NR2B Phosphorylation at Tyrosine 1472 Contributes to Brain Injury in a Rodent Model of Neonatal Hypoxia-Ischemia

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Background and Purpose—The NR2B subunit of the N-methyl-D-aspartate (NMDA) receptor is phosphorylated by the Src family kinase Fyn in brain, with tyrosine (Y) 1472 as the major phosphorylation site. Although Y1472 phosphorylation is important for synaptic plasticity, it is unknown whether it is involved in NMDA receptor-mediated excitotoxicity in neonatal brain hypoxia-ischemia (HI). This study was designed to elucidate the specific role of Y1472 phosphorylation of NR2B in neonatal HI in vivo and in NMDA-mediated neuronal death in vitro.

Methods—Neonatal mice with a knockin mutation of Y1472 to phenylalanine (YF-KI) and their wild-type littermates were subjected to HI using the Vannucci model. Brains were scored 5 days later for damage using cresyl violet and iron staining. Western blotting and immunoprecipitation were performed to determine NR2B tyrosine phosphorylation. Expression of NADPH oxidase subunits and superoxide production were measured in vivo. NMDA-induced calcium response, superoxide formation, and cell death were evaluated in primary cortical neurons.

Results—After neonatal HI, YF-KI mice have reduced expression of NADPH oxidase subunit gp91phox and p47phox and superoxide production, lower activity of proteases implicated in necrotic and apoptotic cell death, and less brain damage when compared with the wild-type mice. In vitro, YF-KI mutation diminishes superoxide generation in response to NMDA without effect on calcium accumulation and inhibits NMDA and glutamate-induced cell death.

Conclusions—Upregulation of NR2B phosphorylation at Y1472 after neonatal HI is involved in superoxide-mediated oxidative stress and contributes to brain injury. (Stroke. 2014;45:3040-3047.)

Key Words: Fyn tyrosine kinase ■ hypoxia-ischemia, brain ■ NR2B NMDA receptor

Hypoxic-ischemic encephalopathy remains a significant cause of death and disability in infants and children with few treatment options.1 In our efforts to understand mechanisms and seek novel interventions, we found that Fyn, a Src family kinase, is associated with neuronal death after neonatal brain hypoxia-ischemia (HI).2,3 Fyn has many substrates in the brain, including the N-methyl-D-aspartate receptor (NMDAR) subunit NR2B, the predominant regulatory subunit in the developing brain. Fyn mediates NR2B phosphorylation on 7 tyrosine (Y) residues in its C terminus, with Y1472 as the major site.4 In adult rats subjected to brain ischemia, phosphorylation of NR2B at Y1472 (pY1472NR2B) is significantly and persistently upregulated.5 Increase in pY1472NR2B is also reported after neonatal HI in postnatal day 7 (P7) rats6 and mice.7 However, the functional role of this specific modification and whether it is directly involved in brain injury are unknown.

Overall, NR2B tyrosine phosphorylation and pY1472NR2B in murine brain are developmentally regulated with a gradual increase with age.4 Y1472 phosphorylation is physiologically important because it is implicated in long-term potentiation and hippocampal synaptic plasticity.5,7 At the molecular level, Y1472 phosphorylation prevents NMDAR internalization,8,9 regulates NMDAR localization between synaptic and extrasympatic membrane,8,10 and affects NR2B downstream signaling transduction.8,11

NMDAR-mediated excitotoxicity and oxidative stress derived from superoxide (O2−) and related reactive oxygen species are important contributors in perinatal HI brain injury.1 Excitotoxicity and oxidative stress are inextricably linked, as evidenced by studies showing that NMDAR-dependent neuronal death results partially from O2− produced largely by NADPH oxidase (NOX) and triggered by calcium influx via NR2B-containing NMDAR.12,13 Neurons mainly express the

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Materials and Method

Animals

C57BL/6 YF-KI mice were bred with wild-type (WT) mice to generate heterozygous animals at the Laboratory Animal Resource Center of the University of California, San Francisco. The YF-KI mice are normal in brain anatomy and hippocampal long-term potentiation. Heterozygous mice were crossed to generate WT and homozygous YF-KI littermates for experiments. Both sexes were used at P7.

Hypoxic-Ischemic Brain Injury

We adapted the Vannucci procedure for neonatal HI with ligation of the right common carotid artery and hypoxia for 40 minutes. Sham-operated animals received anesthesia and exposure of the artery without ligation or hypoxia.

