Cytosolic Receptor Melanoma Differentiation–Associated Protein 5 Mediates Preconditioning-Induced Neuroprotection Against Cerebral Ischemic Injury

Raffaella Gesuete, PhD; Sara N. Christensen, BS; Frances R. Bahjat, PhD; Amy E.B. Packard, PhD; Susan L. Stevens, BS; Mingyue Liu, PhD; Andres M. Salazar, PhD; Mary P. Stenzel-Poore, PhD

Background and Purpose—Preconditioning with poly-α-lysine and carboxymethylcellulose (ICLC) provides robust neuroprotection from cerebral ischemia in a mouse stroke model. However, the receptor that mediates neuroprotection is unknown. As a synthetic double-stranded RNA, poly-ICLC may bind endosomal Toll-like receptor 3 or one of the cytosolic retinoic acid–inducible gene I–like receptor family members, retinoic acid–inducible gene I, or melanoma differentiation–associated protein 5. Activation of these receptors culminates in type I interferons (IFN-α/β) induction—a response required for poly-ICLC–induced neuroprotection. In this study, we investigate the receptor required for poly-ICLC–induced neuroprotection.

Methods—Toll-like receptor 3, melanoma differentiation–associated protein 5–, and IFN-promoter stimulator 1–deficient mice were treated with poly-ICLC 24 hours before middle cerebral artery occlusion. Infarct volume was measured 24 hours after stroke to identify the receptor signaling pathways involved in protection. IFN-α/β induction was measured in plasma samples collected 6 hours after poly-ICLC treatment. IFN-β–deficient mice were used to test the requirement of IFN-β for poly-ICLC–induced neuroprotection. Mice were treated with recombinant IFN-α-A to test the role of IFN-α as a potential mediator of neuroprotection.

Results—Poly-ICLC induction of both neuroprotection and systemic IFN-α/β requires the cytosolic receptor melanoma differentiation–associated protein 5 and the adapter molecule IFN-promoter stimulator 1, whereas it is independent of Toll-like receptor 3. IFN-β is not required for poly-ICLC–induced neuroprotection. IFN-α treatment protects against stroke.

Conclusions—Poly-ICLC preconditioning is mediated by melanoma differentiation–associated protein 5 and its adaptor molecule IFN-promoter stimulator 1. This is the first evidence that a cytosolic receptor can mediate neuroprotection, providing a new target for the development of therapeutic agents to protect the brain from ischemic injury.

Key Words: interferons ■ ischemia ■ ischemia-reperfusion injury ■ poly ICLC ■ toll-like receptors

More than 1 million patients annually are at risk of brain ischemia and reperfusion injury that occurs secondary to life-saving endovascular or cardiac procedures. To date, no treatment is available to confer brain protection in this at-risk patient population. Prophylactic brain protection can be achieved through preconditioning, a phenomenon whereby brief exposure to a potential harmful stimulus induces protection against a subsequent injury. We have previously published that preconditioning with polyinosinic polycytidylic acid stabilized with poly-α-lysine and carboxymethylcellulose (poly-ICLC) protects against cerebral ischemic injury in a mouse model of stroke resulting in reduced ischemic injury and attenuation of stroke-induced neurological deficits. In addition, we have shown that protection is dependent on type I interferon (IFN) signaling. As a synthetic double-stranded RNA, poly-ICLC may induce type I IFN signaling through the binding and activation of multiple different pattern recognition receptor signaling cascades. Toll-like receptor 3 (TLR3), retinoic acid–inducible gene I (RIG-I), and melanoma differentiation–associated protein 5 (MDA5) have all been shown to bind double-stranded RNA, making them potential targets for poly-ICLC. Although TLR3 localizes to the endosome, RIG-I and MDA5 are found in the cytoplasm. Identifying the specific receptor and intracellular signaling pathway engaged by poly-ICLC is important for defining drug delivery strategies for future translational development.
In this study, we investigate the receptor-mediated signaling cascade required for poly-ICLC–induced neuroprotection against ischemic brain injury using TLR3−/−, MDA5−/−, and IFN-promoter stimulator (IPS)-1−/− mice. In addition, using IFN-β−/− deficient mice, we show that IFN-β is not involved in neuroprotection, and that exogenous administration of IFN-α protects against stroke, supporting a role for IFN-α in mediating poly-ICLC–induced protection.

