Toll-Like Receptor 7 Preconditioning Induces Robust Neuroprotection Against Stroke by a Novel Type I Interferon-Mediated Mechanism

Philberta Y. Leung, PhD; Susan L. Stevens, BS; Amy E.B. Packard, PhD; Nikola S. Lessov, MD, PhD; Tao Yang, MS; Valerie K. Conrad, BA; Noortje N.A.M. van den Dungen, BAS; Roger P. Simon, MD; Mary P. Stenzel-Poore, PhD

Background and Purpose—Systemic administration of Toll-like receptor (TLR) 4 and TLR9 agonists before cerebral ischemia have been shown to reduce ischemic injury by reprogramming the response of the brain to stroke. Our goal was to explore the mechanism of TLR-induced neuroprotection by determining whether a TLR7 agonist also protects against stroke injury.

Methods—C57Bl/6, TNF−/−, interferon (IFN) regulatory factor 7−/−, or type I IFN receptor (IFNAR)−/− mice were subcutaneously administered the TLR7 agonist Gardiquimod (GDQ) 72 hours before middle cerebral artery occlusion. Infarct volume and functional outcome were determined after reperfusion. Plasma cytokine responses and induction of mRNA for IFN-related genes in the brain were measured. IFNAR−/− mice also were treated with the TLR4 agonist (lipopolysaccharide) or the TLR9 agonist before middle cerebral artery occlusion and infarct volumes measured.

Results—The results show that GDQ reduces infarct volume as well as functional deficits in mice. GDQ pretreatment provided robust neuroprotection in TNF−/− mice, indicating that TNF was not essential. GDQ induced a significant increase in plasma IFNα levels and both IRF7−/− and IFNAR−/− mice failed to be protected, implicating a role for IFN signaling in TLR7-mediated protection.

Conclusions—Our studies provide the first evidence that TLR7 preconditioning can mediate neuroprotection against ischemic injury. Moreover, we show that the mechanism of protection is unique from other TLR preconditioning ligands in that it is independent of TNF and dependent on IFNAR. (Stroke. 2012;43:00-00.)

Key Words: ischemia • neuroprotection

Toll-like receptors (TLR) are sentinels of the innate immune system that recently have been shown to be involved in stroke injury. In a model of brain focal ischemia, mice deficient in TLR2 or TLR4 showed significantly less brain damage compared to their wild-type counterparts, highlighting a deleterious role for these receptors in ischemic injury.1–5 In accordance with this, TLR2 expression and highlighting a deleterious role for these receptors in ischemic brain damage compared to their wild-type counterparts, mice deficient in TLR2 or TLR4 showed significantly less involvement in stroke injury. In a model of brain focal ischemia, cytokines.6 Thus, finding ways to modulate the TLR response to stroke could provide a potential therapeutic target to reduce ischemic injury.

An important aspect of the TLR family is their ability to autoregulate and cross-regulate the response to subsequent TLR signaling by priming initially with a small amount of TLR ligand. The priming event can lead to suppression and redirection of the subsequent response to stimulation with a secondary TLR ligand. For example, pretreating cultured murine macrophages with a small dose of a TLR4, TLR7, or TLR9 ligand reduces nuclear factor kappa-B (NFkB) activation and tumor necrosis factor (TNF), and enhances interferon (IFN) β, in response to subsequent TLR4 activation.7 Importantly, NFkB and TNF have been shown to play damaging roles in brain ischemia,8–10 whereas IFNβ is neuroprotective.11–13 Taken together with the evidence that TLR play a role in stroke injury, we postulated previously that TLR activation in the setting of ischemia could be redirected via previous stimulation with a TLR ligand. We and others14–18 have shown that exogenous administration of small doses of ligands for at least 3 TLR (TLR2, TLR4, and TLR9) before stroke provides protection. In addition, preconditioning with the TLR4 ligand or TLR9 ligand leads to a reprogrammed response to stroke. The reprogrammed response is characterized by enhanced IFN regulatory factor (IRF)-mediated transcription and increased production of IFN-associated...
genes after ischemia in lipopolysaccharide (LPS)-preconditioned and unmethylated cytosine-phosphate-guanine rich oligonucleotide (CpG)-preconditioned animals.11,19

