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Targeting HMGB1/TLR4 signaling as a novel approach to treatment of cerebral ischemia

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1. ABSTRACT

HMGB1 is a ubiquitous, highly conserved DNA-binding protein with well-established functions in the maintenance of nuclear homeostasis. Much of the recent work about its signaling functions in the brain has focused on its proinflammatory properties and relationship to known inflammatory receptors such as toll-like receptor 4 (TLR4). HMGB1 is massively released into the extracellular space immediately after ischemic insult and that it subsequently induces neuroinflammation in the postischemic brain, indicating that HMGB1 acts as a novel mediator in cerebral ischemic injury. Consistently, numerous reports point to TLR4 as a pivotal player in the ischemic brain. The use of HMGB1 and TLR4 ligand as preconditioning stimulus may be benefit of the outcome of cerebral ischemia. Therefore, this review presents the latest findings supporting the involvement of HMGB1 and TLR4 in cerebral ischemia. Targeting HMGB1/TLR4 signaling may provide a novel therapeutic approach for clinical prevention of cerebral ischemic injury.

2. INTRODUCTION

Stroke is the second most common cause of death worldwide. Despite extensive efforts, thrombolytic medications, like tissue plasminogen activator (tPA), remain the only available intervention proven to enhance functional recovery in humans once a stroke has occurred. Thrombolysis is safe and effective only within 3 hours of the onset of symptoms. This short time window results in a low treatment rate and safer treatment regimes that can be used over a wider time window are desperately needed. After stroke, pathology may spread as toxic factors from the core of the lesion pose a risk for cells in the penumbra. One such factor is cortical spreading depression, which has been shown to promote the enlargement of ischemic lesions (1). In addition, soluble mediators from the necrotic core area may diffuse to the adjacent tissue and elicit a delayed inflammatory response that contributes to neuronal cell death. Recent reports provide evidence that high mobility group box 1 (HMGB1) and its receptor, the toll-like

receptor-4 (TLR4), are pivotal mediators of cerebral ischemic inflammation and injury (2-9).

HMGB1 (also known as amphoterin) is a non-histone DNA-binding protein with high electrophoretic mobility (10, 11). HMGB1 participates in nucleosome formation and regulation of gene transcription (12). The protein binds to the minor groove of duplex DNA and induces conformational changes in chromatin architecture, thereby facilitating supramolecular nucleoprotein complex assembly and transcription factor recruitment (10, 12). In addition to its role in the regulation of nuclear homeostasis, HMGB1 has extracellular signaling functions through interactions with different receptors such as the receptor for advanced glycation end products (RAGE), TLR2, and TLR4 (13-18). Specifically, nuclear HMGB1 can be released into the extracellular space either passively from cells undergoing necrosis or actively from numerous cell types (19). When present in the extracellular milieu, it can activate the innate system and mediate a wide range of physiological and pathological responses (20).

Several studies highlight the role of HMGB1 in cerebral ischemic injury (5-9).HMGB-1 is released from neurons soon after ischemic injury and that it subsequently induces neuroinflammation in the post-ischemic brain (7, 9). For example, HMGB1 down-regulation mediated by short hairpin RNA (shRNA) reduces the severity of lesions in a stroke model (7). Notably, intravenous injection of anti-HMGB1 monoclonal antibody also causes a dramatic reduction in infarct size in stroke model (21, 22). Taken together, these findings indicate that HMGB1 is a proinflammatory cytokine-like factor that contributes to delayed inflammatory processes in the post-ischemic brain. However, how the receptors involved in HMGB1 signaling are activated and the relative contributions of the downstream molecules are still not understood.

TLR4 mediates many of the extracellular functions of HMGB1(14, 16, 23, 24). TLR4 is a signaling receptor known to activate the innate immune system in response to systemic bacterial infection and cerebral injury (25). Recently, TLR4 was shown to play a key role in ischemic brain injury (2-4). Notably, TLR4-deficient mice have smaller cerebral infarctions and a lower level inflammatory response after an experimental stroke, suggesting that TLR4 signaling is involved in brain damage (3). Interestingly, endogenous mediators that have been isolated after brain ischemia have been identified as ligands of TLR4, including HMGB1(26). This indicates that TLR4 may interact with HMGB1 and contribute to neuronal injury in cerebral ischemic brain. This review therefore summarizes the recent advances on the role of HMGB1 in the brain, the putative HMGB1/TLR4 signaling pathways, and the role of HMGB1/TLR4 in cerebral ischemic injury. Finally, the therapeutic potential of inhibitors of HMGB1/TLR4 signaling in the treatment of cerebral ischemia is discussed.

