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Toll-Like Receptor Signaling in Endogenous Neuroprotection and Stroke

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Abstract

Stroke and other cerebral vascular diseases are a leading cause of morbidity and mortality in the United States. Despite intensive research to identify interventions that lessen cerebrovascular injury, no major therapies exist. Development of stroke prophylaxis involves an understanding of the mechanisms of damage following cerebral ischemia, and elucidation of the endogenous mechanisms that combat further brain injury. Toll-like receptors (TLRs) are critical components of the innate immune system that have been shown recently to mediate ischemic injury. Paradoxically, TLR ligands administered systemically induce a state of tolerance to subsequent ischemic injury. Herein we suggest that stimulation of TLRs prior to ischemia reprograms TLR signaling that occurs following ischemic injury. Such reprogramming leads to suppressed expression of pro-inflammatory molecules and enhanced expression of numerous anti-inflammatory mediators that collectively confer robust neuroprotection. Our findings indicate that numerous preconditioning stimuli lead to TLR activation, an event that occurs prior to ischemia and ultimately leads to TLR reprogramming. Thus genomic reprogramming of TLR signaling may be a unifying principle of tolerance to cerebral ischemia.

Keywords

reprogramming; preconditioning; tolerance; lipopolysaccharide; inflammation; Type I interferon

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Preface

Ischemia occurs when blood flow to an organ is inhibited, resulting in a lack of oxygen and nutrient delivery and a decrease in the clearance of toxic metabolic byproducts. Extended periods of ischemia cause significant cellular stress and cell death. The brain is particularly sensitive to ischemia as its energy demands rely on glucose delivered from the blood rather than stored glycogen. Paradoxically, restoration of blood flow to organs exposed to long periods of ischemia can further exacerbate injury by generating oxygen-derived free radicals, and results in even greater amounts of cell death. Brain ischemia, or ischemic stroke, is particularly devastating and is one of the leading causes of morbidity and mortality in the United States. Much of the current research in brain ischemia is focused on revealing mechanisms the body has evolved to protect the brain from ischemic damage. Such endogenous neuroprotective mechanisms, if induced early as a therapeutic approach, may enhance protection and preserve brain tissue. Here, we discuss the critical role of Toll-like receptors in mediating cerebral ischemic injury and suggest endogenous mechanisms that, when induced, redirect this role from detrimental to beneficial. In fact, many diverse neuroprotective paradigms may redirect Toll-like receptor signaling as one mechanism of endogenous protection.

An Introduction to Toll-like Receptors

The Toll-like receptors (TLRs), so-called because of their homology to the *Drosophila* Toll receptor, were first characterized in mammals by their ability to recognize pathogen-associated molecular patterns such as those found in the bacterial cell wall components peptidoglycan (TLR2) and lipopolysaccharide (LPS) (TLR4), as well as viral dsRNA (TLR3), ssRNA (TLR7), and nonmethylated cytosine-guanine (CpG) DNA (TLR9). Recently it has been found that in addition to their role in pathogen detection and defense, TLRs act as sentinels of tissue damage and mediate inflammatory responses to aseptic tissue injury. Host-endogenous molecules associated with damaged cells and tissues have been shown to activate TLRs (Table 1).

Surfactant, HSP60, components of the extracellular matrix, and fibrinogen have all been shown to activate TLR4, while host HMGB1, as well as host mRNA and DNA are endogenous ligands of TLR2 (and TLR4), TLR3 and TLR9, respectively. TLRs, upon activation by either pathogen- or host-derived ligands, induce downstream signals that lead to cytokine and chemokine production and thereby initiate inflammatory responses. TLRs are located on antigen presenting cells such as B cells, dendritic cells, monocytes/macrophages and microglia. In addition, these receptors can be expressed by the cerebral endothelium and by cells within the brain parenchyma such as astrocytes, oligodendrocytes, and neurons (Bsibsi et al., 2002, Singh and Jiang, 2004, Jack et al., 2005, Bsibsi et al., 2006).

The TLRs signal through common intracellular pathways leading to transcription factor activation and the generation of cytokines and chemokines (Figure 1) (Vogel et al., 2003, Takeda and Akira, 2005). Each TLR family member, with the exception of TLR3, initiates intracellular signaling via recruitment of the intracellular Toll-interleukin 1 receptor (TIR)-domain-containing adaptor MyD88. When recruited to plasma membrane-associated TLRs, either directly (TLRs 5 and 11) or via the TIRAP adaptor (TLRs 1, 2, 4, 6), MyD88 enlists members of the IRAK family, including IRAK1, IRAK2, and IRAK4, to begin a process of auto- and cross-phosphorylation among the IRAK molecules. Once phosphorylated, IRAKs dissociate from MyD88 and bind TRAF6, an E3 ligase. TRAF6 in turn activates TAK1 which itself activates the IKK complex and MAPKKs. The IKK complex, composed of IKK α , IKK β and the regulatory subunit IKK γ /NEMO, phosphorylates I κ B proteins. This phosphorylation is necessary for the ubiquitination and proteosomal degradation of I κ Bs and the subsequent nuclear translocation of the transcription factor NF κ B. Members of the MAPK

family phosphorylate and activate components of the transcription factor AP-1. Together, these transcription factors induce inflammatory cytokine production (e.g. TNF α , IL1).

MyD88 is also recruited to the endosomal receptors TLR7 and TLR9 again enlisting members of the IRAK family. Due to the endosomal location of the complex, the phosphorylated IRAKs are able to bind TRAF3 in addition to TRAF6. Activation of TRAF3 leads to phosphorylation, dimerization, and nuclear localization of the transcription factors IRF3, IRF5, and IRF7 with resultant Type I interferon (IFN) production. Hence these endosomal TLRs are capable of signaling to NF κ B, AP-1 and IRFs, resulting in a diverse genomic response.