**Materials and Methods**

**Mice**

C57Bl/6 (wild-type [WT]), TLR3−/− (TLR3−/−), and B6.Cg-Tg(MDA5-C)/J (MDA5−/−) mice were from Jackson Laboratories (West Sacramento, CA). IPS1−/− animals were provided by Dr Edith Janssen (University of Cincinnati, Cincinnati, OH). IFN-β−/− mice were from Dr Tomas Leanderson (Lund University). Studies were performed with male mice between 8 and 14 weeks of age. Mice were given free access to food and water and housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal protocols were approved by the Oregon Health and Science University Institutional Animal Care and Use Committee (OWLAW A3304-01) and met the guidelines set forth by the National Institutes of Health.

**Study Design and Blinding**

For each genotype (WT, TLR3−/−, MDA5−/−, IPS1−/−, and IFN-β−/−), mice were randomly given a subcutaneous injection of either vehicle (carboxymethyl cellulose; Sigma Aldrich) or poly-ICLC (Hiltonol; 60 µg per mouse; gifted from Oncovir, Washington, DC). For stroke studies, mice were treated 24 hours before ischemia. To measure systemic type I IFN induction, mice were treated 6 hours before blood collection. For studies involving IFN-α, mice were injected intraperitoneally with vehicle (saline) or recombinant IFN-α-A (1×104 U/mouse; PBL Biomedical Laboratories) 18 hours before the surgery. Surgery and analysis were performed by investigators blinded to treatment and genotype. A total of 73 WT, 22 TLR3−/−, 25 MDA5−/−, 25 IPS1−/−, and 8 IFN-β−/− mice have been used in these studies.

**Mouse Ischemia-Reperfusion Model**

Focal cerebral ischemia was induced by 60- or 45-minute middle cerebral artery occlusion (MCAO) as described previously. Mice that did not show >80% reduction in cerebral blood flow, monitored with laser Doppler flowmetry (Transonic System Inc.), were excluded. Twenty-four hours after MCAO, infarct volume was measured as previously described.

**Cytokine Quantification**

Plasma concentrations of IFN-α and IFN-β were determined using a Mouse IFN-α/IFN-β ProcartaPlex Multiplex Immunoassay (Affymetrix eBioscience) according to manufacturer instructions. Cytokine concentrations were extrapolated from the standard curve with samples below the standard curve assigned a value half that of the lowest standard.

**Statistical Analysis**

Data are represented as group mean±SEM and statistical analysis performed using GraphPad Prism6 software. Two-way ANOVA with Bonferroni post hoc test was used for infarct analysis and IFN-α/IFN-β measurements.

**Results**

**Poly-ICLC Requires MDA5, but Not TLR3, to Induce Neuroprotection**

Potential cognate receptors for poly-ICLC include TLR3, and the RIG-I–like receptor family members, RIG-I and MDA5 (Figure 1A). RIG-I and MDA5 recognize double-stranded RNA of different sizes, thus we postulated that poly-ICLC (≈500 kDa) would bind to MDA5, which recognizes larger double-stranded RNAs. To determine whether TLR3 or

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**Figure 1.** A, Schematic representation of the potential intracellular signaling pathways activated by poly-l-lysine and carboxymethylcellulose (ICLC). B, Poly-ICLC is dependent on melanoma differentiation–associated protein 5 (MDA5) and interferon-promoter stimulator (IPS)-1 for induction of neuroprotection. Wild-type (WT), Toll-like receptor (TLR)-3−/−, MDA5−/−, and IPS1−/− mice were administered poly-ICLC (60 µg) or vehicle 1 d before MCAO (60 min) and ischemic injury was measured 24 h after middle cerebral artery occlusion. The WT mice used as controls for TLR3−/−, MDA5−/− and IPS1−/− stroke studies were combined because no significant difference between the groups was evident. Data reported as group mean±SEM, n=7 to 27, 2-way ANOVA and Bonferroni post hoc test, ***P<0.0001 vs corresponding vehicle. IKK indicates I kappa B kinase; IRF, INF regulatory factor; RIG-I, retinoic acid–inducible gene-I; TBK, TRAF family member–associated NFKb activator binding kinase 1; TRAF, tumour necrosis factor receptor associated factor; and TRIF, TIR-domain containing adapter-inducing IFN-beta.
MDA5 mediated poly-ICLC neuroprotection, TLR3$^\beta$, and MDA5$^\beta$ mice were preconditioned with poly-ICLC 24 hours before MCAO. As expected, poly-ICLC preconditioning significantly reduced ischemic injury in WT animals (15.18±2.5% versus vehicle 32.57±1.8%; Figure 1B). In addition, poly-ICLC preconditioning significantly reduced ischemic injury in TLR3$^\beta$ mice (4.81±2.1% versus vehicle 29.14±4.1%; Figure 1B). No significant difference between poly-ICLC–treated WT and TLR3$^\beta$ mice was evident. In contrast, poly-ICLC–treated MDA5$^\beta$ mice failed to show a reduction in ischemic volume (29.63±3.1% versus vehicle 32.1±1.8%; Figure 1B). These data indicate that poly-ICLC preconditioning-induced neuroprotection is MDA5 dependent, demonstrating for the first time that a cytosolic receptor can mediate neuroprotection.