3. THE ROLE OF HMGB1 IN THE BRAIN

HMGB1 secretion is not fully understood. Under normal conditions, HMGB1 is localized in the cell nucleus and diffusely distributed in the cell cytoplasm (27). In the nucleus, it binds to DNA (19). Nuclear translocation of HMGB1 is controlled by nuclear localization signal sequences NLS and NLS2 (28). During challenge, HMGB1 becomes acetylated on lysine residues within the nuclear localization signals. Once hyper-acetylated, the NLS is masked and HMGB1 nuclear import is prevented, thereby leading to the cytoplasmic accumulation of the protein. The hyper-acetylated HMGB1 then appears to be loaded into secretory lysosomes; the ultrastructural features of HMGB1-containing organelles suggest that they are secretory lysosomes (28, 29). The fusion of these secretory lysosomes with the plasma membrane liberates HMGB1 into the extracellular environment (30).

HMGB1 has been characterized as a proinflammatory cytokine (15,31). It is secreted by activated macrophages, natural killer cells, and myeloid dendritic cells in response to inflammatory stimuli (15,28,32,33). Recently, several studies have highlighted the proinflammatory characteristics of HMGB1 in the central nervous system (CNS). Intracerebrocentricular administration of HMGB1 increases levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6 in brain and induces fever, anorexia, taste aversion, and weight loss in mice (34, 35). In fact, HMGB1 is found in the nuclei of neurons, astrocytes, and pituicytes in the brain (5, 9, 36). Neurons are one of the principal sources of HMGB-1 release during ischemic injury (9).Studies on rodent brain development suggest that HMGB1 regulates neuronal migration and sprouting (37). In the developing rat brain, HMGB1 localizes to the advancing plasma membrane of filopodia at the leading edges in motile cells, which in turn promotes the outgrowth of neuritis (38, 39). HMGB1 is differentially expressed in the mouse brain at various steps of development and tends to be down-regulated in the adult animal (40). These results taken together, point to an important role of HMGB1 in CNS function.

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4. TLR4 AS A KEY RECEPTOR FOR HMGB1 IN THE BRAIN

HMGB1 appears to signal through multiple receptors such as RAGE, TLR2 and TLR4, and presumably other unknown receptors (41). TLR4 mediates many of the extracellular functions of HMGB1 (14, 16, 23, 24). The extent of TLR4 expression in brain cells has been controversial. A number of studies have demonstrated that TLR4 is mainly expressed on glial cell populations, including microglia, astrocytes, and oligodendrocytes, but not on neurons (42-44). However, several findings suggest that neurons may express at least some TLRs responsive to viral RNA or bacterial proteins (45, 46). During the past four years, evidence for the neuronal expression of TLRs has increased, suggesting a role for this receptor family in neurons during physiological as well as pathological conditions (47, 48). Furthermore, recent expression studies confirm that cerebral ischemia results in the up-regulation of mRNAs for TLR2, TLR4, and TLR9 in the mouse neurons (47-49). Notably, microglial cells expresses all known TLRs. In fact, microglia have been reported to express mRNAs for TLRs 1 through 9, whereas neurons and oligodendrocytes express only mRNA for TLR3 (50,51,52). However, further confirmation using single-cell polymerase chain reaction will be required to exclude contaminating microglia as a source of TLR mRNA. Furthermore, the expression of TLR4 at the protein level remains to be investigated in the brain cells.

