Neurobiology of Disease

Systemic Lipopolysaccharide Protects the Brain from Ischemic Injury by Reprogramming the Response of the Brain to Stroke: A Critical Role for IRF3

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Lipopolysaccharide (LPS) preconditioning provides neuroprotection against subsequent cerebral ischemic injury through activation of its receptor, Toll-like receptor 4 (TLR4). Paradoxically, TLR activation by endogenous ligands after ischemia worsens stroke damage. Here, we define a novel, protective role for TLRs after ischemia in the context of LPS preconditioning. Microarray analysis of brains collected 24 h after stroke revealed a unique set of upregulated genes in LPS-pretreated animals. Promoter analysis of the unique gene set identified an overrepresentation of type I interferon (IFN)-associated transcriptional regulatory elements. This finding suggested the presence of type I IFNs or interferon regulatory factors (IRFs), which upregulate interferon-stimulated genes. Upregulation of IFNβ was confirmed by real-time reverse transcription-PCR. Direct administration of IFNβ intracerebroventricularly at the time of stroke was sufficient for neuroprotection. TLR4 can induce both IFNβ and interferon-stimulated genes through its adapter molecule Toll/interleukin receptor domain-containing adaptor-inducing IFNβ (TRIF) and the IRF3 transcription factor. We show in oxygen glucose deprivation of cortical neurons, an *in vitro* model of stroke, that activation of TRIF after stroke reduces neuronal death. Furthermore, mice lacking IRF3 were not protected by LPS preconditioning in our *in vivo* model. Our studies constitute the first demonstration of the neuroprotective capacity of TRIF/IRF3 signaling and suggest that interferon-stimulated genes, whether induced by IFNβ or by enhanced TLR signaling to IRF3, are a potent means of protecting the brain against ischemic damage.

Introduction

It is increasingly clear that Toll-like receptor (TLR) signaling worsens stroke injury. Mice lacking TLR2 or TLR4 are less susceptible to damage in multiple models of cerebral ischemia (Cao et al., 2007; Lehnardt et al., 2007; Ziegler et al., 2007). TLRs are expressed by microglia, astrocytes, and endothelial cells and are activated by the damage-associated molecules HSP70 (TLR4) and HMGB1 (TLR2 and TLR4), present in the brain after ischemia (Kinouchi et al., 1993a,b; Faraco et al., 2007). TLR activation induces production of the inflammatory molecules tumor necrosis factor α (TNFα), IL1β, and inducible nitric oxide synthase and other cytotoxic mediators that increase tissue damage.

Although TLR4 activation after stroke exacerbates injury, activation of TLR4 before stroke protects the brain from damage. Systemic administration of lipopolysaccharide (LPS), a potent TLR4 ligand of bacterial origin, renders animals tolerant to injury in several models of cerebral ischemia (Tasaki et al., 1997; Rosenzweig et al., 2004; Hickey et al., 2007). LPS-induced tolerance to ischemic injury mirrors the phenomenon of LPS-induced tolerance to LPS. Initial exposure of macrophages to LPS induces proinflammatory TNFα, but, during subsequent exposure to LPS, TNFα production is reduced markedly as a result of disrupted signaling through the TLR4 adaptor molecule MyD88 (West and Heagy, 2002; Fan and Cook, 2004; Liew et al., 2005). Conversely, macrophages produce little interferon β (IFNβ) during initial exposure to LPS but enhance IFNβ production during secondary exposure (Broad et al., 2007), suggesting upregulated TLR4 signaling through the Toll/interleukin receptor domain-containing adaptor-inducing IFNβ (TRIF) adaptor molecule. Thus, pretreatment with LPS may cause cells to switch their dominant TLR4 signaling pathway.

TLR4 signaling through TRIF induces IFNβ via activation of the interferon regulatory factor IRF3. IFNβ, administered systemically, reduces ischemic brain damage (Liu et al., 2002; Veldhuis et al., 2003), likely through activation of interferon-stimulated genes (ISGs). IRF3 itself may have similar neuroprotective effects. IRF3 binds to interferon-stimulated response elements (ISREs) within gene promoters, increasing the expression of many ISGs to the same extent of that elicited by type I IFNs (Nakaya et al., 2001). Hence, activation of IRF3 may independently result in protection from ischemic stroke. Thus, enhanced

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TLR4 signaling to TRIF–IRF3–IFNβ would be expected to contribute to neuroprotection.

We propose that pretreatment or preconditioning with LPS changes the cellular environment such that subsequent activation of TLR4 increases signaling via TRIF to IRF3 and upregulates the neuroprotective cytokine IFNβ. Thus, in this way, LPS preconditioning may reprogram subsequent activation of TLR4 during ischemia, which leads to an increase in neuroprotective type I IFN signaling. Here we provide evidence for such reprogramming and its neuroprotective consequences.

Materials and Methods

Mice. C57BL/6 mice (male, 8–12 weeks, ~25 g) were purchased from The Jackson Laboratory. IFNβ knock-out mice were kindly provided by Dr. Leanderson (Lund University, Lund, Sweden). IRF3 knock-out mice were procured from RIKEN BioResource Center (Tsukuba, Japan). Both strains were backcrossed onto the C57BL/6 background for at least eight generations. All mice were housed in an American Association for Laboratory Animal Care-approved facility. Procedures were conducted according to Oregon Health and Science University, Institutional Animal Care and Use Committee, and National Institutes of Health guidelines.

LPS treatment. Mice were given a 200 μl intraperitoneal injection of saline or LPS [0.2–1.0 mg/kg; *Escherichia coli* serotype *0111:B4*; catalog #L2630, purified by phenol extraction, protein content <3% (Sigma)]. Each new lot of LPS was titrated to determine the optimal dose that confers neuroprotection in the particular strain of mouse being tested.

Middle cerebral artery occlusion. Mice were anesthetized with 4% halothane and subjected to middle cerebral artery occlusion (MCAO) using the monofilament suture method described previously (Stevens et al., 2002). Briefly, a silicone-coated 8-0 monofilament nylon surgical suture was threaded through the external carotid artery to the internal carotid artery to block the middle cerebral artery and maintained intraluminally for 40, 45, or 60 min. Duration of occlusion was based on pilot studies performed to determine the time necessary to obtain an infarct size that is between 35 and 45% in the control groups of mice. It is well known that genetic background can influence ischemic outcome and thereby affect infarct size. The suture was then removed to restore blood flow. Cerebral blood flow (CBF) was monitored throughout surgery by laser Doppler flowmetry. The mean CBF during occlusion was between 10 and 17% of baseline in each of the studies presented. Mice that did not maintain a CBF drop within the norm of the group during the occlusion were excluded (<4% of all animals in the combined studies). Body temperature was maintained at 37°C with a thermostat-controlled heating pad. The survival rate for the MCAO procedure was >80%.

Infarct evaluation. To visualize the region of infarction, 6 × 1 mm coronal midsections were placed in 1.5% 2,3,5 triphenyltetrazolium chloride (TTC) in 0.9% PBS and stained at 37°C for 15 min. The infarct size was determined from computer-scanned images of the hemispheres using NIH Image analyses. To account for edema within the infarct region, infarct area for each section was computed indirectly as follows: 100 × (contralateral hemisphere area − area of live tissue on ipsilateral hemisphere)/(contralateral hemisphere area) (Swanson et al., 1990).