The mechanism by which TLR pre conditioning induces ischemic tolerance and provides protection remains incompletely understood. However, an important role for TNF has been shown for LPS and CpG preconditioning because TNF-deficient mice cannot be protected by either of these TLR ligands.10,18 An important role for an IFN response also exists because mice deficient in either IRF3 or IRF7 failed to be protected with LPS or CpG preconditioning.11,19 To further delineate mechanisms underlying TLR pre conditioning, we investigated the potential for a TLR7 agonist to induce neuroprotection. As discussed, TLR7 has been shown to provide cross-tolerance to a subsequent TLR4 stimulation, and thus we postulated that preconditioning through TLR7 also would provide protection against ischemic injury. In addition, because TLR7 signaling induces a more substantial type I IFN response compared to TLR4 or TLR9, which shows minimal to no induction of type I IFN (IFNo and IFNb),20 we hypothesized that TLR7 preconditioning, through its increase in expression of type I IFN, may provide a route to neuroprotection that is unique from TLR4 and TLR9.

The results provided here are the first evidence to our knowledge that TLR7 preconditioning confers robust protection against focal ischemia. We show that the reduced damage is associated with upregulation of IFN-associated genes, which is similar to our previous findings with TLR4 and TLR9 preconditioning. Surprisingly, we find that TLR7-mediated preconditioning works through a TNF-independent mechanism, which contrasts with TLR4 and TLR9. We found that TLR7 preconditioning required IRF7 for the induction of IFNo to confer neuroprotection. Furthermore, only TLR7 preconditioning required the presence of the cognate receptor for type I IFN (IFNAR), a feature not shared by TLR4 or TLR9 preconditioning. Collectively, these novel findings highlight a new mechanism of TLR preconditioning-induced protection that relies on the production and signaling of type I IFN.

Materials and Methods

Mice

C57Bl/6 and B6.129S-Tnf+/+ (TNF−/−) mice were obtained from Jackson Laboratories (West Sacramento, CA). TLR7−/− mice were purchased from OrientalBioService (Osaka, Japan), IRF7−/− mice were provided by Dr Ian Rifkin (Boston University School of Medicine, Boston, MA), and IFNAR−/− mice were provided by Dr Anthony French (Washington University School of Medicine, St. Louis, MO). These strains were back-crossed ≥8 generations onto C57Bl/6. All studies were performed with male mice between 10 and 14 weeks of age.

All mice were given free access to food and water and were 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 and met the guidelines set forth by the National Institutes of Health.

Drug Treatments

Mice were administered a subcutaneous injection of Gardiquimod (GDQ; 10–40 μg/mouse; Invivogen), ODN 1826 (CpG; 40 μg/mouse; Invivogen), lipopolysaccharide (LPS; 20 μg/mouse; Sigma), or saline. To determine the effective time window of protection, mice were injected from 1 to 14 days with GDQ before middle cerebral artery occlusion (MCAO). For all other experiments, mice were treated 72 hours before MCAO.

Ischemia-Reperfusion Model

Mice were subjected to focal cerebral ischemia by MCAO as described previously.19 The number of animals per group and treatment are reported in the Figures. Cerebral blood flow was monitored throughout the procedure by laser Doppler flowmetry (Transonic System). Body temperature was maintained at 37°C during and after the surgery with a heating pad. After 45 to 60 minutes of occlusion, the monofilament was removed and blood flow was restored (reperfusion). The duration of MCAO was optimized based on the surgeon per study to obtain consistent baseline infarct sizes across studies. Twenty-four hours after MCAO, mice were deeply anesthetized and brains were removed and cut into 1-mm coronal sections for measurement of infarct size as previously described. A total of 179 C57BL/6 mice were used for experiments, with 22 excluded because of early attrition or failure to maintain cerebral blood flow reduction of <20% of baseline during study. For the genetically engineered mice the following were used: TLR7−/−, 16 total and 4 excluded; TNF−/−, 16 total and 2 excluded; IRF7−/−, 19 total and 1 excluded; and IFNAR−/−, 48 total and 4 excluded. There was no effect of genotype or treatment on mortality rate associated with the model.