Endosomal TLR3 is unique among the TLRs because it does not signal through MyD88 but signals instead via recruitment of the TIR domain-containing adaptor inducing IFN β (TRIF). TRIF enlists the non-canonical IKKs, TBK1 and IKK ϵ , which activate IRF3. Further, TRIF recruits TRAF6 and RIP-1, which results in activation of MAPK and IKK α / β . Hence TLR3, like the other endosomal receptors, is capable of activating NF κ B, AP-1 and IRFs.

Of all the TLRs, only TLR4 can recruit either MyD88 (via TIRAP) or TRIF (via TRAM) and can thus induce either the pro-inflammatory cytokines TNF α and IL1 via NF κ B or the anti-viral IFN β via IRF3.

The complement of TLR family members expressed by a cell depends on its identity and its activation status. Constitutive expression of TLRs within the brain occurs in microglia and astrocytes and is largely restricted to the circumventricular organs and meninges—areas with direct access to the circulation (Laflamme and Rivest, 2001, Laflamme et al., 2001, Chakravarty and Herkenham, 2005). Human and murine microglia express TLRs 1–9 and generate cytokine profiles specifically tailored by the TLR stimulated (Bsibsi et al., 2002, Olson and Miller, 2004, Jack et al., 2005). Similarly, human and murine astrocytes express multiple TLRs, with particularly prominent TLR3 expression (Bsibsi et al., 2002, Carpentier et al., 2005, Jack et al., 2005, McKimmie and Fazakerley, 2005, Bsibsi et al., 2006). Microglia and astrocytes respond differently to specific TLR engagement reflective of their distinct roles in the brain. Microglia initiate robust cytokine and chemokine responses to stimulation of TLR2 (TNF α , IL-6, IL-10), TLR3 (TNF α , IL-6, IL-10, IL-12, CXCL-10, IFN β), and TLR4 (TNF α , IL-6, IL-10, CXCL-10, IFN β), yet astrocytes initiate only minor IL-6 responses to all but TLR3 stimulation (Jack et al., 2005). Microglia express TLR3 and TLR4 at the cell surface while astrocytes express these receptors intracellularly (Bsibsi et al., 2002). The cellular location of TLRs affects their downstream signaling cascades (Kagan et al., 2008), which may explain the different responses of these cells to TLR stimulation. The inflammatory milieu also plays a critical role in regulating TLR expression. Microglia stimulated with CpG specifically up-regulate TLR9, whereas those stimulated with a synthetic TLR3 ligand suppress all TLRs except TLR3 (Olson and Miller, 2004). Similarly, astrocytes stimulated with LPS up-regulate TLRs 2 and 3 but suppress TLR4, while astrocytes exposed to RNA viruses up-regulate TLR3 and TLR9 (McKimmie and Fazakerley, 2005). Thus microglia and astrocytes initiate a layered and multifaceted response to TLR engagement.

Oligodendrocytes and endothelial cells express a relatively limited repertoire of TLRs. Oligodendrocytes express TLRs 2 and 3 (Bsibsi et al., 2002), while cerebral endothelial cells constitutively express TLRs 2, 4, and 9 (Constantin et al., 2004) and increase their expression of these TLRs in response to stressful stimuli, including systemic LPS and cerebral ischemia (Singh and Jiang, 2004, Zhou et al., 2007, Ziegler et al., 2007). In response to LPS, endothelial cells up-regulate E-selectin, an NF κ B-dependent molecule, and IFN β , an IRF3-dependent molecule, indicating that these cells utilize the TLR-NF κ B and the TLR-IRF3 signaling pathways (Lloyd-Jones et al., 2008).

Neurons express TLR3 and generate inflammatory cytokines (TNF α , IL-6), chemokines (CCL5, CXCL10) and antiviral molecules (IFN β) in response to dsRNA (Lafon et al., 2006). Neurons also employ TLRs in their development and differentiation. TLRs 3 and 8 are expressed on murine neurons early in development and inhibit neurite outgrowth in a MyD88- and NF κ B-independent manner (Ma et al., 2006). TLR2 and TLR4 have been found on adult neural progenitor cells where they appear to elicit opposing effects. While TLR2 activation stimulates neuronal differentiation of these cells, TLR4 activation decreases proliferation and neuronal differentiation, driving these cells toward an astrocytic fate (Rolls et al., 2007). Curiously, both TLRs exert these endogenous effects in a MyD88-dependent manner, suggesting that these molecules utilize MyD88 in distinct ways (For an extensive review of brain inflammation and adult neurogenesis, please refer to Ekdahl et. al. in this issue). Hence even minor alterations of these fine-tuned endogenous pathways can have profound effects on cellular responses to TLR engagement.

Studies with TLR knockout mice illustrate the endogenous function of TLRs in health and disease. TLR2 and TLR4 have been shown to play detrimental roles in the development of congestive heart failure and cardiac hypertrophy, respectively, by signaling through MyD88 and NF κ B (Shishido et al., 2003, Ha et al., 2005). TLR2 has additionally been found to be proatherogenic in hyperlipidaemic mice (Tobias and Curtiss, 2007), and TLR4 has been shown to produce inflammatory reactions in adipose tissue and thereby mediates obesity and insulin resistance (Tsukumo et al., 2007, Davis et al., 2008). Conversely, TLR2 and TLR4 activation by hyaluronic acid protect lung tissue from non-infectious injury (Jiang et al., 2006), and TLR4 has been shown to help maintain lung integrity, and prevent the development of emphysema, by modulating oxidant generation (Zhang et al., 2006).