TLR4 predominantly recognizes lipopolysaccharide (LPS) from Gram-negative bacteria (53). A number of endogenous proteins, including HMGB1, heat shock proteins 60 and 70, oxidized low-density lipoprotein, surfactant protein A, fibronectin, and defensin, bind to and stimulate TLR4 (54). However, the only molecule known to stimulate TLR4 signaling that has been definitively shown to be involved in ischemic injury is HMGB1 (55). HMGB1 release from cultured hepatocytes was found to be an active process regulated by reactive oxygen species (ROS). Importantly, optimal production of ROS and subsequent HMGB1 release by hypoxic hepatocytes requires intact TLR4 signaling (55). Thus, TLR4 participates not only in the recognition of HMGB1 but also in its release through a mechanism involving ROS production, providing a mechanism by which ischemic tissues notify the immune system of the potential loss of tissue integrity. To date, it is not known whether HMGB1 involvement in cerebral ischemic injury is dependent on TLR4 signaling, however, the interaction of TLR4 and HMGB1 seems certain in the cerebral ischemic process. One of our ongoing studies has demonstrated that the release of HMGB1 by necrotic neurons which triggers the activation of microglia is TLR4 dependent (unpublished data). Moreover, in a mouse model of cerebral ischemia/reperfusion (I/R) injury, TLR4-deficient mice have a lower inflammatory response and less extensive I/R injury after administration with HMGB1 that untreated mice (unpublished data). Further, a recent study showed that RAGE mediates ischemic brain injury induced by HMGB1 (8). These results taken together, point to a key role of HMGB1 in triggering inflammation and injury following brain ischemia.

5. HMGB1/TLR4 SIGNALING PATHWAYS

HMGB1/TLR4 signaling results in the activation and nuclear translocation of nuclear factor-κB (NF-κB), which induces the up-regulation of proinflammatory cytokines, thereby promoting inflammation (56, 57). TLR4 can signal via myeloid differentiation primary response protein 88 (MyD88), IL-1R associated kinase 1 (IRAK), and TNF-receptor associated factor (TRAF) to NF-κB, as well as *via* Rac1 and phosphoinositide 3-kinase (PI3K), and possibly *via* Akt, Erk1/2 and p38 mitogen-activated protein kinases (MAPK) as shown schematically in Figure 1 (56).

MyD88 and Toll/IL-1 receptor-domain containing adaptor protein (TIRAP) are associated with TLR4 after receptor activation by HMGB1(16,57). MyD88 recruits members of the IRAK family which activate the downstream TRAF6 kinase. Dominant negative (dn) MyD88, dnTIRAP, dnIRAK1, and dnIRAK2 inhibit HMGB1-induced NF-κB reporter gene activity, suggesting that these proteins are key players in the TLR4 signaling cascade (16). IRAK1 activates TRAF6, which results in the formation of a complex with transforming growth factor β (TGF-β) activated kinase 1 (TAK1) and TAK1 binding protein 2 (TAB2). However, though expression of dnTRAF6 clearly inhibits the NF-κB reporter gene expression after stimulation of HMGB1, neither the expression of dnTAB2 nor dnTAK1 inhibit NF-κB activation stimulated by HMGB1. Thus, TAB2 and TAK1 are not important in HMGB1 signaling through TLR4 (16).

TLR4 signaling may be either MyD88 dependent or independent (Figure 1) (58). Nevertheless, the most prominent effect of TLR activation is the induction of NF-κB-dependent gene expression. The NF-κB family of transcription factors contains five members that function as hetero- or homodimers. The dimers are sequestered in the cytoplasm in an inactive form by inhibitor of NF-κB (IκB). NF-κB is translocated to the nucleus when IκB is phosphorylated and subsequently degraded. The IκB kinase