Analysis of Serum Cytokine Levels

Mice were deeply anesthetized with isoflurane and blood was collected via cardiac puncture. Enzyme-linked immunosorbent assay kits were used to analyze serum levels of TNF (R&D Systems), IFNo, and IFNb (PBL InterferonSource). Samples were performed in duplicate.

Neurological Evaluation

Twenty-four hours after MCAO, mice were scored on body movement (focal) and physical appearance (general well-being) using a scale designed specifically to assess neurological deficits in mice, as has been previously described.21 Sensornotor deficits were evaluated using the corner test, which measures the extent to which the mouse favors (turns toward) the ipsilateral (right) side after approaching a confining corner. Each mouse was tested 10 times. Naive mice turn to each side equally, whereas after a stroke mice tend to turn preferentially to the side ipsilateral to the stroke (right). All analyses were performed by researchers blinded to treatment to prevent experimental bias.

Tissue Processing and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from the brain cortex using the Qiagen Rneasy Lipid Mini Kit (Qiagen). RNA was reverse-transcribed using an Omniscript Reverse Transcription kit (Qiagen). Quantitative polymerase chain reaction was performed using TaqMan Gene Expression Assays (Applied Biosystems) on an ABI prism 7700. Results were normalized to β-actin expression. The relative quantification was determined using the comparative CT method (2−ΔΔCT).

Statistical Analyses

Data are presented as mean±SEM and were analyzed using Student t test, 1-way ANOVA, or 2-way ANOVA with Bonferroni post hoc test, as indicated in Figures. Differences were considered significant at P<0.05. Prism4 (Graphpad) was used for all statistical analyses.

Results

GDQ Preconditioning Reduces Ischemic Damage in an In Vivo Model of Stroke

To determine whether GDQ could protect against ischemia, mice were pretreated with various doses of GDQ (10–40
μg/mouse, subcutaneous) 72 hours before MCAO (60 minutes) and the infarct size was determined 24 hours later. Results show that GDQ significantly reduced ischemic damage in a dose-dependent manner (Figure 1A), with a maximal protective effect achieved at the dose of 40 μg/mouse (28% ± 3.6% compared with saline at 58% ± 0.94%). In addition, we found that the neuroprotection induced by GDQ preconditioning was still evident 72 hours after MCAO (24.78% ± 2.4% compared with saline-treated 39.72% ± 2.2%; Figure 1B), indicating that GDQ-induced neuroprotection is a sustained effect.

To determine the effective time window of GDQ preconditioning, mice were treated with GDQ 1 to 14 days before MCAO. We found that GDQ preconditioning significantly decreased infarct size when administered 1 day before MCAO (35% reduction in infarct volume), and this effect was still evident when GDQ was administered 7 days before MCAO (20% reduction). However, protection was lost when GDQ was administered 14 days before MCAO (Figure 1C), indicating that the neuroprotective time window of TLR7 preconditioning lasts for at least 1 week. This time window of neuroprotection is comparable to those we have reported previously for LPS and CpG preconditioning.10,18

**GDQ Preconditioning Reduces Ischemia-Induced Neurological Deficits**

To determine whether neurological deficits associated with the stroke injury are attenuated by GDQ preconditioning, we examined mice using focal and general assessment scales.21 Mice pretreated with GDQ scored better in the focal and general categories compared to saline controls, providing evidence that GDQ attenuates neurological deficits as well as reduces infarct size (Figure 1D). To assess sensorimotor deficits, mice were subjected to the corner test after MCAO. Results from this test have been shown to correlate with infarct volume and can reveal the extent of postinfarct recovery.22,23 Mice preconditioned with GDQ showed significantly fewer sensorimotor deficits, represented by a decreased tendency to turn to the right (62.50% ± 8.54%) compared to saline-treated animals (87.50% ± 4.79%; Figure 1E).

**TLR7 Mediates GDQ-Induced Protection Against Ischemic Injury**

We tested whether the neuroprotective effects were specifically exerted through TLR7 because previous work by others showed that some TLR7 agonists were able to signal through adenosine receptors.24 We preconditioned TLR7−/− mice with GDQ 72 hours before subjecting them to MCAO (45 minutes). Infarct size in GDQ-preconditioned TLR7−/− mice (44.38% ± 3.16%) did not differ significantly (P = 0.4) from saline-treated controls (38.52% ± 6.75%), indicating that TLR7−/− mice are not protected by GDQ preconditioning (Figure 2). Thus, GDQ preconditioning-induced neuroprotection is mediated via TLR7 signaling.