The effects of endogenous TLR stimulation are clearly varied, depending on the cell and tissue type in which the receptors are found and on the disease process in which they are involved. The overwhelming and generally damaging inflammatory response of TLRs to aseptic tissue injury may be a consequence of TLR evolution in response to pathogens. In the setting of pathogen invasion, an inflammatory deluge may be the most effective means to clear microorganisms. The activation and influx of leukocytes, with the concomitant release of free radicals and tissue-destroying enzymes, assails not only the invading pathogen but any host cells that harbor the pathogen. However, when this same powerful response is co-opted by the host to clear and resolve tissue damage, it can destroy the very cells it is meant to save. This damage-promoting characteristic is prominently observed following brain ischemia, where inflammation plays a critical role in both injury progression and resolution.

Toll-like receptors and ischemic damage

A significant portion of the damage associated with stroke injury is due to the resultant inflammatory response. This aspect of the innate immune response is exemplified by the fact that some anti-inflammatory strategies have been shown to ameliorate ischemic damage (Relton JK et al., 1996, Hara et al., 1997, Spera et al., 1998). The inflammatory response to stroke is initiated by the detection of injury-associated molecules by local cells such as microglia and astrocytes. The response is further promoted by infiltrating neutrophils and macrophages, resulting in the production of inflammatory cytokines, proteolytic enzymes, and other cytotoxic mediators. In the mouse, leukocytes and brain cells (microglia, astrocytes and neurons) express TLRs (Zarembek and Godowski, 2002, Olson and Miller, 2004). Hence, injury-associated molecules such as HSP60 and HMGB1 may act as endogenous ligands for TLRs, thereby initiating the damaging inflammatory response to stroke.

It is increasingly clear that TLRs do in fact play a role in ischemic damage (Figure 2). The pathogenic role of TLRs in ischemic processes was first demonstrated in a mouse model

myocardial ischemia/reperfusion injury, because mice lacking functional TLR4 incur less damage than wild type mice (Oyama et al., 2004). Since then, TLR2 has also been shown to cause dysfunction following cardiac ischemia and both have been shown to exacerbate renal ischemic damage, in a MyD88-dependent and a MyD88-independent manner (Sakata et al., 2007, Shigeoka et al., 2007). However, the particular pathway responsible for the damaging effects of TLR activation may differ depending on the cell type or organ affected as TLR4 worsens ischemic damage following liver transplant in a MyD88-independent, IRF3 dependent fashion (Zhai et al., 2004, Shen et al., 2005).

Importantly, TLR2 and TLR4 have been shown to play a role in cerebral ischemic damage (Table 2). Mice lacking either functional TLR2 or TLR4 are less susceptible to transient focal cerebral ischemia/reperfusion damage, demonstrating smaller infarcts than wild type controls (Cao et al., 2007, Lehnardt et al., 2007, Ziegler et al., 2007). Further, mice lacking TLR4 incur less damage following global cerebral ischemia and permanent focal ischemia (Caso et al., 2007, Hua et al., 2007). The TLR endogenous ligands HSP 60, HSP70 and HMGB1 are found in the brain following injury (Kinouchi et al., 1993, Faraco et al., 2007, Lehnardt et al., 2008). Hence these molecules may activate TLR2 and TLR4 within the brain itself, leading to the generation of inflammatory mediators such as TNF α , IL1, IL6, and iNOS, all known to be associated with stroke damage.

Toll-like receptors and neuroprotection

In contrast to the detrimental role of TLRs in response to ischemia, stimulation of these receptors prior to ischemia provides robust neuroprotection (Table 3). TLR4-induced tolerance to cerebral ischemia was first demonstrated with low dose systemic administration of LPS (endotoxin), a cell wall component of gram-negative bacteria, which caused spontaneously hypertensive rats to become tolerant to subsequent ischemic brain damage induced by middle cerebral artery occlusion (MCAO) (Tasaki et al., 1997). Since then, LPS-induced tolerance to brain ischemia has been demonstrated in a mouse model of stroke and in a porcine model of deep hypothermic circulatory arrest (Rosenzweig et al., 2004, Hickey et al., 2007) (For additional information on the dual effects of neuro-immune crosstalk, please refer to Kerschensteiner et. al. in this issue).

Neuroprotection induced by LPS is time and dose dependent. Tolerance appears by 24 hours after LPS administration and extends out to 7 days but is gone by 14 days (Rosenzweig et al., 2007). Protective doses of LPS appear to depend on the animal model of stroke and the route of systemic administration, ranging from 0.02 to 1mg/kg (Tasaki et al., 1997, Ahmed et al., 2000, Bordet et al., 2000, Furuya et al., 2005, Hickey et al., 2007, Kunz et al., 2007, Rosenzweig et al., 2007). Tolerance induction has been shown to require new protein synthesis and a modest inflammatory response, as it can be blocked by prior administration of cyclohexamide or dexamethasone (Bordet et al., 2000). Specifically, TNF α has been implicated as a mediator of LPS-induced ischemic tolerance because inhibition of TNF α systemically (Tasaki et al., 1997) or within the brain (Rosenzweig et al., 2007) blocks neuroprotection, and mice lacking TNF α fail to be protected by LPS preconditioning (Rosenzweig et al., 2007).