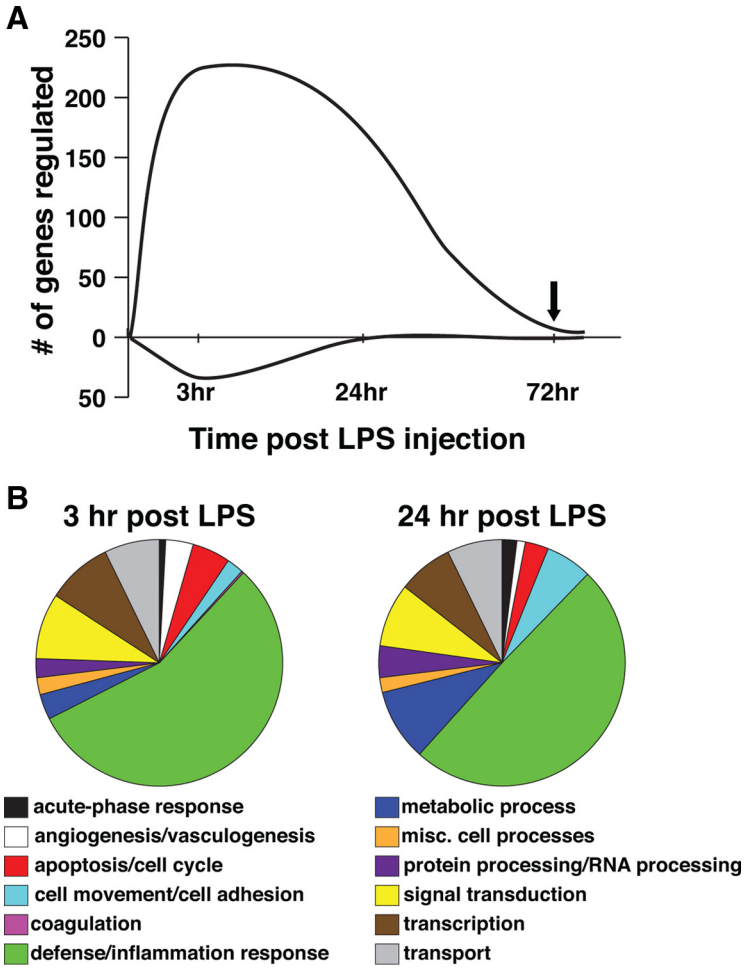

Figure 1. Systemic administration of LPS induces early gene regulation in the brain, consistent with an inflammatory response. C57BL/6 mice were administered saline or LPS (0.2 mg/kg, i.p.). At varying times (3, 24, and 72 h) after treatment, mice ($n = 4$ per time point) were killed, and cortical brain tissue was collected. RNA was isolated and hybridized to Affymetrix gene chips (MOE430). ***A***, Graph represents the number of genes differentially regulated in LPS- or saline-treated mice compared with unhandled controls. Time of subsequent stroke is denoted with a black arrow. ***B***, Putative biological functions were assigned to the regulated genes using available public databases and published literature.

Experimental design for gene expression studies. C57BL/6 mice were divided into 10 groups with four animals per group: groups 1–3 received a saline injection and were killed at 3, 24, and 72 h, respectively. Groups 4–6 received an LPS injection and were killed at 3, 24, and 72 h, respectively. Groups 7 and 8 received a saline injection, followed 72 h later with a 45 min MCAO. Group 9 and 10 received an LPS injection, followed 72 h later with a 45 min MCAO. Groups 7 and 9 were killed at 3 h after start of occlusion with groups 8 and 10 killed 24 h after start of occlusion. At the time of the mice were killed, the animals were anesthetized and then perfused with heparinized saline. One group ($n = 6$) was included as unhandled controls. Under RNase-free conditions, a 1 mm section was removed (4 mm from rostral end) to determine the area of infarct based on TTC staining. The ipsilateral cortex region from the frontal 4 mm was isolated and snap frozen in liquid nitrogen.

RNA isolation. Total RNA was isolated using the Qiagen RNeasy Lipid Mini kit. RNA from individual animals was hybridized to single arrays as described below.

GeneChip expression analyses. Microarray assays were performed in the Affymetrix Microarray Core of the Oregon Health and Science University Gene Microarray Shared Resource. RNA samples were labeled using the NuGEN Ovation Biotin RNA Amplification and Labeling System_V1. Hybridization was performed as described in the Affymetrix technical

manual with modifications as recommended for the Ovation labeling protocol. Labeled cRNA target was quality checked based on yield and size distribution. Quality-tested samples were hybridized to the MOE430 2.0 array. The array image was processed with Affymetrix GeneChip Operating Software. Arrays which did not meet empirically defined cutoffs within the core facility were remade and hybridized to fresh arrays. Data were normalized using the robust multichip average method (Irizarry et al., 2003). The normalized data were then analyzed using a two-way ANOVA model for each gene, using conditions and time as groups. *Post hoc* comparisons were made using the unhandled mice as a control group. *p* values were adjusted for multiple comparisons using the Hochberg and Benjamini method (Hochberg and Benjamini, 1990). Genes were considered significantly regulated if the adjusted *p* value was <0.05, and the fold change in regulation was greater than or equal to 2.

Transcriptional regulatory network analysis. Using the Web-based program Promoter Analysis and Interaction Network Toolset (PAINT) version 3.5 (Vadigepalli et al., 2003), we examined the predicted regulatory elements associated with the unique gene regulation identified by microarray. In brief, using PAINT, we obtained the 5000 bp upstream sequence for the transcripts represented on the MOE430 Affymetrix gene chip (33,635 transcripts were identified with 5000 bp of upstream sequence). PAINT identified putative transcription factor binding sequences [transcriptional regulatory elements (TREs)] in these upstream sequences using the TRANSFAC PRO database version 10.4. This pool of genes and identified TREs was used as our reference comparison group. The statistical component of PAINT (false discovery rate adjusted *p* value set at ≤ 0.2) was used to determine the overrepresented TREs in individual gene clusters compared with the reference comparison group (i.e., uniquely expressed genes in LPS-preconditioned mice compared with 33,635 member reference group).

Intracerebral ventricular injection of IFNβ during MCAO. Recombinant mouse (rm) IFNβ (Cell Sciences) or vehicle [artificial CSF (aCSF)] was injected into the left lateral ventricle as described previously (Meller et al., 2005). Injections (1 μl) of either rmIFNβ (200 U) or aCSF were administered immediately before and after surgery (60 min MCAO). Infarct volume was measured 24 h after stroke.

Quantitative real-time PCR for IFNβ. RNA was treated with DNase and transcribed into cDNA using the Omniscript RT kit (Qiagen). Real-time reverse transcription-PCRs were performed using TaqMan PCR Master Mix (Applied Biosystems). For IFNβ, TaqMan Gene Expression Assay Mix for mouse IFNβ was used (Mm00439546_S1; Applied Biosystems). Primers and probe for β-actin were obtained from Integrated DNA Technologies: forward, 5′-AGAGGGAAATCGTGCGTGAC-3′; reverse, 5′-CAATAGTGATGACCTGGCCGT-3′; probe, CACTGCCGCATCCTCTTCCTCCC. Samples were run on an ABI-Prism 7700 (Applied Biosystems). Results were analyzed using Applied Biosystems sequence detection software. The relative quantitation of IFNβ was determined using the comparative cycle threshold (CT) method ($2^{-\Delta\Delta CT}$) described in Applied Biosystems User Bulletin #2. Results were normalized to β-actin and presented relative to unhandled mice. All reactions were performed in triplicate.

Oxygen glucose deprivation in vitro. Primary mouse mixed cortical cultures were prepared from embryonic day 15 to 17 mouse fetuses. Cortices were dissected and dissociated with trypsin-EDTA (Invitrogen) and plated at a density of 4.5×10^5 cells/ml onto coverslips coated with poly-L-ornithine (15 mg/L). Cells were cultured in Neurobasal media (containing 4.5 g/L glucose; supplemented with Glutamax and B27-AO; Invitrogen) for 5 d before each experiment. Cultures consisted of ~60% neurons (range, 53–66%) as determined by staining for neuronal-specific nuclear protein (Millipore Bioscience Research Reagents), with <5% astrocytes ($GFAP^+$; Sigma) and <5% microglia (tomato $lectin^+$; Vector Laboratories). Oxygen glucose deprivation (OGD) was performed by removal of the culture medium and replacement with Dulbecco's PBS (Invitrogen), followed by incubation in an anaerobic atmosphere of 85% N_2, 10% CO_2, and 5% H_2 at 37°C for 3 h. The anaerobic conditions within the chamber were monitored using an electronic oxygen/hydrogen analyzer (Coy Laboratories). OGD was terminated by replacement of the exposure medium with Neurobasal medium (containing 4.5 g/L glucose; supplemented with Glutamax and B27-AO) and return of the cells to a normoxic incubator. Control plates were kept in the normoxic incubator during the OGD interval.