Evaluation of Brain Injury

Five days after HI, brain damage was scored as described using brain sections stained with cresyl violet and Perl’s iron stain.

Western Blotting

Western blotting was performed with cortical tissue from sham-operated and the ipsilateral side of HI-injured animals. The following primary antibodies were used: NR2B (BD Transduction Laboratories, San Jose, CA), phospho-Y1252 NR2B and phospho-Y1336 NR2B (PhosphoSolutions, Aurora, CO), phospho-Y1472 NR2B and phospho-Y1070 NR2B (Cell Signaling Technology, Boston, MA), α-spectrin (Millipore, Billerica, MA), cleaved-caspase 3 (Cell Signaling), p47phox (Millipore), gp91phox (BD), and p22phox (Invitrogen) was dissolved in DMSO at 10 mg/mL and diluted to 1 μg/mL in saline. It was injected intraperitoneally (5 mg/kg) 3 hours before the mice were euthanized at 24 hours after HI. The mice were transcardially perfused with 4% paraformaldehyde, the brains were cut into 3-mm slices, and the sections stained with cresyl violet and Perl’s iron stain.

Immunoprecipitation

Immunoprecipitations were performed to measure NR2B tyrosine phosphorylation. Two hundred micrograms protein was incubated with 4 μg goat NR2B antibody (Santa Cruz) or 4 μg normal goat IgG as negative control. Eluted immune complexes were applied for Western blotting and probed with mouse 4G10 antiphosphotyrosine (anti-pY) antibody (Millipore), then stripped and reprobed with mouse NR2B (BD) antibody. NR2B tyrosine phosphorylation was expressed as the optical density ratio of phosphotyrosine (pY) to NR2B.

Detection of Superoxide Production In Vivo

Dihydroethidium was used for superoxide measurement because it is oxidized by O$_2^-$ to a fluorescent product. Dihydroethidium (Invitrogen) was dissolved in DMSO at 10 mg/mL and diluted to 1 mg/mL in saline. It was injected intraperitoneally (5 mg/kg) 3 hours before the mice were euthanized at 24 hours after HI. The mice were transcardially perfused with 4% paraformaldehyde, the brains were dissected and postfixed for 2 hours followed by cryoprotection with 30% sucrose. Cryosections (12 μm) were evaluated for oxidized dihydroethidium (510/590 nm excitation/emission), and the fluorescence intensities were measured with Velocity software (Improvision). We analyzed 3 sections of each brain at the levels of injured cortical regions from 3 animals of each genotype. The results are presented as mean dihydroethidium fluorescence intensity.

Immunofluorescent Staining

Cryosections were treated with 2 N HCl for 10 minutes at 37°C and then with 0.5 mol/L boric acid (pH 8.4) for 10 minutes at room temperature. After blocking, cleaved-caspase-3 (Cell signaling) antibody in blocking solution with 2.5% goat serum was applied overnight at 4°C. Secondary antibody (Alexa Fluor 568; Invitrogen) was applied for 1 hour at room temperature, and the nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI). Images were captured with Zeiss Axiovert 100 microscope.

Primary Cortical Neuronal Culture

Cultures were prepared from the cortices of embryonic day 14 C57BL/6 mice and plated in poly-d-lysine coated 24-well plates or glass coverslips to a fluorescent product. Dihydroethidium was used for superoxide measurement because oxidation is presented as raw ethidium fluorescence normalized between 340 and 380 nm (emission, >510 nm). Calcium levels were expressed as changes in the 340/380 nm signal ratio relative to baseline fluorescence (ΔF/F0) before stimulus and quantified by integrating the change over baseline for the 30-minute observation period. Regions of interest were drawn around neuronal cell bodies. Measurements were made from 3 dissections with 2 coverslips per experiment and 10 to 12 neurons per coverslip for a total of 60 to 72 neurons for each cell type.

 Calcium Imaging in Primary Neurons

To monitor changes in intracellular calcium, neurons were loaded for 30 minutes with either 4 μmol/L Fura 4F-AM or Fura FF-AM (Molecular Probes, Grand Island, NY), washed once with balanced salt solution, and allowed to recover for 20 minutes at 37°C before addition of NMDA. The cells were exposed to 100 μmol/L NMDA for 30 minutes, and the images were acquired at either 10-s intervals for Fura4F or 30-s intervals for Fura FF, using excitation that alternated between 340 and 380 nm (emission, >510 nm). Calcium levels were expressed as changes in the 340/380 nm signal ratio relative to baseline fluorescence (ΔF/F0) before stimulus and quantified by integrating the change over baseline for the 30-minute observation period. Regions of interest were drawn around neuronal cell bodies. Measurements were made from 3 dissections with 2 coverslips per experiment and 10 to 12 neurons per coverslip for a total of 60 to 72 neurons for each cell type.