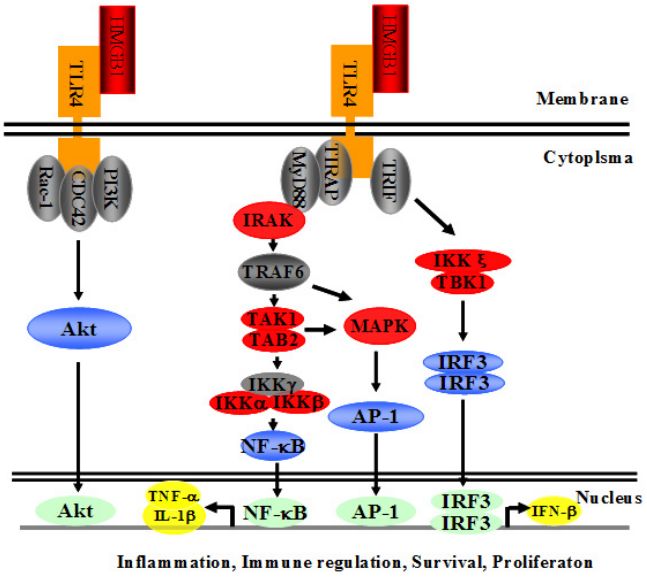

Figure 1. HMGB1/TLR4 signaling pathways. HMGB1 binds TLR4 and signals through adaptor molecules via the Toll/IL-1 receptor-domain. TLR4 can then signal via MyD88, IRAK, and TRAF to NF-κB, as well as *via* Rac1, PI3K, and CDC42, and possibly *via* Akt and MAPKs. The signaling cascades that lead to release of NF-κB from the inhibitory IKK complex, consisting of IKKα, IKKβ, and IKKγ, are the main activators of NF-κB. After activation, NF-κB translocates into the nucleus and induces the transcription of proinflammatory immune mediators, such as TNF-α and IL-1β. TLR signaling is also mediated through another adaptor, TIR domain-containing adaptor protein (TRIF), which activates TANK-binding kinase1 (TBK1) and IKKξ, leads to the activation of IFN regulatory factor 3 (IRF3), and thereby activates interferon-β (IFN-β) and IFN-β-inducible genes.

(IKK) complex, consisting of IKKα, IKKβ, and IKKγ, is the main activator of NF-κB and is itself activated by diverse upstream signals (Figure 1) (59).

How activation of TLR4 mediates the phenotypic effects of HMGB1 is still unclear. HMGB1 may directly interact with TLR4, causing cell activation and the NF-κB-dependent transcription of proinflammatory cytokines (14). HMGB1 also binds to cytokines, in particular IL-1β (60). HMGB1 also catalytically disaggregates LPS and transfers it to soluble CD14 and to leukocytes (61). This enhanced access sustains a positive feedback amplificatory loop, facilitating TLR4-mediated inflammatory responses (61). Interestingly, in a recent study, we observed cerebral I/R injury in MyD88 knockout mice (4). Compared with wild-type mice, 6 hours of cerebral ischemia followed by 24 hours of reperfusion did not significantly change the cerebral infarction area and neurologic impairment scores in MyD88 knockout mice, suggesting that TLR4 may contribute to cerebral I/R injury through an MyD88-independent signal pathway (4). Moreover, a most recent study indicates that the TIR domain-containing adaptor protein(TRIF)-dependent signaling pathway is not required for cerebral I/R injury in mice (62).Therefore, TLR4-mediated cerebral ischemia injury may be either MyD88 or TRIF independent.

6. ROLE OF HMGB1 IN CEREBRAL ISCHEMIA

Very recently, HMGB1 has been implicated in the mechanism of ischemic brain damage (5-9, 21, 63). In patients with ischemic stroke, the serum or plasma levels of HMGB1 are dramatically higher than those in controls (8, 63). In an animal model of ischemic stroke, the serum levels of HMGB1 are increased 4 hours after ischemia and HMGB1 is released into the extracellular space immediately after ischemic insult where it subsequently induces the release of inflammatory mediators in the post-ischemic brain (6, 7). The relocation dynamics of HMGB1 in the neuronal cells are intriguing: HMGB1 is initially expressed in the nucleus of neurons and astrocytes of the mouse brain. It predominantly translocates into the cytoplasm of neurons within the ischemic brain and then rapidly disappears from neurons (5,6,7,9). HMGB1 is translocated from neuronal nuclei to the cytoplasm and subsequently depleted from neurons 1 hour after middle cerebral artery occlusion (MCAO) (6,9). These data indicate that HMGB1 is an early inflammatory mediator that contributes to the initial stages of the inflammatory response in the ischemic brain.