Table 1. Genes differentially regulated 72 h after LPS preconditioning (time of stroke)

Title	Symbol	Fold change
Serum amyloid A3	Saa3	6.48
Topoisomerase (DNA) II α	Top2a	2.14
UDP glucuronosyltransferase 2 family, polypeptide B37	Ugt2b37	3.53
RIKEN cDNA 4930440C22 gene		2.24
RIKEN cDNA 4930554P06 gene		2.35

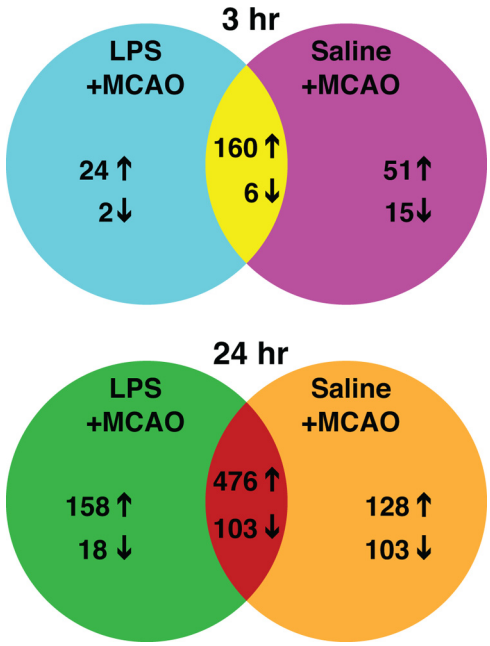

Figure 2. LPS preconditioning induces a unique set of genes in response to MCAO. C57BL/6 mice were preconditioned with LPS (0.2 mg/kg) or saline 72 h before MCAO (45 min). At 3 or 24 h after MCAO, mice ($n = 4$ per time point) were killed, and the ipsilateral cortical brain tissue was collected. RNA was isolated and hybridized to Affymetrix gene chips (MOE430). Venn diagram showing the number of genes differentially regulated in each condition compared with unhandled controls. Arrows indicate the direction of regulation.

Cell death evaluation in vitro. Cell death *in vitro* was examined 24 h after OGD by means of fluorescent, cell-permeable, DNA-binding dyes: propidium iodide (PI), as an indicator of cell death, and 4′,6-diamidino-2-phenylindole (DAPI), as an indicator of the total number of cells. Coverslips were incubated with PI (1.5 μg/ml; Sigma) for 5 min, washed with PBS, and fixed for 30 min in 10% Formalin. Coverslips were mounted on slides with Fluoromount-G mounting medium containing DAPI (Southern Biotechnology Associates). Stained cells were visualized with a fluorescent microscope (Leica) and analyzed using Metmorph7 software (Molecular Devices). The number of PI- and DAPI-stained cells were counted in two random fields of view on each coverslip, and percentage death was calculated as mean (PI)/(DAPI) × 100 per field of view. Each treatment was performed with triplicate coverslips within an experiment, and the entire experiment was repeated three or more times.

Results

Systemic administration of LPS induces an inflammatory response in the brain

As we have shown previously, systemic administration of LPS (0.2 mg/kg) given 3 d before MCAO substantially attenuates ischemic damage (Rosenzweig et al., 2004, 2007). To begin to elucidate possible mechanisms of neuroprotection, we isolated RNA from the cortex of LPS-treated and control mice at time points

Table 2. Genes regulated 24 h after stroke only in LPS-preconditioned mice

	Symbol	LPS at 3 h[a,b]	LPS at 24 h	LPS at 72 h	LPS + MCAO at 3 h	LPS + MCAO at 24 h
Apoptosis/cell cycle						
CD274 antigen	Cd274	11.60	NS	NS	NS	2.48
GLI pathogenesis-related 1 (glioma)	Glipr1	NS	NS	NS	NS	2.34
Caspase 8	Casp8	NS	NS	NS	NS	2.01
Cell movement/cell adhesion						
Filamin binding LIM protein 1	Fblim1	NS	NS	NS	NS	2.44
Selectin, endothelial cell	Sele	1.98	NS	NS	1.66	2.37
Neurogenic differentiation 4	Neurod4	NS	1.60	NS	NS	2.33
Kelch-like 6 (Drosophila)	Klhl6	NS	NS	NS	NS	2.18
Glycoprotein (transmembrane) nmb	Gpnmb	NS	NS	NS	NS	2.11
Claudin 1	Cldn1	NS	NS	NS	NS	2.03
M-phase phosphoprotein 1	Mphosph1	NS	NS	NS	NS	2.02
PDZ and LIM domain 5	Pdlim5	NS	NS	NS	1.73	2.01
Protocadherin 20	Pcdh20	NS	NS	NS	NS	−2.20
Protocadherin 21	Pcdh21	NS	NS	NS	NS	−2.37
Coagulation						
Coagulation factor V	F5	NS	NS	NS	NS	3.12
Coagulation factor XIII, A1 subunit	F13a1	NS	NS	NS	NS	2.60
Hepatocyte growth factor	Hgf	NS	NS	NS	NS	2.60
Protein S (α)	Pros1	NS	NS	NS	NS	2.41
Defense response						
Radical *S*-adenosyl methionine domain containing 2	Rsad2, VIPERIN	33.06	3.01	NS	NS	3.84
Killer cell lectin-like receptor subfamily B member 1F	Klrb1f	NS	4.77	NS	NS	3.77
Interferon inducible GTPase 1	Iigp1	23.33	NS	NS	NS	3.56
CD52 antigen	Cd52	NS	2.37	NS	NS	3.33
Fc receptor, IgG, high affinity I	Fcgr1	NS	2.77	NS	NS	3.09
Interferon-induced protein with tetratricopeptide repeats 1	Ifit1	19.84	4.03	NS	NS	3.06
Guanylate nucleotide binding protein 3	Gbp3	7.09	2.35	NS	NS	2.96
SLAM family member 9	Slamf9	NS	1.53	NS	NS	2.92
Protein tyrosine phosphatase, receptor type, C	Ptprc, B220	NS	1.94	NS	1.58	2.65
Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)	Tap1	3.42	1.78	NS	NS	2.65
Histocompatibility 2, Q region locus 1	H2-Q1	7.45	2.84	NS	2.48	2.60
PYD and CARD domain containing	Pycard	NS	2.11	NS	NS	2.58
Lymphocyte cytosolic protein 2	Lcp2	1.79	NS	NS	NS	2.51
Fc receptor, IgE, high affinity I, γ polypeptide	Fcer1g	NS	1.87	NS	NS	2.49
2′-5′ oligoadenylate synthetase-like 2	Oasl2	5.05	3.87	NS	NS	2.48
Phospholipid scramblase 2	Plscr2	3.46	NS	NS	NS	2.47
Interferon γ-induced GTPase	Igtp	4.35	1.78	NS	NS	2.46
Neutrophilic granule protein	Ngp	NS	NS	NS	NS	2.44
Interferon-induced transmembrane protein 6	Ifitm6	NS	NS	NS	NS	2.44
Complement component 1, q subcomponent, β polypeptide	C1qb	NS	1.54	NS	NS	2.42
Toll-like receptor 4	Tlr4	NS	NS	NS	NS	2.42
Myxovirus (influenza virus) resistance 1	Mx1	11.10	2.43	NS	NS	2.34
Lymphocyte cytosolic protein 1	Lcp1	NS	1.60	NS	NS	2.29
DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	Ddx58, RIG1	3.59	1.79	NS	NS	2.23
Interleukin 1 β	Il1b	4.69	NS	NS	2.08	2.20
Leukocyte Ig-like receptor, subfamily B member 3	Lilrb3	NS	1.54	NS	NS	2.13
Histocompatibility 2, D region	H2-L	1.55	2.03	1.63	1.61	2.13
Stabilin 1	Stab1	NS	NS	NS	NS	2.12
Histocompatibility 2, K1, K region	H2-K1	2.01	2.38	1.74	1.86	2.11
Protein tyrosine phosphatase, non-receptor type 6	Ptpn6,Shp1	NS	NS	NS	NS	2.11
E74-like factor 1	Elf1	NS	1.98	NS	1.63	2.09
Interferon-induced protein 35	Ifi35	3.27	2.06	NS	NS	2.08
SAM domain, SH3 domain and nuclear localization signals, 1	Samsn1	1.91	NS	NS	1.54	2.08
Histocompatibility 2, D region locus 1	H2-D1	NS	2.05	1.62	1.60	2.08
H-2 class I histocompatibility antigen, Q7 α chain precursor	QA-2	5.26	3.89	NS	3.08	2.08
C-type lectin domain family 14, member a	Clec14a	−2.36	NS	NS	NS	2.07
C-type lectin domain family 5, member a	Clec5a	NS	NS	NS	NS	2.06
Interferon-induced protein with tetratricopeptide repeats 3	Ifit3	5.41	2.42	NS	NS	2.06
Proteosome subunit, β type 8	Psmb8	2.56	2.03	NS	NS	2.05
Neutrophil cytosolic factor 1	Ncf1	1.51	NS	NS	NS	2.03
Lymphocyte antigen 6 complex, locus A	Ly6a	1.79	1.88	NS	NS	2.01
Signal peptide, CUB domain, EGF-like 1	Scube1	NS	NS	NS	NS	−2.03
Corticotropin releasing hormone	Crh	NS	NS	NS	NS	−2.10

