF-κB TFD (lane 4). Loading with mutated TFD (lane 5) or scrambled TFD (lane 6) resulted in slight reduction.

Human endothelial cells, particularly brain endothelial cells, are difficult to transfect with DNA. Our cells could not be transfected unless the cells were exposed to mannitol or PEG-sucrose (data not shown). Mannitol effectively and rapidly allowed transfection of the TFD into brain endothelial cells. There are reports of mannitol causing endothelial cell apoptosis.25 In that study, however, apoptosis was detected after exposure of the cells to mannitol for 6 hours. Our exposure times were much lower (10 minutes). We did not observe any cell death in our cultures under our experimental conditions.

Besides being useful as tools in the study of gene regulation in vitro and in vivo, TFDs have potential therapeutic uses in human stroke. TFDs have been used successfully in a myocardial ischemia-reperfusion model. Transfection of FITC-labeled oligonucleotides by infusion in the rat left main coronary artery resulted in widely distributed fluorescence in the coronary microvascular endothelial cells.23 The use of hemagglutinating virus of Japan liposomes was required to deliver the decoys into endothelial cells in vitro and in vivo. Transfection of NF-κB–specific TFDs before coronary occlusion as well as immediately after reperfusion significantly reduced myocardial infarct size compared with scrambled decoy. These results underscore the feasibility and potential efficacy of TFDs in reperfusion injury.

Intra-arterial mannitol has been used clinically to disrupt the blood-brain barrier and enhance delivery of chemotherapeutic agents into the brain.26 After administration of intra-arterial mannitol, virus-sized iron oxide particles can be delivered across the blood-brain barrier and into neurons.27 Mannitol also promotes the delivery of replication-deficient...
adenovirus containing the *Escherichia coli* β-galactosidase gene into perivascular astrocytes. The mechanism of action of mannitol in breaching the blood-brain barrier has been theorized to involve shrinkage of cerebral endothelial cells and opening of the tight junctions. However, careful pathological studies have not demonstrated any morphological change in the tight junctions after intra-arterial mannitol. Mannitol affects signal transduction pathways in endothelial cells, suggesting that increased pinocytosis may be at least partly responsible for the effect on the blood-brain barrier. Our in vitro studies suggest that mannitol may be a useful agent to deliver oligonucleotides into cerebral endothelial cells in vivo. With the use of mannitol, a NF-κB TFD could be administered directly into the distal internal carotid or middle cerebral artery after intra-arterial administration of prourokinase and delivered directly into the ischemic zone.

There is considerable evidence that NF-κB plays a role in apoptosis in neurons; some evidence suggests NF-κB activation is associated with cell survival and other evidence suggests a death-promoting role. Schneider et al demonstrated that mice with a knockout of the p50 subunit of NF-κB had smaller infarcts than wild-type mice. However, other investigators have shown that p50 knockout mice are more susceptible to excitotoxic injury. Therefore, further studies and attempts to block NF-κB in cerebral ischemia will need to distinguish between effects on endothelial cells, neurons, and astrocytes. A strategy of selectively inhibiting NF-κB in endothelial cells and not neurons may be advantageous.

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**References**


The cerebral endothelial cells (ECs) are a primary target of hypoxic or ischemic brain insults. Postischemic activation or injury of ECs may compromise cerebral blood flow and cause the breakdown of blood-brain barrier (BBB), leading to increased vascular permeability and vasogenic brain edema. EC injury may also enhance leukocyte adhesion and promote postischemic inflammatory reactions. Postischemic inflammatory events involve activation of proinflammatory transcription factors such as NF-κB, which in turn transactivate proinflammatory genes, including cytokines, adhesion molecules, iNOS, COX-2, and others. A number of therapeutic interventions have been explored to suppress postischemic inflammatory reaction in attempts to reduce secondary injury following cerebral ischemia/reperfusion. In the preceding article, Hess and colleagues explored a novel approach to modulate NF-κB activation in order to regulate the expression of downstream genes in ECs that may contribute to inflammatory reaction. The target gene selected for this study is an adhesion molecule, ICAM-1. Endothelial expression of ICAM-1 leads to increased leukocyte adhesion and its infiltration across blood-brain barrier. The authors designed an oligonucleotide decoy that carries consensus DNA binding sequence for NF-κB. Excessive amounts of this decoy competed for NF-κB binding to prevent its translocation and binding to the cognate sequence in the nucleus. Reduction in the transcription activity of NF-κB by this decoy diminished its transactivation of ICAM-1. Similar strategy has been used to block NF-κB transactivation of iNOS in cerebral ECs.

Another innovative approach in the present study by Hess and associates was the application of mannitol to facilitate the delivery of the oligonucleotide decoy into ECs. High concentrations of mannitol may be cytotoxic, which leads to EC apoptosis. However, brief exposure to this osmotic agent did not cause EC death in the current study. This innovative in vitro study offers insight into a potential tool to modulate transcription regulation of proinflammatory genes that may be of clinical utility in future therapies directed at suppression of postischemic inflammatory reaction.

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