Figure 1. Gardiquimod (GDQ) preconditioning reduces ischemic injury. A, C57Bl/6 mice were preconditioned with escalating doses of GDQ (N = 8; 10, 20, or 40 μg per mouse, subcutaneous) or saline (N = 5) 72 hours before 60 minutes of middle cerebral artery occlusion (MCAO). Infarct size was determined 24 hours after MCAO. B, C57Bl/6 mice were pretreated with GDQ (N = 8; 20 μg/mouse, subcutaneous) or saline (n = 6) 72 hours before MCAO. Infarct size was determined 72 hours after MCAO. C, C57Bl/6 mice were pretreated with GDQ (N = 5–6; 20 μg/mouse, subcutaneous) or saline (n = 6) at various times before MCAO. Infarct size was determined 24 hours after MCAO. Two-way analysis of variance (ANOVA), Bonferroni post hoc, *P < 0.01, **P < 0.01, ***P < 0.001 vs saline controls. D and E, C57BL/6 mice were treated with GDQ (N = 5; 40 μg/mouse, subcutaneous) or saline (N = 6) 72 hours before MCAO (60 minutes). Mice were then examined using the (D) neurological score (focal and general) and (E) corner test to determine neurological and sensorimotor deficits 24 hours after MCAO. Student t test: *P < 0.01, **P < 0.01, ***P < 0.001 vs saline controls.
To determine whether TLR7-mediated protection depends on TNF, we measured serum levels of TNF in GDQ-treated mice at 1, 3, and 24 hours after injection. GDQ did not induce any measurable changes in TNF levels (Figure 3A). It should be noted that although our low protective dose of GDQ did not induce an increase in serum TNF levels, previous studies have shown that higher doses of other TLR7 ligands (e.g., Imiquimod) can induce serum TNF. The unaltered TNF serum levels in mice treated with a protective dose of GDQ suggest that TNF may not be critical to GDQ-induced protection.

To determine whether TNF is required for GDQ-induced neuroprotection, we examined the effects of preconditioning TNF$^{-/-}$ mice with GDQ. TNF$^{-/-}$ and TNF$^{+/+}$ mice were preconditioned with GDQ 72 hours before MCAO (50 minutes). GDQ-treated TNF$^{+/+}$ mice had significantly reduced infarcts (39.43% ± 3.66%) compared to saline controls (50.8% ± 2.92%). Interestingly, TNF$^{-/-}$ mice preconditioned with GDQ were also protected (saline 45.35% ± 2.94% versus GDQ 36.02% ± 1.21%), indicating that TNF does not play a role in GDQ-induced neuroprotection (Figure 3B). This contrasts sharply with LPS and CpG preconditioning and suggests a novel TNF-independent mechanism through which TLR7 mediates neuroprotection against ischemia.

**GDQ Administration Increases IFNα but not IFNβ**

TLR7 signaling activates the transcription factor NFκB and pro-inflammatory cytokines, as well as IRF and induction of type I IFN (IFNα and IFNβ). We postulated that GDQ preconditioning relies on the IFN response because our results showed that TLR7-induced neuroprotection was independent of TNF. To determine the role of type I IFN in TLR7 preconditioning, we measured the changes in serum levels of IFNα and IFNβ after treatment with GDQ as well as with the TLR4 and TLR9 ligands, LPS, and CpG. GDQ induced a dramatic increase in IFNα levels at 1 hour and 2 hours (5-fold and 10-fold, respectively) but returned to baseline levels by 24 hours after injection. CpG induced only a modest increase in IFNα, whereas no increase in IFNα was observed with LPS (Figure 4A). The same doses of GDQ, LPS, and CpG failed to induce detectable levels of serum IFNβ (data not shown). As expected, TLR7$^{-/-}$ mice showed no increase in IFNα after GDQ preconditioning compared to saline-treated mice (data not shown). Thus, GDQ preconditioning causes a robust increase in IFNα in the systemic circulation, whereas CpG and LPS preconditioning induces little to no expression of type I IFN.