In addition to its neuroprotective effects, LPS preconditioning has vasculoprotective efficacy. Nitric oxide appears to play a critical role in the protective effects of LPS. Mice lacking iNOS expression fail to be protected by LPS pretreatment (Kunz et al., 2007), and eNOS expression within the brain is directly correlated to the time window of LPS-induced neuroprotection (Furuya et al., 2005). LPS pretreatment has further been shown to prevent the impairment of endothelial and smooth muscle relaxation normally induced by ischemia/reperfusion injury (Bastide et al., 2003), resulting in normalization of cerebral blood flow in peri-infarct regions lasting out to 24 hours after MCAO (Dawson et al., 1999, Furuya et al., 2005).

LPS-induced ischemic protection requires an inflammatory response *prior* to the ischemic event, yet protection occurs through modulation of the inflammatory response *following* ischemia. Rosenzweig and colleagues have shown that LPS preconditioning changes the response of circulating leukocytes to stroke, attenuating stroke-induced neutrophilia, lymphopenia, and monocyte activation. This altered inflammatory responses extends into the brain itself. LPS preconditioning attenuates activation of microglia after stroke and reduces neutrophil infiltration into the ischemic hemisphere (Rosenzweig et al., 2004). Hence, LPS-induced preservation of microvascular function following MCAO may be due to suppressed lymphocyte adhesion to activated endothelium, either by TNF α -induced suppression of endothelial activation and adhesion molecules (Ginis et al., 1999, Ahmed et al., 2000) or by prevention of cellular inflammatory responses to ischemia (Rosenzweig et al., 2004).

One hallmark of LPS preconditioning is suppression of cytotoxic TNF α signaling following stroke. Mice that have been preconditioned with LPS prior to ischemia display a pronounced suppression of the TNF α pathway following stroke, as evinced by reduced TNF α in the serum, decreased levels of cellular TNFR1, and enhanced levels of neutralizing soluble-TNFR1. These mice are thus protected from the cytotoxic effects of TNF α after cerebral ischemia (Rosenzweig et al., 2007). Collectively, these mechanisms lead to a muted TNF α response to ischemic injury and increased cell survival.

Recently a new TLR ligand has been shown to induce tolerance to brain ischemia. As with TLR4 and LPS, stimulation of TLR9 by systemically administered CpG oligodeoxynucleotides induces robust protection against brain ischemia in a time and dose dependent manner. CpG pretreatment protects neurons in both *in vivo* and *in vitro* models of stroke (Stevens et al., 2008). Notably, the protection afforded by CpG depends on TNF α , as systemic CpG administration acutely and significantly increases serum TNF α , and TNF α knock-out mice fail to be protected by CpG preconditioning.

Similarities among the known TLR signaling pathways and their shared ability to induce TNF α , itself a potent preconditioning stimulus, suggests that stimulation of TLR4 and TLR9 may induce ischemic tolerance by similar means. The neuroprotective potential of other TLRs has yet to be explored, but this family of molecules may be a rich source of therapeutic targets.

Mechanisms of neuroprotection—reprogramming TLR signaling

The finding that TLRs are mediators of ischemic injury provides insight into the potential mechanisms of LPS- and CpG-induced neuroprotection. In fact, TLR-induced tolerance to subsequent ischemia may occur by the same mechanisms that govern a very similar phenomenon—that of LPS-induced tolerance to subsequent LPS exposure. The latter phenomenon is known as “endotoxin tolerance” and occurs when pretreatment with a low dose of LPS renders cells or whole animals tolerant to the normally detrimental effects of a second, higher dose of LPS. Cells that are tolerant to LPS are defined by their inability to generate TNF α in response to TLR4 activation. Upon TLR4 ligation, LPS tolerant cells, unlike naive cells, do not recruit MyD88 to TLR4, and fail to activate IRAK-1 and NF κ B (Medvedev et al., 2002). The TLR4-NF κ B signaling axis becomes decommissioned following a primary exposure to LPS via an elaborate negative feedback loop that involves known inhibitors of TLR signaling. Among those inhibitors are Ship-1, which prevents TLR4-MyD88 interaction, IRAK-M, a non-functional IRAK decoy, and TRIM30 α , which destabilized the TAK1 complex (Kobayashi et al., 2002, Sly et al., 2004, Shi et al., 2008). Thus, subsequent signaling of TLR4 to NF κ B is blocked and inflammatory cytokine production is suppressed. Conversely, secondary exposure causes *enhanced* IFN β release, suggesting increased signaling via the TLR4-IRF3 axis (Broad et al., 2007). Thus, pretreatment with LPS causes cells to switch their

transcriptional response to TLR4 stimulation by enhancing the IRF3- induced cytokine IFN β and suppressing the NF κ B-induced cytokine TNF α .

Similar to endotoxin tolerance, priming TLR9 with its ligand, CpG, induces a state of hypo-responsiveness to subsequent challenge with CpGs (Dalpke et al., 2005). Interestingly, cross-tolerance between the two receptors has also been reported, as ligands for TLR9 induce tolerance against a subsequent challenge with a TLR4 ligand (Bagchi et al., 2007, Broad et al., 2007). CpG-pretreated cells not only produce less TNF α when secondarily challenged with LPS, they also produce significantly higher levels of IFN β (Broad et al., 2007).