HMGB1 proinflammatory signaling appears to begin in the first hour after focal ischemia and may continue for 24 hours thereafter (5). HMGB1 is localized in soma and processes of ischemic neurons but levels are rapidly (after 1-3 hours) reduced in ischemic brain tissue and remain inhibited up to 24 hour after ischemia (5). These findings, together with the evidence that HMGB1 levels increase in the cerebrospinal fluid and serum after brain ischemia in the rat, suggest that the protein translocates from the nuclei of ischemic neurons to the extracellular space and then to the liquor (7). Importantly, this may provide a wide window for therapeutic intervention when HMGB1 is targeted.

Table 1. Targeting HMGB1/TLR-4 signaling in cerebral ischemia

Strategy	Actions and effects	References
Anti-HMGB1 antibodies	Neutralizing of HMGB1; HMGB1 MAb or pAb treatment ameliorates brain infarction in rats.	8,21,22
HMGB1 box A	Antagonist of HMGB1; Box A ameliorates ischemia brain damage in rats.	8
LPS preconditioning	TLR-4 agonist; LPS-induced tolerance to brain ischemia in mouse models of stroke.	66,69,80
Minocycline	A tetracycline antibiotic, inhibits activated microglia expressing HMGB1; Minocycline decreases neurologic impairment induced by cerebral ischemia in mice.	85
Edaravone	A free radical scavenger, attenuates the release of HMGB1; Edaravone rescues rats from cerebral infarction.	86
Cannabidiol	A psychoactive cannabinoid, attenuates the release of HMGB1; Cannabidiol ameliorates brain infarction in mice.	87

Indeed, down-regulation of HMGB1 in brain using shRNA correlates with diminished infarct volumes in the rat (7). Inhibition of HMGB1 expression by shRNA reduced ischemia-dependent microglia activation and induced TNF-α and IL-1β expression in the ischemic brain (7). Moreover, HMGB1 promotes microglia activation (5,7). Therefore, evidence that inflammation plays an active role of in the pathogenesis of ischemic brain injury, along with the cytokine functions of HMGB1 suggest that the detrimental effect of the protein during brain ischemia is due, at least in part, to its capability to promote neuroinflammation.

7. ROLE OF TLR4 IN CEREBRAL ISCHEMIA

TLR4 plays a key role in the innate immunity of the CNS (43). Numerous studies demonstrate that TLR4 participates in cerebral injury upon ischemic stroke. Several expression studies confirm that cerebral ischemia results in the up-regulation of TLR4 mRNA in neurons as early as 1 hour after initiation of ischemia *in vivo* (4, 47). Importantly, cortical neuronal cultures from TLR4-deficient mice show increased survival after glucose deprivation (47). Mice lacking TLR4 exhibit reduced infarct size compared with wild-type mice after cerebral ischemia injury (2, 3, 47, 64, 65). TLR4 mutant mice subjected to MCAO or animals suffering global cerebral ischemia exhibit improved neurological behavior and reduced edema, as well as reduced levels of secretion of proinflammatory cytokines such as TNF-α and IL-6 (2, 3, 65). In addition, mice lacking TLR4 have reduced expression of inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX2), and interferon γ (3, 65). Likewise, a TLR4 mutation confers protection against MCAO (64). Moreover, after MCAO, loss of TLR4 function is associated with reduced expression of p38 and Erk1/2 in damaged neurons, implicating TLR4 in MCAO injury (2, 3, 47, 64). Taken together, these studies indicate that TLR4 signaling modulates the severity of ischemia-induced neuronal damage and suggests TLR4 should be a target of stroke therapy.

Interestingly, several lines of evidence suggest that TLR4 might also be involved in the preconditioning-induced protective effect against ischemic brain injury (66, 67). First, TLR4 signaling activates nuclear NF-κB and induces expression of mediators such as iNOS and COX-2, which participate in ischemic tolerance (68). Second, preconditioning with LPS, a TLR4 ligand, produces ischemic tolerance through a TNF-α-dependent process (69). TLR4-mediated activation of NF-κB leads to the expression of TNF-α, iNOS, and COX-2 and induces neuroprotective effects (66). Therefore, as ischemic preconditioning activates endogenous signaling pathways that culminate in marked protection against ischemic brain damage, drugs that stimulate TLR4 might protect against cerebral ischemic injury.