**IRF7 Is a Critical Mediator for GDQ-Induced Neuroprotection**

TLR7 mediates the induction of IFNα through the transcription factor IRF7. Thus, mice deficient in IRF7 should not increase IFNα in response to GDQ and could be used to explore whether IFNα plays a critical role in GDQ preconditioning. We confirmed that GDQ-stimulated IFNα induction required IRF7 activation (Figure 4B), because the increase in IFNα observed in IRF7$^{+/+}$ mice (6.5-fold over saline) was absent in IRF7$^{-/-}$ mice (no significant increase).
To determine whether IRF7 is a critical effector of GDQ-mediated protection, we treated IRF7−/− and IRF7+/+ mice with GDQ (40 μg/mouse) 72 hours before MCAO (45 minutes) and measured infarct size 24 hours later. GDQ-treated IRF7−/− mice were not protected by GDQ preconditioning (42.11%±2.87%), showing no significant difference in infarct size compared to saline controls (38.94%±2.43%; Figure 4C). Hence, IRF7 is essential for the protective effects of GDQ preconditioning, an effect that may likely occur through IFNα.

IFNAR Mediates GDQ-Induced Neuroprotection

To further determine whether IFNα plays a novel role in TLR7-mediated neuroprotection, we used mice deficient in the IFNα/β receptor (IFNAR−/− mice). Mice were preconditioned with GDQ, CpG, or LPS 72 hours before MCAO (45 minutes). We found that IFNAR−/− mice preconditioned with GDQ displayed a significant reduction in protection compared to IFNAR+/+ mice (Figure 5; P<0.05), with no significant decrease in infarct size compared to saline-treated mice (P>0.05; Figure 5). In contrast, CpG and LPS precon-
Evidence of a potential neuroprotective state that is similar to response to stroke in the context of GDQ preconditioning is evident from the genomic response to stroke injury.11,19 This suggests that although multiple TLR ligands can induce neuroprotection through genomic reprogramming and induction of type I IFN genes, the molecular pathways leading to the protective phenotype are not identical.

We have recently published that LPS and CpG preconditioning depend on the transcription factors IRF3 and IRF7,11,19 which are key modulators of the type I IFN response.27,28 Thus, we postulated that because TNF was not required for GDQ preconditioning and TLR7 stimulation leads to robust production of IFNα, the mechanism underlying TLR7 preconditioning may be based on IFN regulation. We found that our preconditioning dose of GDQ induced a significant increase in serum IFNα, and that the increase in IFNα was functionally relevant because IFNAR−/− mice were not protected by GDQ preconditioning. Importantly, the IFNAR−/− mice could be protected by preconditioning with either LPS or CpG, implying that the mechanism of protection involving IFNAR is unique to TLR7. Further, we report that IRF7 is required for GDQ-induced neuroprotection. We suggest this occurs through TLR7-driven activation of IRF7 and subsequent induction of IFNα, because IRF7−/− mice did not have induced IFNα and were unable to be protected against ischemia in response to GDQ. These results implicate a new mechanism of TLR-induced preconditioning in which TLR7 initiates a pathway of protection driven by IRF7 induction of IFNα and activation of the type I IFN receptor, culminating in a reprogrammed TLR response to injury.

The mechanism by which IFNα is involved in the TLR7-mediated reprogramming of the response to ischemic injury is unclear. However, work in macrophages may provide some insight. Similar to our current results, it previously has been shown that TLR4 signaling in response to LPS was altered after IFNα treatment, wherein type I IFN and IRF gene regulation were enhanced.29 The alteration of TLR4 signaling was induced by pretreatment of macrophages with IFNα, which resulted in increased TRIF as well as downstream molecules IKKα and IRF7. Such regulation is similar to our findings showing the effect of GDQ preconditioning on the genomic response to stroke injury. In addition, systemic IFNα can induce central nervous system upregulation of IRF genes,30 suggesting that IFNα may be able to cross the blood–brain barrier to elicit these responses. Thus, in our model, GDQ preconditioning-induced neuroprotection may occur through the induction of systemic IFNα that, in turn, crosses the blood–brain barrier to affect the endogenous TLR4 response to ischemia by the brain.