Together, the aforementioned studies suggest the intriguing possibility that TLR stimulation prior to stroke may reprogram ischemia-induced TLR activation (Figure 3). Specifically, administration of LPS or CpG may activate TLR4 and TLR9, respectively, causing a small inflammatory response, with an initial rise in TNF α . Cells would then regulate their inflammatory response through expression of negative feedback inhibitors of the TLR4-NF κ B signaling axis that remain present when cells are subsequently exposed to endogenous TLR ligands generated from ischemia-injured tissue. Within this new cellular environment, stimulated TLRs such as TLR2 and TLR4 would be unable to activate NF κ B-inducing pathways. Because of this, stroke-induced TLR2 signaling may be blocked completely leading to reduced injury, and stroke-induced TLR4 signaling would shift from NF κ B induction to IRF3 induction. Suppression of NF κ B induction would be expected to protect the brain, as mice lacking the p50 subunit of NF κ B suffer less cerebral ischemic damage than wild type mice (Schneider et al., 1999). Enhancement of IRF signaling would also be expected to protect the brain, as IFN β , a downstream product of IRF3 induction, has been shown to act as an acute neuroprotectant (Liu et al., 2002, Veldhuis et al., 2003a).

IFN β , best known for its anti-viral effects, has potent anti-inflammatory activities as well. Several studies have shown that IFN β can stabilize the blood-brain barrier, potentially by reducing matrix metalloprotease production by activated glia (Veldhuis et al., 2003b, Kraus et al., 2004, Liuzzi et al., 2004) (For an extensive review of matrix metalloproteases and the blood-brain barrier, please refer to Candelario-Jalil et al. in this issue). Similarly, it has been shown to inhibit monocyte migration across human brain-derived endothelial cells (Seguin et al., 2003) and reduce cellular infiltration into damaged brain regions (Veldhuis et al., 2003b). On a cellular level, IFN β has been shown to reduce reactive oxygen species (Lopez-Collazo et al., 1998, Stewart et al., 1998, Hua et al., 2002), suppress inflammatory cytokine production and induce IL-1Ra (Bosca et al., 2000, Palmer et al., 2004), promote nerve growth factor production by astrocytes (Boutros et al., 1997) and protect neurons from toxicity induced by activated microglia (Jin et al., 2007). In addition, systemic administration of IFN β has been shown to reduce infarct damage in rat and rabbit models of ischemic stroke (Liu et al., 2002, Veldhuis et al., 2003a). Therefore, in the setting of LPS preconditioning, upregulation of this cytokine following stroke would be expected to contribute to neuroprotection.

IFN β may not be the only neuroprotective molecule downstream of IRF signaling. TLR3 signals exclusively through the TRIF-dependent pathway and stimulation of TLR3 in human astrocyte cultures induces the expression of several neuroprotective molecules such as brain derived neurotrophic factor, neurotrophin 4, pleiotrophin, and TGF β 2 (Bsibsi et al., 2006), all of which have been implicated in endogenous neuroprotection (Yeh et al., 1998, Endres et al., 2000, Zhang et al., 2005). Astrocytic TLR3 stimulation also results in production of the anti-inflammatory cytokine IL-10 (Bsibsi et al., 2006). Conditioned media from these cultures enhances neuronal survival and suppresses astrocyte growth in slice cultures. Interestingly, LPS stimulation of macrophages has been shown to upregulate TLR3 expression (Nhu et al., 2006), inviting the possibility that LPS preconditioning may upregulate TLR3 in the brain, further enhancing stroke-induced IRF signaling.

We suggest that pretreatment with TLR ligands reprograms the brain's response to ischemia and alters endogenous stroke-induced TLR signaling by suppression of the NF κ B-inducing pathway and upregulation of the IRF-inducing pathway. Reprogramming causes a finely controlled shift in the balance of proinflammatory and anti-inflammatory cytokines, and represents an endogenously orchestrated mechanism that protects the organism from additional damage. We further suggest that reprogramming of endogenous TLR signaling, with the subsequent generation of neuroprotective Type I IFNs, is a unifying property of the neuroprotected phenotype.

Reprogramming TLRs—a common neuroprotective mechanism?

The brain has evolved numerous mechanisms that allow it to withstand the shortage of energy and the oxidative stress caused by ischemia. This tolerant state can be induced by prior exposure to LPS or CpG, or by prior exposure to other non-damaging (i.e., sub-threshold) noxious stimuli. For example, mild exposure to ischemia, excitotoxic stimuli, or inflammatory mediators can precondition the brain to better tolerate a subsequent injurious ischemic event. These mild preconditioning exposures herald impending danger and, as such, induce endogenous protective strategies in anticipation of injury.

Though the final outcome of tolerance induction is the same—protection of brain tissue from ischemic injury—the effector mechanisms employed by the brain are as diverse as the preconditioning stimuli that induced them. In fact, the phenotype of neuroprotection may be specifically tailored by the nature of the preconditioning stimulus (Stenzel-Poore et al., 2007). For example, preconditioning events that deprive the brain of oxygen or glucose for a short time lead to conservation of energy regulation and mitochondrial integrity during the injurious ischemic episode (Stenzel-Poore et al., 2003, McFalls et al., 2006). Further, as we have described above, preconditioning events that invoke a small inflammatory response lead to altered inflammatory responses to damaging ischemia (Rosenzweig et al., 2004, Rosenzweig et al., 2007). It should be emphasized that although significant overlap exists in the cellular processes induced by these diverse stimuli, the pathways that dominate each response are distinct.