**Poly-ICLC–Induced Neuroprotection Is Mediated Through IPS1**

MDA5 signals through the adaptor molecule IFN-β-promoter stimulator (IPS1) leading to the induction of type I IFNs (Figure 1A). Therefore, we tested the involvement of IPS1 in poly-ICLC preconditioning using IPS1$^\beta$ mice. IPS1$^\beta$ mice preconditioned with poly-ICLC failed to show a significant reduction in ischemic injury (40.8±2.5% versus vehicle 37.11±2.0%; Figure 1B), demonstrating that poly-ICLC preconditioning depends on both MDA5 and IPS1 for the induction of neuroprotection.

**Poly-ICLC Induction of Type I IFNs Requires MDA5 and IPS1, Not TLR3**

We have previously shown that poly-ICLC requires type I IFN signaling for protection. Therefore, we postulate that MDA5 and IPS1 mediate poly-ICLC induction of IFN-α and/or IFN-β. We measured plasma IFN-α and IFN-β levels 6 hours after poly-ICLC treatment in WT, TLR3$^\beta$, MDA5$^\beta$, and IPS1$^\beta$ mice. Poly-ICLC significantly increased plasma levels of IFN-α in both WT and TLR3$^\beta$ mice (WT vehicle, 1.72 versus poly-ICLC, 596.37±181.8 pg/mL; TLR3$^\beta$ vehicle, 1.72 versus poly-ICLC, 720.88±371.8; Figure 2A). However, MDA5$^\beta$ and IPS1$^\beta$ mice showed no significant induction of IFN-α in response to poly-ICLC (Figure 2A). Similar results were obtained for IFN-β (Figure 2B) with TLR3$^\beta$ and WT mice inducing equivalent levels of IFN-β (WT, 33.17±14.2 versus TLR3$^\beta$, 60.53±40.3 pg/mL; Figure 2B), whereas no increase in IFN-β was detected in MDA5$^\beta$ and IPS1$^\beta$ mice. These results are consistent with poly-ICLC–mediating protection through binding of the MDA5 receptor, signaling through IPS1 and subsequent induction of type I IFNs, independent of TLR3 signaling.

**IFN-β Is Not Required for Poly-ICLC Neuroprotection**

IFN-β has been shown to provide protection against cerebral ischemic injury in experimental models of stroke, thus we hypothesized that IFN-β may be the key mediator of neuroprotection. To test this, we preconditioned IFN-β$^\beta$ mice with poly-ICLC 24 hours before ischemia. We found that IFN-β$^\beta$ mice were significantly protected against ischemic brain injury (poly-ICLC, 21.54±5.3% versus vehicle, 41.91±5.8%; Figure 3A), indicating that poly-ICLC–induced neuroprotection is independent of IFN-β.

**Systemic IFN-α Protects Against Stroke**

The previous data indicate that IFN-β is not required, indicating that another type I IFN receptor ligand, such as IFN-α, may mediate the protective response. To test this, IFN-α was administered 18 hours before MCAO. This timing corresponds

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**Figure 2.** Poly-l-lysine and carboxymethylcellulose (ICLC) induction of interferon (IFN-α/IFN-β) is dependent on melanoma differentiation-associated protein 5 (MDA5) and IFN-promoter stimulator (IPS)-1. IFN-α (A) and IFN-β (B) were measured in plasma samples collected from wild-type (WT), Toll-like receptor (TLR)-3$^-\beta$, MDA5$^-\beta$, and IPS1$^-\beta$ mice 6 h after vehicle or poly-ICLC (60 μg) treatment. Data reported as group mean±SEM, n=3 to 4, 2-way ANOVA and Bonferroni post hoc test, **P<0.01 and *P<0.05 vs corresponding vehicle.