(*Table continues*.)

Table 2. Continued

	Symbol	LPS at 3 h[a,b]	LPS at 24 h	LPS at 72 h	LPS + MCAO at 3 h	LPS + MCAO at 24 h
Metabolic processes						
Klotho	Kl	NS	NS	NS	NS	2.48
Hexokinase 2	Hk2	NS	NS	NS	NS	2.26
Ethanolamine kinase 1	Etnk1	NS	NS	NS	2.89	2.24
Carbonic anhydrase 13	Car13	NS	NS	NS	NS	2.16
Centromere protein A	Cenpa	NS	NS	NS	NS	2.12
Phosphodiesterase 3A, cGMP inhibited	Pde3a	NS	2.19	NS	NS	2.08
Folate receptor 1 (adult)	Folr1	NS	NS	NS	NS	2.04
AMP deaminase 3	Ampd3	NS	NS	NS	NS	2.02
Miscellaneous cell processes						
Schlafen 2	Slfn2	4.00	2.18	NS	NS	3.35
Calmodulin-like 4	Calml4	NS	NS	NS	NS	2.50
Ecotropic viral integration site 2b	Evi2b	NS	NS	NS	NS	2.49
Estrogen receptor 1 (α)	Esr1	NS	NS	NS	2.35	2.09
Luc7 homolog (*Saccharomyces / cerevisiae*)-like	Luc7l	NS	NS	NS	NS	2.04
Protein/RNA processing						
Ubiquitin specific peptidase 18	Usp18	12.83	3.83	NS	NS	2.89
RIKEN cDNA 5430435G22 gene		NS	NS	NS	NS	2.75
Ribosomal protein L7	Rpl7	NS	2.11	NS	NS	2.75
Heat shock protein 8	Hspb8	NS	NS	NS	1.70	2.42
Serine (or cysteine) peptidase inhibitor, clade H, member 1	Serpinh1	NS	NS	NS	1.70	2.33
ST6 (α -*N*-acetyl-neuraminyl-2,3-β -galactosyl-1,3)-*N*-acetyl-galactosaminide α -2,6-sialyltransferase 2	St6galnac2	NS	NS	NS	NS	2.31
UDP-GlcNAc: β Gal β -1,3-N-acetylglucosaminyltransferase 5	B3gnt5	NS	NS	NS	NS	2.27
Phospholipase A2, group IVA	Pla2g4a	NS	NS	NS	NS	2.27
Z-DNA binding protein 1	Zbp1	3.50	3.40	NS	NS	2.27
A disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 5	Adamts5	NS	NS	NS	NS	2.13
Translocating chain-associating membrane protein 2	Tram2	NS	NS	NS	NS	2.08
IMP4, U3 small nucleolar ribonucleoprotein	Imp4	NS	NS	NS	NS	2.08
Ribosomal protein S25	Rps25	NS	NS	NS	NS	2.08
Ubiquitin-like, containing PHD and RING finger domains, 1	Uhrf1	NS	NS	NS	NS	2.06
cDNA sequence BC099439	BC099439	NS	NS	NS	NS	2.05
DnaJ (Hsp40) homolog, subfamily B, member 5	Dnajb5	NS	NS	NS	NS	−2.04
Signal transduction						
Receptor transporter protein 4	Rtp4	3.83	3.46	NS	NS	2.92
Component of Sp100-rs	Csprs	NS	2.95	NS	NS	2.36
Rho GTPase activating protein 30	Arhgap30	NS	NS	NS	NS	2.33
Adenylate cyclase 7	Adcy7	NS	NS	NS	NS	2.28
Guanine nucleotide binding protein, α 14	Gna14	NS	NS	NS	NS	2.28
Vomeronasal 1 receptor, A1	V1ra1	NS	2.52	NS	NS	2.22
Fibrinogen-like protein 2	Fgl2	NS	NS	NS	NS	2.14
Ras homolog gene family, member C	Rhoc	1.66	NS	NS	1.55	2.12
Pleckstrin homology, Sec7 and coiled/coil domains 4	Pscd4	NS	1.52	NS	NS	2.07
Pleckstrin homology domain containing, family G member 2	Plekhg2	NS	NS	NS	NS	2.05
Transcription						
Reduced expression 2	Rex2	NS	NS	NS	NS	2.28
MyoD family inhibitor domain containing	Mdfic	NS	NS	NS	NS	2.24
Leucine rich repeat (in FLII) interacting protein 1	Lrrfip1	NS	NS	NS	2.05	2.16
Bromodomain adjacent to zinc finger domain 1A	Baz1a	NS	NS	NS	2.28	2.05
Ladybird homeobox 1 homolog (*Drosophila*) corepressor 1	Lbxcor1	NS	NS	NS	1.87	2.04
Annexin A11	Anxa11	NS	NS	NS	NS	2.02
Tripartite motif protein 30	Trim30	8.15	2.13	NS	NS	2.02
Inversin	Invs	NS	2.11	NS	NS	2.02
Transport						
Transthyretin	Ttr	NS	NS	NS	NS	12.74
Translocator protein	Tspo	NS	2.16	NS	NS	2.76
Stanniocalcin 2	Stc2	NS	NS	NS	1.97	2.64
Transient receptor potential cation channel, subfamily M, member 3	Trpm3	NS	NS	NS	NS	2.63
Potassium voltage-gated channel, Isk-related subfamily, gene 2	Kcne2	NS	NS	NS	NS	2.41
Transferrin	Trf	NS	NS	NS	NS	2.37
Chloride channel calcium activated 1///chloride channel calcium activated 2	Clca1///Clca2	NS	NS	NS	NS	2.26
Mannose receptor, C type 1	Mrc1	NS	NS	NS	NS	2.25
Cysteine-rich hydrophobic domain 2	Chic2	NS	NS	NS	NS	2.10
Exocyst complex component 6	Exoc6	NS	NS	NS	NS	2.10
SEH1-like (*S. cerevisiae*)	Seh1l	NS	NS	NS	NS	2.08

(*Table continues*.)