Ethidium Imaging of Superoxide Production in Primary Neurons

On DIV10, the medium was exchanged for balanced salt solution containing 1.2 mmol/L CaCl$_2$, 0.8 mmol/L MgSO$_4$, 5.3 mmol/L KCl, 0.4 mmol/L KH$_2$PO$_4$, 137 mmol/L NaCl, 0.3 mmol/L NaHPO$_4$, 5 mmol/L glucose, and 10 mmol/L 1,4-piperazinedinedethanesulfonate buffer, pH 7.2. When NMDA or glutamate was added, the MgSO$_4$ concentration in balanced salt solution was dropped to 0.4 mmol/L.

Cell Death Measurement

Dead and live neurons were identified with fluorescence markers, propidium iodide, and calcein-AM, which were added to the cultures 24 hours after NMDA exposure. In another set of experiment, the neurons were exposed to 100 μmol/L glutamate for 30 minutes and cell death was determined 24 hours thereafter. Live and dead neurons were counted in 3 randomly chosen fields in a minimum of 4 wells per plate.

Statistical Analysis

Brain injury scores are presented as median and interquartile range using Prism 4 nonparametric tests for ANOVA (Kruskal–Wallis test). Western blotting results are presented as mean±SD and were evaluated using SAS Wilcoxon–Mann–Whitney test. For cell culture experiments, 1-way ANOVA (Tukeys post hoc test) was used and data are presented as mean±SEM. Differences were considered significant at P<0.05.
Results

YF-KI Mice Have Decreased Brain Injury After Neonatal HI

YF-KI mice had decreased overall brain injury when compared with WT animals (median=16.25; range, 11.5–19 in WT [n=18]; median=11; range, 7.5–15 in YF-KI [n=23]; WT versus YF-KI, P=0.034; Figure 1A and 1B). The cortex showed a significant decrease in brain injury in YF-KI mice when compared with WT and the striatum showed a trend toward a significant decrease in brain injury (cortex P=0.013; striatum P=0.067; Figure 1C). There were no differences in injury in the hippocampus (Figure 1C). There were no sex differences in WT or YF-KI mice (Figure 1D).

YF-KI Mice Have Less Activity of Calpain and Caspase and Less Cell Death at 24 Hours After HI

We assessed cell death by examining the activity of protease calpain and caspase-3 for their substrate α-spectrin. Calpain

Figure 1. YF-KI mice have decreased brain injury after neonatal hypoxia-ischemia. A, Brain sections were stained with Cresyl violet and Perl’s iron stain. Arrows indicate patches of cell loss, and arrowheads show iron accumulation in similar injured areas. B, Composite injury score and (D) composite injury score by sex. C, Regional injury scores in cortex, hippocampus, and striatum. The horizontal lines represent the median. WT indicates wild-type. *P<0.05.
cleavage of α-spectrin produces a breakdown product of 150 and 145 kDa, whereas caspase cleavage of α-spectrin produces a 120-kDa fragment. In WT mice, we found increased calpain and caspase activity, as measured by α-spectrin cleavage, at 1, 6, and 24 hours after injury. However, YF-KI mice did not differ from sham animals in calpain and caspase activity (Figure 2A). Consistently, cleaved (activated)-caspase 3 protein levels were elevated in WT mice at 6 and 24 hours after injury but not in YF-KI mice (Figure 2A). Immunofluorescent staining showed markedly reduced cleaved-caspase-3 expression in the cortex of YF-KI mice when compared with WT mice at 24 hours after HI, in line with the extension of neurodegeneration as revealed by Fluoro-Jade B staining (Figure 2B).

pY1472 Affects NR2B Tyrosine Phosphorylation at Specific Sites

Next, we determined the phosphorylation status of the NR2B subunit because YF-KI mice are hypotyrosine phosphorylated in the amygdala. The overall NR2B tyrosine phosphorylation was significantly decreased in the YF-KI mice in sham (reduced 70%) and HI-injured animals (Figure 3A), confirming that Y1472 is the major tyrosine phosphorylated on NR2B. About Fyn-mediated other sites, pY1336NR2B and pY1252NR2B were increased after HI in WT mice (Figure 3B). There was a small increase in pY1070, whose function has not been characterized, at 15 minutes in WT animals (Figure 3B).