The first demonstration that a short period of oxygen deprivation could protect the brain from a subsequent extended period of hypoxia occurred in 1943 (Noble, 1943). Since then, hundreds of studies have been undertaken to better understand the underlying mechanisms of “ischemic preconditioning”. Though several endogenously protective pathways are induced by the initiating ischemic event, one particular theme is emerging—that of mitochondrial maintenance and energy conservation (Dirnagl and Meisel, 2008). The priming ischemic episode appears to induce cellular pathways that protect mitochondria against stroke-induced deficits in the electron transport chain (Dave et al., 2001). These pathways protect mitochondrial membrane potential (Wu et al., 2004) preserve mitochondrial cytochrome c (Zhan et al., 2002), increase mitochondrial sequestration of Ca⁺ and increase Ca⁺-ATPase activity. In addition, ischemic preconditioning appears to suppress molecules that regulate ion channels, leading to channel arrest—i.e. reduction in ion permeability through the plasma membrane—which has been shown to reduce the amount of ATP required to maintain ionic homeostasis (Buck and Hochachka, 1993, Stenzel-Poore et al., 2003). Finally, a decrease in the overall cellular metabolic rate limits the stressful effects of oxygen deprivation. The preconditioning stimulus suppresses the expression of genes involved in protein turnover, proteasomal degradation, and energy metabolism (Stenzel-Poore et al., 2003). Although ischemic preconditioning has also been shown to help maintain protein structure and function and to suppress the damaging inflammatory response to stroke, it is increasingly clear that sustaining mitochondrial integrity and conserving energy are important mechanisms driving endogenous ischemic tolerance.

Several studies have shown that the priming ischemic event induces HSP70 within the brain (Truettner et al., 2002). In addition to its role in stabilizing protein structure, HSP70 acts as an endogenous ligand of TLR4. In fact, extracellular HSP70 has been shown to induce endotoxin tolerance (Aneja et al., 2006). Hence TLRs may be stimulated in the course of ischemic preconditioning, resulting in a reprogrammed TLR response to subsequent injurious ischemia. One of the molecular consequences of reprogrammed TLR signaling is an increase in IFN β . Notably, IFN β has been shown to aid in the maintenance of mitochondrial integrity. For example, treatment of astrocytes with IFN β prevents neuronal mitochondrial respiratory chain damage (Stewart et al., 1998) and mitigates IFN γ induced nitric oxide synthase (Stewart et al., 1997). Thus reprogrammed TLR signaling may help shape the phenotype of ischemia-induced tolerance.

The phenomenon of inflammation-induced cross-tolerance to ischemia is not limited to LPS, but extends to TNF α as well. Nawashiro and colleagues were the first to demonstrate that intracisternal administration of TNF α protects the brain from subsequent ischemic challenge (Nawashiro et al., 1997). This protection is correlated to a decrease in CD11b immunoreactivity, suggesting a decrease in the inflammatory response to ischemia in the setting of preconditioning. Consistent with this observation, TNF α pretreatment of astrocytes and endothelial cells, through its signaling intermediate ceramide, produces a state of hyporesponsiveness as pretreated cells fail to upregulate ICAM-1 during subsequent hypoxia (Ginis et al., 2002). The decrease in ICAM-1 does not reflect global cellular suppression, but instead signifies a reprogrammed genomic response to stroke, as the hypoxia-induced expression of cytoprotective MnSOD is not affected by preconditioning. Evidence for a reprogrammed genomic response to ischemia is supported by the observation that TNF α preconditioning prevents hypoxia-induced phosphorylation of the proinflammatory transcription factor component NF κ Bp65, thereby preventing its interaction with the transcriptional activator p300. Taken together, these data indicate that pretreatment with TNF α reprograms the cellular environment and hence alters inflammatory reactions in response to ischemia.

Just as TNF α can induce tolerance to subsequent ischemic exposure, it can induce tolerance to subsequent LPS exposure (Porter et al., 1998, Ferlito et al., 2001, Murphey and Traber, 2001). Hence TNF α preconditioning has the potential to induce a state of cross-tolerance to TLR ligands, and thereby reprogram the TLR response to stroke. IFN β has been shown to cause many of the effects observed in TNF α -induced ischemic tolerance, such as suppression of inflammatory cytokine production, including TNF α itself, and reduction of cellular infiltration into ischemic brain regions (Veldhuis et al., 2003a).

Together, these studies suggest that multiple preconditioning stimuli may cause a reprogrammed TLR response to stroke. IFN β , produced secondary to this reprogrammed response, may aid in maintaining mitochondrial stability and in dampening the inflammatory responses to injurious ischemia.

Conclusion and Prospective

The studies described above demonstrate an emerging role for TLRs in ischemic damage and in ischemic prophylaxis. The use of TLR ligands as ischemic prophylactic therapy provides a powerful new paradigm for stroke therapeutics, with the potential to improve the quality of life of thousands of high-risk patients each year.

Cardiovascular and cerebrovascular diseases are the leading causes of death and disability in the United States (Murray and Lopez, 1997, Yusuf et al., 2001a, b). In recent years, explosive progress in endovascular and surgical procedures has substantially improved the life expectancy and quality of life of patients with coronary-artery disease and atherosclerosis;

more than 4 million diagnostic and therapeutic cardiovascular procedures are performed each year in the USA (AHA Heart and Stroke Statistics: 2005 update). Unfortunately, many of these procedures result in significant periprocedural morbidity and mortality due to ischemic brain injury. Coronary artery bypass grafting (CABG) (Bucerius et al., 2003, McKhann et al., 2006), cardiac valve replacement (Bucerius et al., 2003), carotid endarterectomy (Bond et al., 2004), aortic repair (McKhann et al., 2006) and carotid stenting (Yadav et al., 2004) have all been shown to put patients at risk of acute ischemic brain lesions. Prophylactic neuroprotection of these high-risk patient populations has the potential to protect patients from devastating neurological complications and even death. By setting the stage for improved ischemic outcome, should an ischemic event occur, TLR reprogramming offers a low-risk, high-benefit opportunity to combat brain injury in the event of cerebral ischemia.