Table 2. Continued

	Symbol	LPS at 3 h[a,b]	LPS at 24 h	LPS at 72 h	LPS + MCAO at 3 h	LPS + MCAO at 24 h
Aquaporin 1	Aqp1	NS	NS	NS	NS	2.06
Poly (ADP-ribose) polymerase family, member 9	Parp9	3.34	1.60	NS	NS	2.04
RAB20, member RAS oncogene family	Rab20	1.98	NS	NS	1.95	2.04
Triadin	Trdn	NS	NS	NS	NS	−2.04
Solute carrier family 2 (facilitated glucose transporter), member 5	Slc2a5	NS	NS	NS	NS	−2.13
Function unknown						
RIKEN cDNA 1700040G22 gene		NS	3.10	NS	NS	2.86
RIKEN cDNA 1500015O10 gene		NS	NS	NS	NS	2.70
RIKEN cDNA 9330175E14 gene		2.32	2.11	NS	1.62	2.62
BM241008		NS	2.28	NS	NS	2.61
RIKEN cDNA 1810053B01 gene		NS	NS	NS	NS	2.57
RIKEN cDNA 1810032O08 gene		NS	NS	NS	NS	2.50
Hypothetical LOC433632		NS	NS	NS	NS	2.44
RIKEN cDNA 3830408D24 gene		NS	NS	NS	NS	2.38
Expressed sequence AI451617		3.29	2.95	NS	NS	2.34
Expressed sequence AU020206		NS	1.79	NS	NS	2.33
Expressed sequence AU045094		NS	2.54	NS	NS	2.32
RIKEN cDNA 2410131K14 gene		NS	NS	NS	NS	2.29
Stefin A2 like 1	Stfa2l1	NS	NS	NS	NS	2.29
RIKEN cDNA 2810007J24 gene		NS	NS	NS	NS	2.27
BG072508		2.54	3.00	NS	NS	2.27
Transcribed locus AW215795		NS	1.68	NS	NS	2.26
RIKEN cDNA 4632434I11 gene		NS	NS	NS	NS	2.23
RIKEN cDNA 2610305J24 gene		NS	NS	NS	NS	2.22
RIKEN cDNA 5430416N02 gene		NS	NS	NS	NS	2.21
Predicted gene, EG240327		4.26	2.20	NS	NS	2.21
RIKEN cDNA 1110018M03 gene		NS	NS	NS	NS	2.20
Similar to phospholipid scramblase 1	LOC331000	NS	2.10	NS	NS	2.19
cDNA sequence BC039210		NS	NS	NS	NS	2.18
RIKEN cDNA 4632432E15 gene		NS	NS	NS	NS	2.16
RIKEN cDNA 1700010N08 gene		NS	2.27	NS	NS	2.15
Expressed sequence AI447904		1.58	1.89	NS	NS	2.13
BB548602		NS	NS	NS	NS	2.13
RIKEN cDNA 2410006H16 gene		NS	NS	NS	NS	2.12
Testis expressed gene 15	Tex15	NS	NS	NS	NS	2.12
AV227312		NS	1.66	NS	NS	2.10
BG068057		NS	2.43	NS	NS	2.10
Expressed sequence AU022436		NS	NS	NS	NS	2.09
Expressed sequence AU022479		NS	NS	NS	2.86	2.08
AV354139		NS	NS	NS	NS	2.08
BG068123		NS	NS	NS	NS	2.04
C85463		NS	2.40	NS	NS	2.03
RIKEN cDNA 2810026P18 gene		NS	NS	NS	NS	2.02
RIKEN cDNA 4632409D06 gene		NS	1.64	NS	2.60	2.02
Similar to EH-domain containing 2	LOC673251	NS	1.52	NS	1.87	2.02
BB485297		6.11	NS	NS	NS	2.01
Transcribed locus, BF148780		NS	NS	NS	NS	2.00
RIKEN cDNA E530001K10 gene		NS	NS	NS	NS	−2.01
LEM domain containing 1	Lemd1	NS	NS	NS	NS	−2.01
Expressed sequence AI449310		NS	NS	NS	NS	−2.05
AV118079		NS	NS	NS	NS	−2.07
RIKEN cDNA 9630014M24 gene		NS	NS	NS	NS	−2.08
Transmembrane protein 107	Tmem107	NS	NS	NS	NS	−2.09
RIKEN full-length enriched library, clone:D130065D02		NS	NS	NS	NS	−2.10
RIKEN cDNA 1700040D17 gene		NS	NS	NS	−1.75	−2.11
RIKEN cDNA 9630002A11 gene		NS	NS	NS	NS	−2.11
BE957247		NS	NS	NS	NS	−2.19
BB335621		NS	NS	NS	NS	−2.36

[a]Fold change compared with unhandled baseline group.

[b]NS, Not significantly regulated; false discovery rate adjusted p value >0.05.

before MCAO. Using Affymetrix oligonucleotide microarrays, we identified 263 genes (228 increased, 35 decreased) significantly regulated 3 h after LPS injection and 176 genes (174 increased, 2 decreased) at 24 h after LPS treatment. However, within 72 h after LPS administration, most of the genomic changes had subsided to baseline with the exception of five differentially regulated genes that remained increased (Fig. 1*A*; Table 1). The saline-treated controls showed no statistically

Table 3. TREs identified as significantly overrepresented in genes induced 24 h after stroke in LPS-preconditioned mice

	Adjusted *p* values for overrepresentation	
Transcriptional regulatory element	LPS group	Saline group
c-Rel/V$CREL_01	0.00	>1.00
IRF/V$IRF_Q6	0.00	>1.00
IRF/V$IRF_Q6_01	0.01	>1.00
NF-kappaB (p65)/V$NFKAPPAB65_01	0.01	0.86
RREB-1/V$RREB1_01	0.01	>1.00
IRF-8/V$ICSBP_Q6	0.03	0.99
NF-Y/V$NFY_Q6	0.03	>1.00
ISRE/V$ISRE_01	0.07	>1.00
STAT5B (homodimer)/V$STAT5B_01	0.07	>1.00
IRF-7/V$IRF7_01	0.15	>1.00
COMP1/V$COMP1_01	0.17	0.37
Freac-3/V$FREAC3_01	0.17	>1.00
Muscle TATA box/V$MTATA_B	0.17	>1.00
Ik1/V$IK1_01	0.199	0.64
S8/V$S8_01	>1.00	0.00
E2/V$E2_01	0.69	0.0024
C/EBPβ /V$CEBPB_02	>1.00	0.13
HNF-1/V$HNF1_C	>1.00	0.13
Myogenin/NF-1/MYOGNF1_01	0.94	0.19

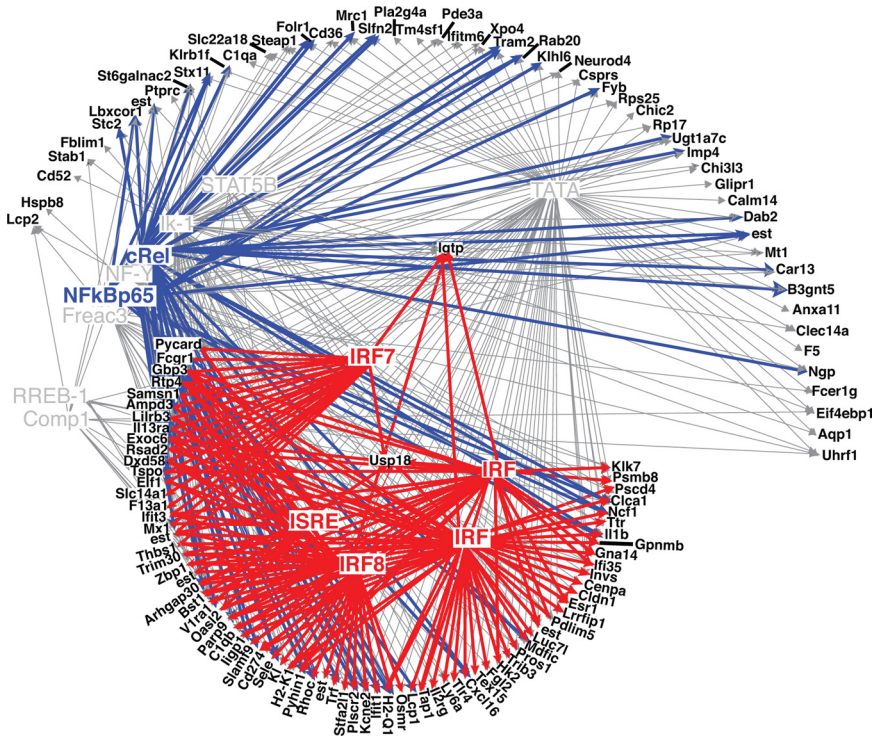

Figure 3. Interferon- and NFκB-related TREs identified in the majority of genes increased after stroke in LPS-preconditioned mice. Hypothetical gene–TRE network showing the relationship of the identified TREs to the genes increased after stroke in LPS-preconditioned mice. Genes are depicted in black, interferon-associated TREs are represented in red, NFκB-associated TREs are in blue, and other TREs are in gray. *p* value threshold set at 0.2.

significant gene regulation at any time point when compared with the unhandled baseline group.