However, several basic questions still need to be answered before the involvement of TLR4 in cerebral ischemia injury is completely clarified. Thus far, studies on TLRs in ischemic brain stroke have mainly focused on ischemic damage in TLR4 mutant mice and, to a lesser extent, TLR2 mutant mice. Although this approach has provided some understanding of the relevance of TLR signaling in ischemic brains, it has not enabled an understanding of the role of TLR signaling in specific cell types. This issue is of high importance because of the involvement of many different cells in the pathology of ischemic brain injury, including for example, neurons, astrocytes, microglial, endothelial cells, and invading immune cells.

8. HMGB1/TLR4-TARGETED THERAPIES IN CEREBRAL ISCHEMIA

HMGB1 has been demonstrated to be a significant contributor to ischemia injury through a TLR4-dependent mechanism (55). Importantly, although HMGB-1 clearly contributes to the early stages of inflammation in brain ischemia, it may also contribute to later stages of inflammation by activating microglia (7). Therefore, HMGB-1 may exert multiple proinflammatory effects in the ischemic brain, which means that the time frame for clinical intervention against cerebral ischemic injury would be extended relative to the 3-hour window for thrombolytic therapy. Similarly, the use of TLR4 ligands as ischemic prophylactic therapy provides a powerful new paradigm that should be explored for stroke therapy (Figure 2 and Table.1).

8.1. HMGB1 as a target of therapy in cerebral ischemia

HMGB1 has proven to be an excellent therapeutic target in experimental models of infectious and inflammatory disorders, including sepsis, trauma, cancer, and rheumatoid arthritis (70-72). The concept of counteracting HMGB1 in the context of cerebral ischemic injury has so far been explored with strategies based on HMGB1 neutralization, inhibition of HMGB1 synthesis, and prevention of extracellular HMGB1 release. Therapeutic agents to treat cerebral ischemia may include anti-HMGB1 antibodies, HMGB1 A box protein, and HMGB1 preconditioning.