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Toll-like receptor signaling

Figure 1.
Toll-like receptor signaling

Cerebral ischemia induces Toll-like receptor signaling

Figure 2.
Cerebral ischemia induces Toll-like receptor signaling

Preconditioning up-regulates inhibitors of TLR proinflammatory pathways

Figure 3.
Preconditioning up-regulates inhibitors of TLR proinflammatory pathways

Preconditioning reprograms TLR signaling following cerebral ischemia

Figure 4.
Preconditioning reprograms TLR signaling following cerebral ischemia

Table 1

Endogenous Toll-like receptor ligands

TLRs	Endogenous Ligand	Ligand Source	Response	Citation
TLRs 2 and 4	Hsp60	Necrotic cells	TNF-alpha and NO in macrophages	Ohashi K, et al. J Immunol. 2000; 164(2): 558–61. Vabulas RM, et al. J Biol Chem. 2001; 276(33): 31332–9. Lenhardt S, et al. J Neurosci. 2008; 28(10): 2320–31
	Hsp70	Necrotic cells	IL-12 and ELAM-1 (endothelial cell-leukocyte adhesion molecule-1) in macrophages	Asea A, et al. J Biol Chem. 2002; 277(17): 15028–34. Vabulas RM, et al. J Biol Chem. 2002; 277(17): 15107–12.
	gp96	Necrotic cells	IL-12 in DCs	Vabulas RM, et al. J Biol Chem. 2002; 277(23): 2084–53.
	High mobility group box 1 (HMGB1)	Released from nucleus in inflammation	Lethality in sepsis reperfusion injury	Parks JS, et al. J Biol Chem. 2004; 279(9): 7370–7.
	Urate crystal	Deposition in joints and bursal tissues in gout	TNF-alpha, IL-1beta, TGF-beta1 in macrophages	Liu-Bryan R, et al. Arthritis Rheum. 2005; 52(9): 2934–46.
	Biglycan	Released from ECM	TNF-alpha, MIP in macrophages/DCs	Schaefer L, et al. J Clin Invest. 2005; 115(8): 2223–33.
	Hyaluronan	Degraded from larger species in ECM	Chemokine production in macrophages, activation of DCs via TLR4	Jiang D, et al. Nat Med. 2005; 11(11): 1173–9. Termeer C, et al. J Exp Med. 2002; 195(1): 99–111.
TLR 3	mRNA when complexed with lipofectin	Necrotic cells	Activation and TNF-alpha production by human DCs	Kariko K, et al. J Biol Chem. 2004; 279(13): 12542–50.
TLR 4	Hsp22 (HspB8)	Synovial fluid in rheumatoid arthritis	IL-6, TNF-alpha, upregulation of co-stimulatory molecules in DCs	Roelofs MF, et al. J Immunol. 2006; 176(11): 7021–7.
	Fibronectin extradomain A	Tissue damage	MMP-9 in human macrophages	Okamura Y, et al. J Biol Chem. 2001; 276(13): 10229–33.
	Surfactant protein-A	Lung surfactant	TNF-alpha and IL-10 in macrophages	Guillot L, et al. J Immunol. 2002; 168(12): 5989–92.
	Fibrinogen	Extravated from vasculature in response to endothelial cell retraction	Chemokine production in macrophages	Smiley ST, et al. J Immunol. 2001; 167(5): 2887–94.

TLRs	Endogenous Ligand	Ligand Source	Response	Citation
	Heparin sulfate	Released from ECM, cell membranes	Maturation and up-regulation of co-stimulator molecules in DCs	Johnson GB, et al. J Immunol. 2002; 168(10): 5233–9.
	Beta-defensin 2- fusion protein (rmDF-2/rfv)	Epithelial antibacterial peptides	Maturation and up-regulation of co-stimulator molecules in DCs	Birgyn A, et al. Science. 2002; 298(5595): 1025–9.
	Mimimally modified (oxidized) low density lipoprotein	Pro-inflammatory and pro-atherogenic protein	Actin polymerization and spreading of macrophages	Miller YI, et al. J Biol Chem. 2003; 278(3): 1561–8.
	Pancreatic elastase	Pancreatic elastase	TNF-alpha secretion in THP-1 cells	Hietaranta A, et al. Biochem Biophys Res Commun. 2004; 323(1): 192–6.
	Alpha-A crystallin	Necrotic cells	Activation of DCs	Roelofs MF, et al. J Immunol. 2006; 176(11): 7021–7.
TLR 7	RNA immune complex	Necrotic cells	IFN-alpha production by PDCs	Barrat FJ, et al. J Exp Med. 2005; 202(8): 1131–9.
TLR 7 and 8	siRNAs when encapsulated into liposomes	Necrotic cells	Induction of TNF-alpha and IL-6 in PBMCs	Sioud M. J Mol Biol. 2005; 348 (5): 1079–90.
TLR 9	Chromatin immune complex	Necrotic cells	DC activation	Boule MW, et al. J Exp Med. 2004; 199(12): 1631–40.
	DNA immune complex	Necrotic cells	IFN-alpha production by PDCs	Barrat FJ, et al. J Exp Med. 2005; 202(8): 1131–9.