We determined putative functions for the modulated genes using the Affymetrix Netaffx website, the Stanford-Online Universal Resource for Clones, and expressed sequence tagged website (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch) and the published literature. A large fraction (~50%) of the genes regulated at both 3 and 24 h are involved in the defense/inflammation response, which includes genes associated with both the innate and adaptive immune response as well as genes involved in stress and wound responses (Fig. 1*B*). Thus, a low dose of LPS given systemically induces genomic regulation of the inflammatory response in the brain as early as 3 h after administration, which is resolved at the genomic level of RNA expression by 72 h.

LPS preconditioning induces a novel genomic response to stroke

We compared the transcriptional response with MCAO in LPS-preconditioned and control mice. The majority of genes regulated (~70%) at both 3 and 24 h after MCAO were independent of the preconditioning stimulus. However, a significant number of genes were uniquely regulated based on LPS preconditioning. At 3 h after MCAO, 66 genes (29%) were unique to the saline-pretreated animals, whereas 26 genes (14%) were only seen in mice preconditioned with LPS (Fig. 2, purple and blue regions, respectively). Only one of the 26 genes unique to the LPS-preconditioned mice was regulated at the time of ischemia. Saa3, an acute phase responder, was increased sixfold over unhandled controls at 72 h after LPS injection. At 3 h after MCAO, Saa3 remained increased (5.5-fold) in mice preconditioned with LPS, suggesting that this increased level was attributable to the preconditioning stimulus that had occurred 3 d earlier. The general absence of unique gene regulation just before ischemia is in contrast to the presence of unique gene regulation that occurs after ischemia and suggests that previous LPS preconditioning modifies the genomic response to ischemia.

The distinct responses to stroke are also evident at the 24 h time point, at which 231 genes (29%) are uniquely regulated in the saline-pretreated animals and 176 genes (23%) are unique to the LPS-preconditioned mice (Fig. 2, gold and green regions, respectively). Table 2 shows the regulation of each of these 176 genes before and after MCAO in LPS-preconditioned mice. These genes appear to be regulated in response to the MCAO, because there is little (less than twofold) or no regulation for each identified gene at the time of stroke (72 h after injection). Hence, after stroke, LPS preconditioning induces the regulation of a unique set of genes as early as 3 h after stroke that is not evident in saline-pretreated mice. These findings suggest that LPS preconditioning reprograms the genomic response to stroke in LPS-preconditioned mice.

Interferon transcriptional regulatory elements are associated with LPS preconditioning

We identified TREs associated with the unique gene regulation detected in the LPS and saline preconditioned animals using the Web-based program PAINT version 3.5. We compared the TREs identified in the cluster of genes uniquely increased in LPS-preconditioned mice 24 h after stroke (158 genes) (Fig. 2) to a reference cluster consisting of ~33,000 transcripts from the

MOE430 gene chip. This allowed the determination of overrepresented TREs associated with the genes in the preconditioned cluster. We performed the same comparison using the cluster of genes uniquely increased in the saline-pretreated mice 24 h after stroke (128 genes) (Fig. 2). Analysis of the LPS-preconditioned group identified 14 TREs with an adjusted p value <0.2, whereas the saline-pretreated cluster revealed only five overrepresented TREs (Table 3). Five of the 14 identified TREs in the LPS-preconditioned cluster are interferon associated [IRF (V$IRF_Q6 and V$IRF_Q6_01), IRF8, ISRE, and IRF7], and two are nuclear factor-κB (NFκB) components (cRel, NF-kappaBp65). A network depiction of interactions between the identified TREs and the genes in the LPS-preconditioned cluster is displayed in Figure 3. The interferon-associated TREs (in red) are linked to a substantial number of the genes shown (60%; 76 of 127). In fact, a large number of the genes with identified IFN TREs have been reported in the literature to be induced by type I interferons (Fig. 4, red asterisks). NFκB regulatory elements were also overrepresented; these sequences were found on 54 of the 127 genes (42%) (Fig. 3, blue), with 30 of those also sharing IFN TREs (Fig. 4). It has been reported recently that cRel directly binds to the promoter and regulates several ISG genes during IFN stimulation (Wei et al., 2008). Thus, cRel/NFκB may play an integral role in the interferon fingerprint associated with LPS preconditioning. Collectively, 79% (100 of 127) of the genes induced 24 h after stroke in LPS-preconditioned mice contain a regulatory sequence for IFN or NFκB (Fig. 4). The predominance of the type I interferon signature prompted us to pursue the possible role of enhanced type I interferon signaling in LPS-induced neuroprotection.

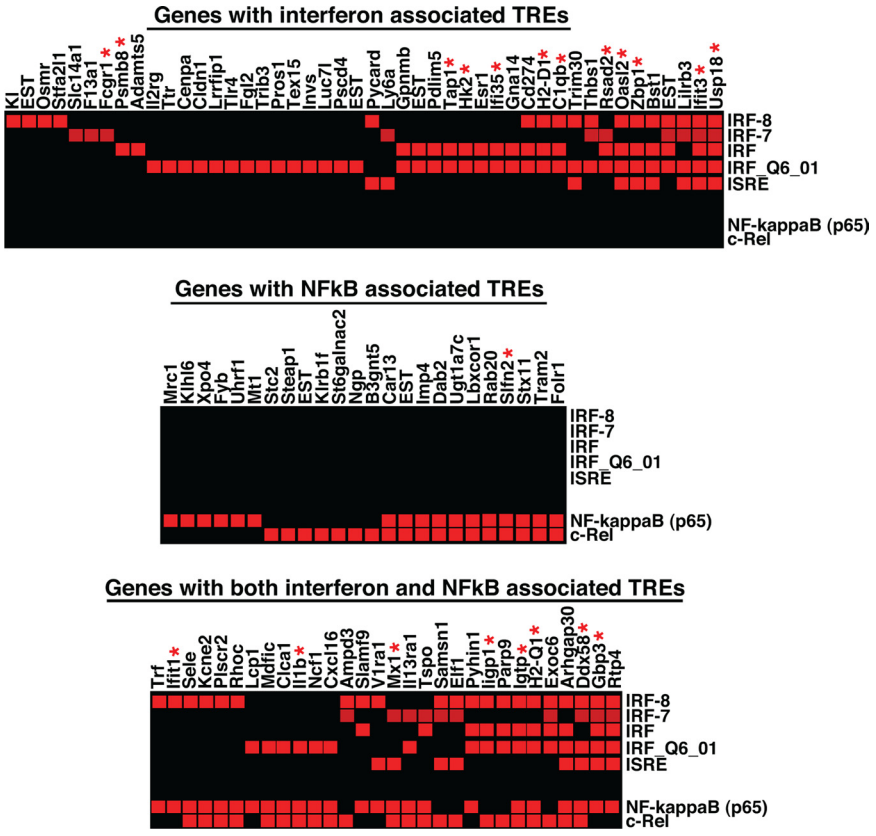

Figure 4. Genes induced in LPS-preconditioned mice after stroke with identified interferon- or NFκB-related TREs. Matrices depicting the occurrence of IFN and NFκB regulatory sites within the genes induced 24 h after stroke in LPS-preconditioned mice. Genes are listed down the right side, with TREs listed across the top. Identification of a TRE within the 5′ sequence of a gene is denoted with a red box. Red asterisks show genes induced by type I interferons as reported in the published literature.