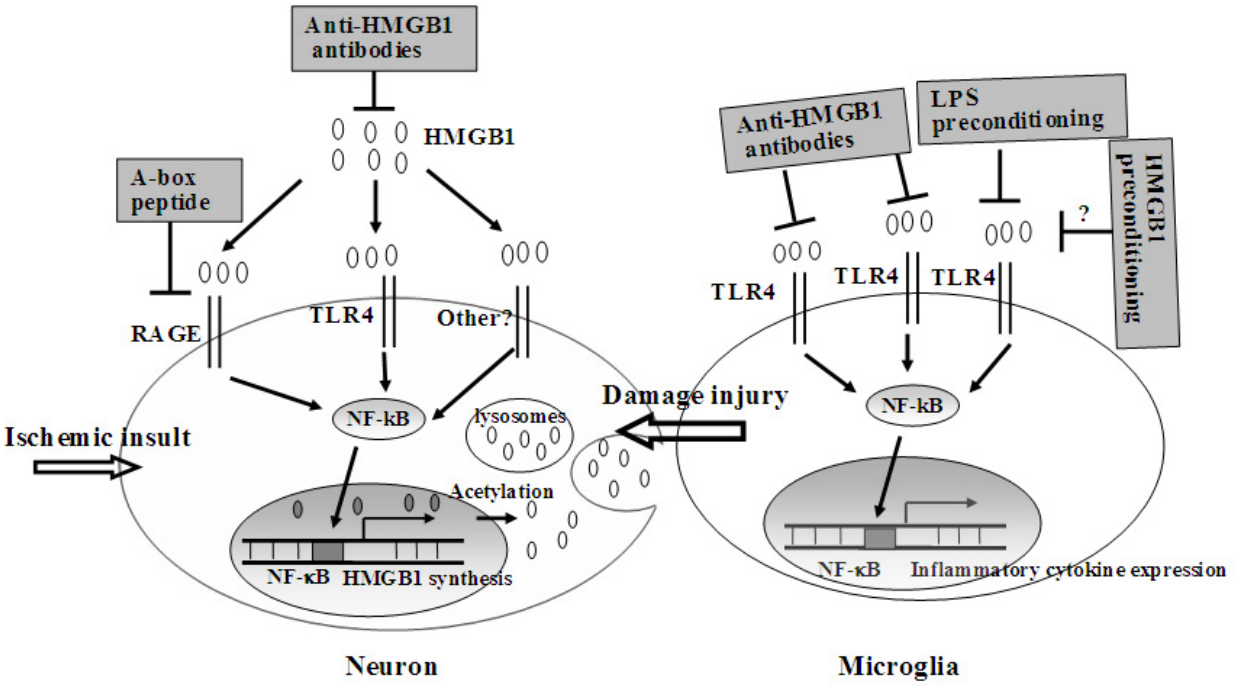

Figure 2. Schematic representation of therapeutic strategies of HMGB1/TLR4 signaling for cerebral ischemia. Ischemic insult induces HMGB1 synthesis and acetylation in nucleus of neuronal cells and then HMGB1 is released into the extracellular space, thereby allowing its interactions with RAGE and TLR2/4 or other unknown receptors in neuronal cells and in microglia. The common signaling pathway involves activation of NF-κB to induce expression of HMGB1 and inflammatory mediators, which ultimately results in neuronal injury in cerebral ischemia. HMGB1 and TLR4 may be therapeutic targets for the treatment of cerebral ischemia. Therapeutic strategies include anti-HMGB1 antibodies, HMGB1 A box protein, and LPS preconditioning. LPS preconditioned microglial cells do not recruit MyD88 to TLR4 and fail to activate IRAK-1 and NF-κB, and importantly, do not express NF-κB-dependent inflammatory genes (87). However, how HMGB1 preconditioning protects from ischemic injury remains unkown. ,"actively secreted" acetylated HMGB1; , "passively released" HMGB1.

The HMGB1 mAb that has been evaluated in a rat ischemic model is directed to the end of the repetitive C-terminal tail of HMGB1 (21). Mori *et al.* reported that treatment with HMGB1 mAb remarkably ameliorated brain infarction in rats, even when the mAb was administered after the start of reperfusion (22). Furthermore, the accompanying neurological deficits in locomotor function were significantly improved (22). Additionally, some biochemical markers such as the expression of TNF-α, iNOS and matrix metalloproteinase-9 were altered by mAb injection (22). Similarly, intervention with neutralizing polyclonal anti-HMGB1 antibodies can prevent the progression of disease due to established stoke in rodents (8). Neutralizing strategies are reported to specifically inhibit extracellular HMGB1 activity rather than prevent its secretion, therefore, current efforts are focused on developing anti-inflammatory strategies that inhibit HMGB1 secretion (32).

HMGB1 box A is a functional antagonist of the proinflammatory effects mediated by full-length HMGB1 protein through competitive bind to RAGE (73). HMGB1 box A has been used as a pharmacological tool to inhibit the effects of HMGB1 that are mediated through RAGE (73, 74). HMGB1 box A protein therapy confers significant clinical protection in experimental inflammatory conditions, including lethal endotoxemia and arthritis (73, 75). In a study of stroke, systemic administration of HMGB1 box A protein significantly ameliorated ischemic brain injury (8). Thus, there may be therapeutic potential in using genetically engineered peptides to decrease inflammation.

Recently, the use of HMGB1 as a preconditioning stimulus has been explored (76). Exogenous HMGB1, given as a pharmacologic pretreatment, has been shown to protect against hepatic ischemia injury (77). Mice were pretreated with a single HMGB1 injection ranging from 2 to 20 μg, administered systemically 1 hour prior to ischemia of the liver. Protection was demonstrated by decreased levels of circulating alanine transaminase. In addition, levels of circulating TNF-α and IL-6 were also decreased (77). The study using HMGB1 as a preconditioning agent also investigated the role of TLR4 in preconditioning (77).The pretreatment with HMGB1 did not confer any additional protection in TLR4 mutant mice compared to wild-type mice (77). Therefore, the mechanism of HMGB1 preconditioning is a TLR4-dependent phenomenon. Taken these together indicate HMGB1 preconditioning can be

protective in ischemia. However, to date, the use of HMGB1 as a preconditioning pharmacologic treatment in cerebral ischemic injury has not been explored.