Interestingly, YF-KI mice had a significant decrease in the expression of pY1070 (70% reduction), pY1252 (50% reduction), and pY1336 (20% reduction) in sham animals (P<0.05, WT versus YF-KI; Figure 3B). After HI, YF-KI mice had significantly less pY1252 at 15 minutes and less pY1070 for up to 6 hours when compared with WT mice (Figure 3B). pY1336 had a trend toward decreased expression at 15 minutes after HI in YF-KI mice when compared with WT (P=0.083; Figure 3B).

Figure 2. YF-KI mice have less activity of calpain and caspase and less cell death at 24 hours after hypoxia-ischemia (HI). A, Western blots using anti-α-spectrin, cleaved-caspase 3, and β-actin with cortical lysates from sham and HI animals. Expression of spectrin breakdown product (SBDP) 150/145; SBDP 120; and cleaved-caspase 3 was normalized to β-actin. Data (n=4) were normalized to wild-type (WT) sham values (*P<0.05). B, Representative images of brain sections stained with Fluoro-Jade (green) and cleaved caspase-3 (red) at 24 hours after HI.

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YF-KI Mice Have Decreased Superoxide Generation After Neonatal HI

To find out whether pY1472NR2B is functionally linked to superoxide generation, we measured superoxide after neonatal HI in YF-KI mice. At 24 hours after HI, the oxidized dihydroethidium increased significantly only in the WT mice but not in the YF-KI mice (Figure 4A). Consistently, expression of p47phox, the regulatory subunit required for NOX2 assembly, and gp91phox, the catalytic subunit of the enzyme, peaked at 24 hours in the WT mice, whereas in the YF-KI mice, there was substantially less p47phox and gp91 phox expression at 24 hours (P<0.05; Figure 4B).

YF-KI Neurons Have Decreased Superoxide Production and Less Cell Death in Response to NMDA Without Differences in Calcium Response

NMDA treatment of WT neurons caused a significant increase in superoxide production, which was diminished substantially in YF-KI neurons (Mean peak ethidium fluorescence; WT neurons 2.75±0.09 versus YF-KI neurons 1.34±0.04; P<0.001; Figure 5A). Exposure of cortical neurons to NMDA resulted in a 1.6-fold increase in cell death relative to control (P<0.01) and a 1.3-fold increase in response to glutamate (P<0.05). However, cell death was remarkably reduced in YF-KI neurons when treated with NMDA or glutamate (P<0.01; Figure 5B). Interestingly, there was no difference in the increase of total intracellular Ca2+ ([Ca2+]i) induced by NMDA between the WT and the YF-KI neurons (mean peak fluorescence; WT neurons 1.55±0.07 versus YF-KI neurons 1.53±0.057; P=0.875; Figure 6). YF-KI neurons showed a trend of slower [Ca2+]i increase than WT neurons as assessed by Fura4F to capture the initial transient accurately (WT neurons took an average of 16.3±1.31 minutes to reach the peak fluorescence and YF-KI neurons took 18.3±0.94 minutes to reach the peak); however, the difference was not statistically significant.

Discussion

We show for the first time that a mutation of NR2B tyrosine 1472 to phenylalanine (YF-KI) results in neuroprotection from cell death in vivo and in vitro, implicating phosphorylation at this site in the pathogenesis of injury after HI. Our study identifies a previously unrecognized role of Y1472 phosphorylation of NR2B as a mediator of superoxide-associated...
oxidative damage during excitotoxicity, an effect that is independent of intracellular Ca\(^{2+}\) increase.