Increased levels of IFNβ after stroke in LPS-preconditioned mice

The increase in interferon-inducible genes and overrepresentation of interferon-associated TREs suggested that IFNβ may be present in the brain cortex after stroke in LPS-preconditioned mice. Using real-time PCR, we examined the levels of IFNβ transcript in the brain after stroke in LPS-preconditioned and saline-treated mice. IFNβ levels were increased after stroke in the preconditioned and non-preconditioned mice compared with unhandled controls (Fig. 5). However, levels in LPS-preconditioned mice were ninefold higher at 3 h (LPS treated, 59.4 ± 22 vs saline treated, 6.7 ± 3; $p < 0.001$) and 3.5-fold higher at 24 h (LPS treated, 45.3 ± 23 vs saline treated, 11.7 ± 6; $p < 0.001$) after stroke. We examined levels of IFNβ just before MCAO (72 h after injection) to confirm that the increase in IFNβ after stroke was independent of any residual increase of IFNβ resulting from the preconditioning LPS injection. Levels of IFNβ in LPS- and saline-treated mice were statistically equivalent to unhandled controls (1.49 ± 1.4 and 0.74 ± 0.6, respectively) (data not shown). Thus, after stroke, mice preconditioned with LPS mount a more robust IFNβ response to ischemic injury.

IFNβ does not play a role in the endogenous response of the brain to ischemia

IFNβ after stroke in non-preconditioned mice was increased 6.7-fold over unhandled mice (Fig. 5). Although not as robust as in LPS-preconditioned mice, this does suggest that IFNβ may play a protective role in the endogenous response to stroke. To determine whether the increase after stroke alone could be protective, IFNβ knock-out mice were subjected to 40 min MCAO, followed by 72 h of reperfusion. Wild-type and IFNβ knock-out mice displayed infarcts of similar size (38.5 ± 2 vs $39.5 \pm 1\%$; $p = 0.7$). Thus, IFNβ itself does not appear to play a critical role in the usual response by the brain to ischemia.

IFNβ protects against ischemic injury

To determine whether the more robust increase in IFNβ (ninefold) (Fig. 5) after stroke in LPS-preconditioned mice would effect ischemia, we tested the effect of exogenous IFNβ administration in the brain after MCAO. We injected C57BL/6 mice intracerebroventricularly with recombinant mouse IFNβ immediately before and after MCAO and measured infarct size 24 h later. Animals treated with IFNβ showed a significant reduction in infarct volume versus vehicle-treated mice (31.9 ± 4 vs $49.4 \pm 2\%$; $p < 0.001$). This result supports the notion that increased expression of IFNβ within the brain would confer protection from ischemic injury.

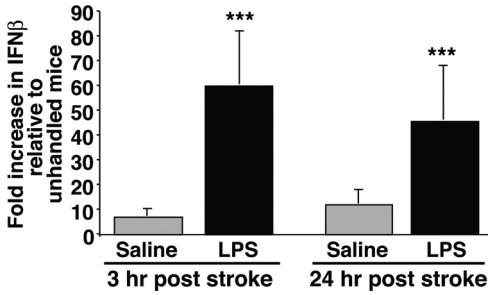

Figure 5. Increased levels of IFNβ after MCAO in LPS-preconditioned mice. Real-time PCR analysis was performed on RNA derived from the cortices after MCAO (3 and 24 h) of mice preconditioned with either LPS or saline. Data were normalized to β-actin. Results are presented as fold increase relative to unhandled controls. $n = 3–4$ mice per group; data are group means $\pm$ SEM; ***$p < 0.001$ by two-way ANOVA with Bonferroni's *post hoc* test.

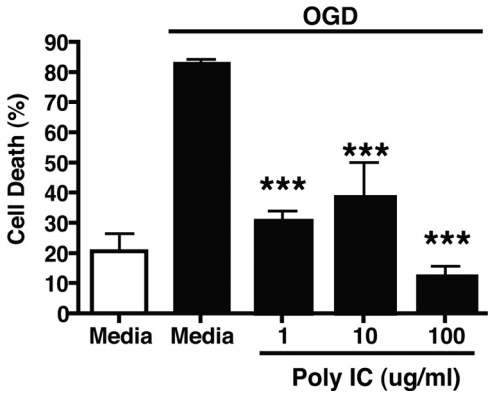

Figure 6. TRIF signaling after oxygen glucose deprivation induces neuroprotection. Mixed cortical cultures were exposed to 3 h OGD, followed by treatment with increasing doses of Poly I:C. Cell death was assessed by PI staining performed 24 h after. Mean $\pm$ SEM are shown; ***$p < 0.001$ versus media-treated OGD control. $n = 2–4$ individually repeated experiments. Poly I:C treatment at the highest dose without exposure to OGD did not induce cell death (data not shown).

TRIF-mediated signaling is protective in ischemia modeled *in vitro*

The increase in IFNβ and type I interferon-associated genes in response to stroke in the LPS-preconditioned mice mirrors the secondary response to LPS in classic endotoxin tolerance and supports a possible reprogramming of the TLR response to stroke, resulting in a TRIF-mediated event. To determine whether signaling via TRIF after stroke would induce protection, we used the synthetic TLR3 ligand Poly I:C (InvivoGen), which signals exclusively via a TRIF-dependent pathway. We reasoned that activation of this pathway at the time of ischemia would provide acute protection from ischemic damage. To test this, mixed cortical cultures from mice were exposed to OGD for 3 h, followed by treatment with varying doses of Poly I:C and subsequently returned to normoxic conditions. Twenty-four hours later, cell death was determined. Acute Poly I:C treatment after OGD significantly reduced OGD-mediated cell death at all three doses tested (Fig. 6). Thus, signaling via TRIF after ischemia provides protection against damage *in vitro*, which suggests that TRIF-mediated signaling in the brain after stroke could reduce ischemic injury.

IRF3 is required for LPS-induced protection from brain ischemia

To further explore the TRIF–IRF3 pathway, we tested whether IRF3 is a critical effector of LPS-induced ischemic protection.

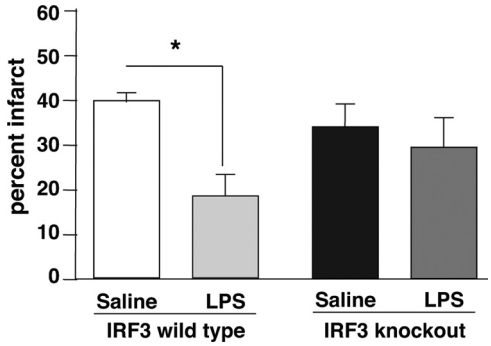

Figure 7. IRF3 is an essential mediator of LPS preconditioning. IRF3 knock-out and wild-type mice were pretreated with LPS (0.4 mg/kg) or saline 72 h before 40 min MCAO. Infarct volume was measured 24 h after surgery using TTC staining. Data shown are group means $\pm$ SEM; *$p < 0.05$ by two-way ANOVA with Bonferroni's *post hoc* test; $n = 7–10$ per group.

First, we determined whether IRF3 is involved in the endogenous response by the brain to stroke. IRF3 knock-out mice were subjected to 40 min MCAO, followed by 72 h of reperfusion. IRF3 knock-out mice displayed infarcts of similar size to wild-type mice (41.2 $\pm$ 5 vs 43.4 $\pm$ 4%; $p = 0.8$). Thus, IRF3 does not play a critical role in the usual response by the brain to ischemia. Next we determined whether IRF3 is a required effector of LPS-induced tolerance to ischemia. IRF3 knock-out mice were pretreated with LPS (0.4 mg/kg) 72 h before 40 min MCAO and killed 24 h later. Figure 7 shows that IRF3 knock-out mice fail to be preconditioned with LPS (17.3 vs 53.2% reduction). Hence, IRF3 is required for the protective effects of LPS pretreatment.