In conclusion, we describe the novel finding that tolerance to ischemic brain injury can be induced by previous systemic preconditioning that allows the brain to respond to ischemic injury by inducing a similar genomic response to stroke injury. This suggests that the mechanism of neuroprotection is not evident in preconditioning via TLR4 or TLR9.

**Discussion**

Mice deficient in either TLR4 or TLR2 exhibit smaller infarcts when subjected to focal cerebral ischemia than wild-type mice, implicating a damaging role for TLR activation in stroke.1–5 Inhibiting or altering this TLR damaging effect would provide a potential means of reducing stroke injury. In macrophages, pretreatment with a TLR ligand, including TLR4, TLR7, and TLR9 ligands, before stimulation with a TLR4 ligand reprograms TLR signaling to suppress the NFκB response and to enhance IFNβ.7,26 We have reported evidence of a similar reprogrammed response in LPS and CpG preconditioning-induced neuroprotection. In particular, we have shown that LPS preconditioning suppressed NFκB and enhanced IRF3 activation after stroke,26 and preconditioning with either LPS or CpG enhanced the type I IFN genomic response to stroke injury.11,19 This suggests that TLR4 and TLR9 preconditioning-induced neuroprotection reprograms the brain’s damaging TLR4 response to stroke, leading to a protective effect. We postulated that a TLR7 ligand would also provide protection from brain ischemia because it induces similar reprogramming of TLR4 signaling in macrophages.

Here, we show that systemic administration of the TLR7 ligand, GDQ, before stroke reduced ischemic injury and induced the IFN-associated genes (Usp18, Oasl2, Isg15, Ifit1) previously identified after stroke in LPS-preconditioned and CpG-preconditioned mice.11,19 Thus, as with LPS and CpG preconditioning, GDQ appears to reprogram the TLR response to stroke, resulting in enhanced induction of type I IFN gene regulation. The presence of this IFN-dominated response to stroke in the context of GDQ preconditioning is evidence of a potential neuroprotective state that is similar to that induced via TLR4-mediated and TLR9-mediated preconditioning.

The precise molecular mechanism initiated by preconditioning that enables the reprogramming of the TLR response is not clear. Whereas LPS and CpG preconditioning depend on the induction of TNF,10,19 we show a preconditioning dose of GDQ failed to induce TNF and, more importantly, TNF-deficient mice preconditioned with GDQ displayed a similar reduction in infarct size as wild-type mice. Thus, although TLR7 signaling induces reprogramming and provides neuroprotection against brain ischemia, TNF is not required. This suggests that although multiple TLR ligands can induce neuroprotection through genomic reprogramming and induction of type I IFN genes, the molecular pathways leading to the protective phenotype are not identical.

**Figure 5.** Type I interferon receptor (IFNAR) signaling is required for TLR7-mediated neuroprotection. IFNAR+/+ and IFNAR−/− mice were injected with Gardiquimod (GDQ; N=6–8), unmethylated cytosine-phosphate-guanine rich oligonucleotide (N=8–11), lipopolysaccharide (LPS; N=8–11), or saline (N=6–9) 72 hours before middle cerebral artery occlusion (MCAO; 45 minutes). Infarct size was determined 24 hours after MCAO. The data are presented as percent damage normalized to saline plus MCAO. Two-way analysis of variance (ANOVA), Bonferroni post hoc: ***P<0.001 vs saline control for respective genotype; #P<0.05 vs IFNAR+/+ for respective treatment.
administration of the TLR7 ligand, GDQ. TLR7-mediated preconditioning results in new IFN-associated gene regulation in response to ischemic injury, which mirrors the TLR-reprogrammed response to stroke that we have previously reported for TLR4 and TLR9 preconditioning. These findings support the postulate that TLR reprogramming is an endogenous process capable of providing protection against subsequent TLR-mediated stroke injury. However, in contrast to TLR4 and TLR9 preconditioning that depend on the proinflammatory cytokine TNF, TLR7-induced neuroprotection is independent of TNF. Instead, TLR7-induced neuroprotection relies on a novel mechanism of IRF7-mediated induction of IFNα and signaling through the type I IFN receptor. These findings demonstrate that at least 2 different pathways participate in TLR-induced protection against ischemic injury, providing 2 distinct targets for the development of therapeutic interventions against stroke injury.

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Disclosures
None.

References


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