Studies have demonstrated that NOX2 is the main source of superoxide after NMDAR activation in cortical cultures and in hippocampus in vivo.\(^\text{12}\) We found substantial superoxide production at 24 hours after HI in the WT mice, with concomitant increase of key NOX2 subunit p47\(^{phox}\) and gp91\(^{phox}\), which are required for NOX2 assembly and activation. This suggests that NOX2 might be associated with superoxide formation after neonatal HI, which is in agreement with another HI study in P7 rats,\(^\text{17}\) as well as an ibotenate-induced excitotoxic injury model in P5 mice.\(^\text{18}\) However, we cannot rule out other O\(^{2-}\) sources including those produced in mitochondria or by other enzymes, such as xanthine oxidase and lipoxygenase. These increases are dramatically attenuated in the YF-KI mice in vivo and in vitro, indicating that pY1472 NR2B regulates O\(^{2-}\) formation under HI and excitotoxicity. It should be noted that in vivo both neurons\(^\text{17}\) and microglia\(^\text{18}\) could be the sources of NOX2 activation/O\(^{2-}\) production. Microglia express functional NMDARs, including the NR2B subunit, in murine and human brain although at significantly lower levels than the neurons. Activation of microglia NMDAR triggers inflammation and neuronal cell death in the neonatal and mature brain.\(^\text{19}\) We have not determined the cell-type–specific distribution and expression of pY1472NR2B, so we cannot exclude the role of tyrosine phosphorylation of microglia NR2B in O\(^{2-}\) formation and brain injury.
Our in vitro studies with pure cortical neurons clearly show that NR2B phosphorylation at Y1472 is essential for NMDA-induced superoxide formation and the resultant neuronal death. Calcium influx is required for NMDAR-mediated superoxide production, but our results indicate that calcium rise alone is not sufficient for this effect because YF-KI neurons have similar Ca2+ accumulation in response to NMDA but diminished superoxide generation when compared with WT cells. Other Ca2+-independent mechanisms linking Y1472 phosphorylation and NOX2 superoxide formation remain to be elucidated. Although phosphorylation of this site inhibits NMDAR internalization, there are no convincing data demonstrating a change in NR2B surface expression, in total NMDAR number at synapses, or in NMDAR subunit composition in the YF-KI mice. Our Ca2+ influx results, together with data showing normal basic properties of synaptic transmission in YF-KI mice, suggest that the suppressed superoxide production does not result from reduced NMDAR activity in YF-KI neurons but rather from altered downstream signaling or protein–protein interactions that mediate excitotoxicity.

Mutation of NR2B Y1472 does not cause compensation of tyrosine phosphorylation of other NR2B residues. On the contrary, in neonatal cortex, phosphorylation of at least 3 other Fyn-targeted residues on NR2B-Y1336, Y1252, and Y1070 was decreased in YF-KI mice. Two previous studies did not find changes in phosphorylation of Y1336 or Y1252 in YF-KI mice in amygdala or spinal cord. These discrepancies may be because of differences in brain regions studied or brain maturity. Although the function of pY1070 and pY1252 is unknown, pY1336 mediates the interaction of NR2B with the p85 subunit of PI-3 kinase. In addition, YF-KI mice have decreased Ca2+/calmodulin-dependent protein kinase II (CaMKII) and α-actinin associated with NR2B in amygdala. Therefore, it is likely that changes in multiple tyrosine phosphorylation sites affect recruitment of proteins to the NR2B complex both in the naive state and after injury. How Y1472 phosphorylation regulates protein composition of NR2B complex merits further investigation.

pY1472 NR2B is enriched in synaptic membranes in neonatal cortex and increases after HI. Although there has been debate over the function of synaptic and extrasynaptic NMDARs in cell survival versus death, several recent studies suggest a role for synaptic NMDAR in mediating NMDA-induced neurotoxicity and hypoxic excitotoxic death. Another report found that the C-terminal domain of NR2B is linked to excitotoxic cell death. Therefore, pY1472 is situated to affect cell death processes synthaptically through its ability to modify proteins associated with the C-terminal domain of NR2B. In addition, YF-KI mutation affects synaptic localization of NR1 and NR2B (shifting to the periphery of PSD and the perisynaptic regions), but the consequences of this improper localization are unknown.

Taken together, we provide a mechanistic basis for injury after HI in the neonatal brain through increased NR2B tyrosine phosphorylation by Fyn. Although pY1472NR2B is important for brain physiology, sustained upregulation may initiate downstream cell death signaling. Because neonatal brain is more vulnerable to free radical injury than the mature brain, these findings could advance our understanding of mediators of oxidative damage in the immature brain and may have significant implications for neonatal hypoxic-ischemic encephalopathy therapies.

**Figure 5.** YF-KI neurons have decreased superoxide production in response to N-methyl-d-aspartate (NMDA) and are protected from NMDA- and glutamate-induced cell death. A, Ethidium (Eth) fluorescence before NMDA application (0 minutes), at the time of NMDA addition (5 minutes) and then at 5-minute intervals in wild-type (WT) or YF-KI neurons. Representative Eth fluorescence transients (right) showed superoxide production in WT neurons after NMDA application (arrow) when compared with YF-KI neurons. **P<0.001. B, Propidium iodide labeled dead neurons 24 hours after NMDA or glutamate treatment in WT or YF-KI neurons. **P<0.05, ***P<0.01; n=3.


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