8.2. TLR4 as a target of therapy in cerebral ischemia

A number of studies have demonstrated that TLR4 is expressed on all brain cells, including microglia, astrocytes, oligodendrocytes, and neurons (42-44, 47, 48). In cerebral ischemia, TLR4 is significantly up-regulated and may modulate the severity of neuronal damage, suggesting TLR4 may be a target of stroke therapy (48). Our previous study demonstrated that systematic administration of TLR-4 mAb remarkably ameliorated cerebral infarction and edema in cerebral I/R mice. Moreover, treatment with TLR-4 mAb significantly improved the neurological deficits and the expressions of inflammatory mediators as TNF-α, IL-6, IL-1 and MMP-9 were dramatically reduced (78).

TLR4-induced tolerance to cerebral ischemia was first demonstrated with low dose systemic administration of LPS, which causes spontaneously hypertensive rats to become tolerant to subsequent ischemic brain damage induced by MCAO (79). Since then, LPS-induced tolerance to brain ischemia has been demonstrated in mouse models of stroke (66,69, 80).Neuroprotection induced by LPS is time and dose dependent. Tolerance appears by 24 hour after LPS administration and extends through 7 days but is gone by 14 days (69). Protective doses of LPS appear to depend on the model and the dose.(69, 81, 82). Tolerance induction has been shown to require new protein synthesis and a modest inflammatory response (83). Specifically, TNF-α has been implicated as a mediator of LPS-induced ischemic tolerance because inhibition of TNF-α systemically or within the brain blocks neuroprotective effect and mice lacking TNF-α are not protected by LPS preconditioning (66,69,79). Therefore, the use of LPS preconditioning may prove beneficial in cerebral ischemic injury.

9. PERSPECTIVE AND CONCLUSIONS

Considering that the release of HMGB1 takes place early after the cerebral ischemia, new therapies, like neutralizing antibodies or drugs with antagonist characteristics, that inhibit HMGB1 signaling should produce a neuroprotective effect. Indeed, a recent study demonstrates that minocycline, a semisynthetic tetracycline antibiotic, attenuates both oxygen-glucose deprivation-induced HMGB1 release and HMGB1-induced cell death (84). Moreover, administration of minocycline for 14 days inhibits activated microglia expressing HMGB1 and decreases neurologic impairment induced by cerebral ischemia (85). Intriguingly, edaravone, a free radical scavenger clinically used for the treatment of cerebral infarction, was recently reported to rescue rats from cerebral infarction by attenuating the release of HMGB1 in neuronal cells (86). Another novel clinical medicine, cannabidiol, decreases both cerebral infarction and HMGB1 levels in the early ischemic phase (87).Together, these findings open new therapeutic possibilities for treatment of cerebral ischemic injury via an HMGB1-inhibiting mechanism.

A growing body of evidence points to a potential novel and pivotal role for HMGB-1 in the TLR4 signaling pathway that links the very early stage responses to cerebral ischemic injury with the activation of local neuroinflammatory responses. Likely, efficient inhibition of HMGB1/TLR-4 signaling will ameliorate inflammatory injury in cerebral ischemia. However, the complexity of inflammatory signaling in cerebral ischemic injury raises the question whether targeting HMGB-1 or TLR4 alone will be effective. Moreover, the mechanism by which HMGB1 exerts its proinflammatory cytokine-like effects in the cerebral ischemia is still unknown. Additionally, the therapeutic window of time during which treatments targeting HMGB-1 or TLR4 must be initiated has not yet been determined.

To summarize, HMGB1 and TLR4 play important roles in cerebral ischemic injury and in ischemic prophylaxis. Potential drugs candidates might target the HMGB1 signaling pathway at different levels in order to prevent HMGB1 release and inhibit HMGB1 binding to its receptors. HMGB1 preconditioning may also be a useful therapeutic strategy. The use of TLR4 ligands as ischemic prophylactic therapy also provides powerful new paradigm that should be explored for stroke therapy.

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