Table 2
The endogenous role of Toll-like receptors in cerebral ischemia

TLR knock-out	Stroke model	Animal model	Outcome	Proposed mechanism	Citation
TLR 2	MCAO via filament, 60 min, 48h reperfusion	mouse, C57BL/6J, male, 10-12 week, TLR 2 -/-	decreased infarct in TLR2 -/-	TLR 2 signaling	Ziegler G, et al. Biochem Biophys Res Commun. 2007; 359(3): 574-9.
	MCAO via filament, 60min, 24 and 72h reperfusion	mouse, C57BL/6J, male, 13-15 week, TLR 2 -/-	decreased infarct in TLR2 -/-	TLR 2 in microglia	Lehnardt S, et al. J Neuroimmunol. 2007; 190(1-2): 28-33.
	MCAO via filament, 60 min, 72h reperfusion	mouse, TLR 2 -/-	decreased infarct in TLR2 -/-		Tang SC, et al. Proc Nat Acad Sci USA. 2007; 104(34):13798-803.
TLR 4	permanent MCAO via electrocoagulation, 24h and 7d reperfusion	mouse, male, adult, TLR 4 -/- (C3H/HeJ and C57BL/10ScCr)	decreased infarct in TLR4 -/-	decreased inflammatory response, i.e. MMP9	Caso JR, et al. Circulation. 2007; 115(12): 1599-608.
	MCAO, 6h, embolism, 24h reperfusion	mouse, C3H/HeJ, female, 8 week, TLR 4 -/-	decreased infarct in TLR4 -/-	decreased inflammatory cytokines i.e. TNF- alpha, IL-6	Cao CX, et al. Biochem Biophys Res Commun. 2007; 353(2):509-14.
	MCAO via filament, 60 min, 72h reperfusion	mouse, TLR 4 -/-	decreased infarct in TLR4 -/-		Tang SC, et al. Proc Nat Acad Sci USA. 2007; 104(34):13798-803.
	Global cerebral ischemia/reperfusion via occlusion of CCA, LSA and RSA, 12 min, 6h reperfusion	mouse, C57BL/10ScCr, male, 8-12 week, TLR 4 -/-	decreased infarct in TLR4 -/-	decrease phospho-IkB and NFkB	Hua F, et al. J Neuroimmunol. 2007; 190(1-2): 101-11.
	permanent MCAO via cauterization, 24h reperfusion	mouse, male, adult, TLR 4 -/- (C3H/HeJ and C57BL/HeN)	decreased infarct in TLR4 -/-, decreased subacute stress response	decreased iNOS and COX-2 in response to immobilization stress	Caso JR, et al. Stroke 2008; 39 (4): 1314-20.

Table 3
Toll-like receptor ligands precondition against cerebral ischemia

Ligand (TLR)	Treatment	Stroke model	Animal model	Outcome	Proposed mechanism	Citation
LPS (TLR 4)	0.9 mg/kg, IV, 1-7d prior	permanent MCAO via electrocoagulation, 24h reperfusion	Rat, adult, male, spontaneously hypertensive	Reduced infarct in LPS preconditioned	IL-1, TNF-alpha	Tasaki K, et al. Brain Res. 1997; 748(1-2): 267-70.
	0.9 mg/kg, IV, 72h prior	permanent MCAO via electrocoagulation, 24h reperfusion	Rat, adult, male, spontaneously hypertensive	Reduced infarct in LPS preconditioned	Maintenance of microvascular patency	Dawson DA, et al. J Cereb Blood Flow Metab. 1999; 19(6): 616-23.
	0.05 mg/kg, SC, 24h prior	transient MCAO, 6 and 24h reperfusion	Rat, adult, male	Reduced infarct in LPS preconditioned	protection despite increased PMN infiltration	Ahmed SH, et al. Stroke 2000; 31(1):193-9.
	0.5 mg/kg, IP, 72h prior	transient MCAO, 24h reperfusion	Rat, adult, male	Reduced infarct in LPS preconditioned	protein synthesis and inflammation dependent	Bordet R, et al. J Cereb Blood Flow Metab. 2000; 20(8):1190-6.
	0.3 mg/kg, IP, 72h prior	transient MCAO, 24h reperfusion	Rat, adult, male	Reduced infarct in LPS preconditioned	maintenance of endothelial and smooth muscle relaxation	Bastide M, et al. J Cereb Blood Flow Metab. 2003;23(4):399-405.
	0.2 mg/kg, IP, 48h prior	MCAO via filament, 60 min, 48h reperfusion	C57BL/6 male mice	Reduced infarct in LPS preconditioned	suppression of cellular inflammation	Rosenzweig HL, et al. Stroke. 2004; 35(11): 2576-81.
	0.9 mg/kg, IV, 72h prior	focal cerebral ischemia, 6h, 24h, 7d and 14d reperfusion	Rat, spontaneously hypertensive	Reduced infarct in LPS preconditioned	preservation of local cerebral blood flow, upregulation of eNOS	Furuya K, et al. J Neurosurg. 2005; 103(4): 715-23.
	0.2 mg/kg, IP, 72h prior	MCAO via filament, 60 min, 24h reperfusion	C57BL/6 male mice	Reduced infarct in LPS preconditioned	suppression of TNF-alpha signaling	Rosenzweig HL, et al. J Cereb Blood Flow Metab. 2007; 27(10): 1663-74.
	0.02 mg/kg, IV, 72h prior	Deep hypothermic circulatory arrest	neonatal piglets	Reduced infarct in LPS preconditioned	upregulation of inflammation inhibitors	Hickey EJ et al. J Thorac Cardiovasc Surg. 2007; 133(6):1588-96.
	0.5 mg/kg, IP, 24h prior	MCAO via filament, 25min, 72h reperfusion	C57BL/6 male mice	Reduced infarct in LPS preconditioned	iNOS-induced preservation of neurovascular function and CBF	Kunz A, et al. J Neurosci. 2007; 27(27): 7083-93.
CpG (TLR 9)	20 ug, IP, 72h prior	MCAO via filament, 60 min, 24h reperfusion	C57BL/6 male mice	Reduced infarct in CpG preconditioned	Reprogramming of TLR9 signalling pathway	Stevens SL, et al. J Cereb Blood Flow Metab. 2008; 28(5): 1040-7.