**Figure 3.** Poly-l-lysine and carboxymethylcellulose (ICLC) neuroprotection is independent of interferon (IFN)-γ, whereas IFN-α treatment confers protection against stroke. A, IFN-β$^-\beta$ mice were administered poly-ICLC (60 μg) or vehicle 1 d before middle cerebral artery occlusion (MCAO; 60 min), and ischemic injury was measured 24 h after MCAO. B, Mice were administered recombinant IFN-α (1×104 U/mouse) or vehicle 18 h before MCAO (45 min), and ischemic injury was measured 24 h after MCAO. Data reported as group mean±SEM, n=3 to 6, unpaired t test, *P<0.05 vs vehicle.
with the increase in serum levels of IFN-α induced by preconditioning with poly-ICLC. We found that IFN-α–treated mice showed a significant reduction in ischemic volume (IFN-α, 18.11±4% versus vehicle, 33.19±5.1%; Figure 3B), indicating that poly-ICLC induction of systemic IFN-α may mediate protection against stroke.

Discussion
We demonstrate that poly-ICLC preconditioning mediates neuroprotection through the cytoplasmic receptor MDA5 and its adapter IPS1. We have previously published that the type I IFN receptor is required for protection.4 Here we show that, although systemic levels of both IFN-α and IFN-β are increased in response to poly-ICLC preconditioning, the protective response is independent of IFN-β whereas direct treatment with IFN-α protects the brain from ischemic injury, supporting a key role for IFN-α in poly-ICLC neuroprotection. This broadens our knowledge of endogenous targets for the induction of neuroprotection against cerebral ischemic injury. In particular, the localization of these mediators of protection (both the receptor and its adapter molecule) in the cytoplasm may offer alternative drug delivery strategies for the translational development of therapeutic agents for stroke.

We found that neuroprotection induced by poly-ICLC preconditioning is mediated exclusively through MDA5 and independent of TLR3. This is in contrast to a recent study using native poly-IC in which acute neuroprotection against brain ischemic injury was mediated through TLR3.10 The apparent difference may relate to the chemical modification of poly-ICLC, which is a version of poly-IC that has been stabilized with poly-s-lysine and carboxymethylcellulose to improve pharmacokinetics. These alterations may influence the structure and trafficking of the compound resulting in different responses between poly-ICLC and native poly-IC. Consistent with this postulate, we found that although poly-ICLC was able to induce IFN-α and β in TLR3−/− mice (Figure 2), poly-IC was unable to induce either IFN in the TLR3−/− mice (data not shown). This difference is likely the result of altered trafficking of the 2 poly-IC compounds into the cell. This is supported by recent data by Dann et al11 demonstrating that native (noncomplexed) poly-IC is a ligand for TLR3, whereas poly-IC in complex with poly-ethylenimine targets the cytoplasmic receptor MDA5.12 Poly-IC complexation with polyethylenimine changes its cell delivery by making it available for the cytosolic receptors. We propose that the LC component of poly-ICLC modifies the cell delivery as well, making poly-ICLC a specific ligand for cytosolic MDA5.

The association between stimulation of RIG-I–like receptors and reduction of central nervous system inflammation was previously reported by Dann et al,11 who showed that activation of RIG-I and MDA5 reduced macrophage and lymphocyte infiltration into the spinal cord in an experimental model of multiple sclerosis. Similar to our results, this process was dependent on type I IFN receptor signaling.4,11

We and others have shown that prophylactic administration of TLR ligands induces neuroprotection against MCAO in mice.13 Here, we demonstrate for the first time that the pattern recognition receptor, MDA5, is involved in neuroprotection. This may indicate the existence of a conserved feature shared among pattern recognition receptors for conferring protection against brain ischemic injury. This discovery has real significance in neuroprotection because it broadens substantially the possibilities of new approaches to achieve neuroprotection, particularly in the realm of preconditioning modalities.

Conclusions
Our results demonstrate that cytoplasmic receptors play an important role in preconditioning, and that MDA5 and its adapter IPS1 are required for poly-ICLC–induced neuroprotection. These findings increase our understanding of the neuroprotective mechanisms associated with poly-ICLC, furthering its development as a prophylactic treatment against ischemic injury. Furthermore, the results reported here expand our knowledge of potential endogenous targets for the development of new therapeutic agents for stroke.

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Disclosures
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