Discussion

We propose a molecular model of LPS-induced neuroprotection from ischemic injury wherein systemic LPS preconditioning reprograms TLR4 signaling in response to stroke, directing it toward a neuroprotective pathway. Administration of systemic LPS induces an early inflammatory genomic response in the brain that has receded by 72 h. However, the response to stroke in these LPS-preconditioned mice is altered, and a new pattern of gene regulation is induced as early as 3 h after exposure to ischemia. The genomic changes in the brain in response to LPS suggest a possible activation of brain TLRs before the stroke, which go on to respond with an altered signaling pathway after activation by the stroke event. The fact that IFN regulatory elements are overrepresented among the unique genes induced after stroke in LPS-preconditioned animals suggests that TLR signaling in this setting may be altered. To pursue this, we examined the possibility that increased signaling via the TRIF-dependent pathway could serve as a neuroprotective mechanism associated with LPS preconditioning.

The induction of IFNβ is a hallmark of the TRIF-dependent TLR4 cascade (Biswas et al., 2007). In accordance with this, we found that LPS preconditioning increased IFNβ within the brain 3 and 24 h after stroke. We then tested whether the increase in IFNβ was required to confer neuroprotection. Mice lacking IFNβ displayed infarcts of similar size to wild-type mice, suggesting that endogenous IFNβ does not protect the brain from ischemic injury. However, exogenous administration of IFNβ intracerebroventricularly at the time of stroke conferred significant protection against ischemic damage, indicating that local upregulation of this cytokine may be neuroprotective. Our *in vitro* studies using modeled ischemia showed that activation of the TRIF–IRF3 pathway with the TLR3 ligand Poly I:C after OGD

reduced neuronal death, thus supporting the notion that TRIF-dependent signaling can provide protection to ischemic injury.

The role of the TRIF–IRF3 cascade in the natural response of the brain to stroke appears relatively minor because mice lacking IRF3 displayed infarcts of similar size to wild-type mice. However, there appears to be a critical role for IRF3 in LPS preconditioning because IRF3-deficient mice could not be protected from ischemic injury in the setting of LPS preconditioning. Because these mice are deficient in IRF3 during the induction of tolerance as well as after the ischemic event, we cannot rule out a requirement for IRF3 during the induction phase as well as the resolution phase. In preliminary studies, we found that Poly I:C preconditioning is protective in stroke (our unpublished observation), which suggests that TRIF signaling may be sufficient for the induction of tolerance. In models of endotoxin–endotoxin (LPS) tolerance in which previous exposure to low-dose endotoxin provides protection against subsequent high-dose exposure, there is substantial support for the notion that TRIF–IRF3 signaling is required. Biswas et al. (2007) showed that endotoxin tolerance was induced in MyD88-deficient mice but not in TRIF- or IRF3-deficient mice (Biswas et al., 2007). Thus, although TRIF-dependent signaling is likely involved in the induction of tolerance, the enhancement of IRF3-dependent genes after stroke in LPS-preconditioned mice and the protective response elucidated with Poly I:C after OGD supports a role for the TRIF–IRF3 pathway in the protective phenotype as well.

We postulate that LPS preconditioning against ischemic injury results in redirected TLR4 signaling that resembles endotoxin tolerance. Cells made tolerant to endotoxin (LPS) are known to suppress the proinflammatory MyD88–TNFα pathway by upregulating pathway inhibitors, namely IRAK-M, Tollip, Ship1, and Trim30α, among others (Lang and Mansell, 2007; Shi et al., 2008), which results in decreased inflammatory cytokine responses during secondary exposure to TLR4 ligands. Inhibition of these pathways shunts subsequent TLR4 signaling down the TRIF–IRF3–IFNβ pathway and results in enhanced production of IFNβ (Bagchi et al., 2007). Similarly, LPS preconditioning may upregulate inflammatory pathway inhibitors in the brain that shunt subsequent TLR signaling down the TRIF–IRF3–IFNβ pathway. Thus, in the setting of ischemia, release of endogenous TLR ligands would be expected to lead to TLR signaling that is shunted down the TRIF–IRF3–IFNβ pathway and result in upregulation of IFNβ.

Our data support a model of redirected TLR4 signaling after stroke in preconditioned animals. Unlike control animals, LPS-preconditioned mice demonstrate a significant upregulation of IFNβ- and IFN-associated genes after stroke, which, based on promoter region analyses, are likely produced via the TLR4–TRIF–IRF3 pathway. Hirotani et al. (2005) have shown in endotoxin tolerance, using TRIF-deficient mice, that induction of type I interferon-associated genes after a secondary challenge is exclusively dependent on TRIF (Hirotani et al., 2005). Together, these data suggest that the type I IFN "fingerprint" is generated downstream of TLR4–TRIF–IRF3 and supports the concept of TLR4 reprogramming.

The potential for the reprogrammed TLR response to be protective is evinced by the TRIF-mediated neuronal protection in our *in vitro* model and via the protective effect of IFNβ directly administered in the brain. Others have shown that systemic administration of IFNβ reduces tissue damage in both a rat and rabbit model of ischemic stroke (Liu et al., 2002; Veldhuis et al., 2003). In these studies, the protective effects of IFNβ may have been mediated through leakage across the blood–brain barrier (BBB) after stroke wherein IFNβ may have then acted directly on the brain parenchyma. A recent study by Maier et al. (2006) in which BBB integrity is believed to be preserved failed to show attenuation of ischemic brain injury after systemic administration of IFNβ (Maier et al., 2006). Thus, as with our data, these results support the notion that the neuroprotective effects of IFNβ occur centrally.

We show that IRF3 is required for LPS-induced neuroprotection and that this is most likely through its induction of IFNβ and other ISGs. Two potential ISG modulators of the protective effect, Trim30 and Ifit1, were identified in our microarray analysis. Trim30 has been shown to negatively regulate LPS-induced TNFα and IL6 expression via inhibition of TLR4-induced NFκB activation (Shi et al., 2008). In the brain, we found increased mRNA levels of Trim30 at 3 and 24 h after LPS treatment and at 24 h after stroke in LPS-preconditioned mice. This induction could play a role in the suppression of inflammatory cytokines in the brain after stroke. Although Shi et al. (2008) reported that Trim30 expression depends on NFκB, the promoter sequence also contains an ISRE site (Fig. 4). In addition, we find induction of Ifit1 after LPS administration and again 24 h after stroke in LPS-preconditioned mice. The closely related gene Ifit2 has been shown recently to suppress the LPS-mediated induction of TNFα and IL6 (Berchtold et al., 2008), although it has not been directly linked to suppression of TLR signaling. The induction of these two ISG genes and IFNβ in LPS-preconditioned mice support the hypothesis of a reprogrammed TLR response to stroke resulting in a neuroprotective state.

Our data suggest that systemic administration of LPS reprograms TLR4-expressing cells within the brain. TLR4 is widely expressed in the brain (Lehnardt et al., 2002; Olson and Miller, 2004; Chakravarty and Herkenham, 2005), and many studies have shown that peripheral LPS induces a proinflammatory response within the brain (Chen et al., 2005; Qin et al., 2008). However, it is unclear whether LPS crosses the BBB and/or whether it induces peripheral cytokines, which in turn, cross into the brain. Recent evidence suggests that systemic LPS elicits TLR4 signaling in the brain independent of peripheral cytokine responses (Chakravarty and Herkenham, 2005; Gosselin and Rivest, 2008). However, other researchers have failed to find LPS within the brain parenchyma after systemic administration (Singh and Jiang, 2004). It is clear that LPS binds to cerebral endothelial cells (Singh and Jiang, 2004; Verma et al., 2006). Because these cells are an interface between the systemic circulation and the brain parenchyma, they may help integrate information from both compartments. Hence, reprogramming of TLR4 may occur within the cerebral endothelium.

In summary, we have shown that LPS preconditioning reprograms the cellular response to stroke and causes a type I IFN response, with a critical and protective role for IRF3. These reprogramming events may exemplify endogenous processes that protect the brain against additional injury and suggest that LPS preconditioning fundamentally changes the cellular response to stroke. This is the first demonstration that a preconditioning stimulus results in an interferon fingerprint after the ischemic event and the first report of a neuroprotective role for TRIF–IRF3 signaling after injury.

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