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Role of Reactive Oxygen Species in Modulation of Nrf2 following Ischemic Reperfusion Injury

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Abstract

The transcriptional factor Nrf2 has a unique role in various physiological stress conditions, but its contribution to ischemia/reperfusion injury has not been fully explored. Therefore, wildtype (WT) and Nrf2 knockout (Nrf2^{-/-}) mice were subjected to 90-min occlusion of the middle cerebral artery (MCA) followed by 24-h reperfusion to elucidate Nrf2 contribution in protecting against ischemia/reperfusion injury. Infarct volume, represented as percent of hemispheric volume, was significantly ($P < 0.05$) larger in Nrf2^{-/-} mice than in WT mice ($30.8 \pm 6.1\%$ vs $17.0 \pm 5.1\%$). Furthermore, neurological deficit was significantly greater in the Nrf2^{-/-} mice. To examine whether neuronal protection was mediated by Nrf2, neurons were treated with various compounds to induce excitotoxic or oxidative stress. Translocation of Nrf2 into the nucleus was increased by the free-radical donor *tert*-butylhydroperoxide, but not by glutamate or NMDA. In addition, a common Nrf2 inducer, *tert*-butylhydroquinone, significantly attenuated neuronal cell death induced by *tert*-butylhydroperoxide (83.6 ± 1.6 vs $62.0 \pm 7.7\%$) but not as substantially as when excitotoxicity was induced by NMDA (91.9 ± 1.6 vs $79.3 \pm 3.3\%$) or glutamate (87.8 ± 1.5 vs $80.2 \pm 2.6\%$). The results suggest that Nrf2 reduces ischemic brain injury by protecting against oxidative stress.

Keywords

free radicals; MCA occlusion/reperfusion; NF-E2-related factor 2; stroke

Oxidative stress from reactive oxygen species (ROS) enhances inflammatory responses during tissue injury, possibly through activation of redox-sensitive chemokines and transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 has been reported to be a key

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regulator in cell survival mechanisms (Owuor and Kong, 2002). It induces expression and up-regulation of cytoprotective and antioxidant genes that attenuate tissue injury (Zhang et al., 2005). Under basal conditions, cytoplasmic Nrf2 is bound to the Kelch-like ECH-associated protein 1 (Keap 1), but when cells are exposed to oxidative or xenobiotic stress, Nrf2 dissociates and traverses to the nucleus (Wakabayashi et al., 2003; Li et al., 2004a).

Nrf2 is considered to be a multi-organ protector (for review see Lee et al. (Lee et al., 2005)) and is widely viewed as a mediator of neuroprotection. The small-molecule activator sulforaphane, which induces the expression of multiple Nrf2-responsive genes, has been shown to reduce infarct volume following focal cerebral ischemia in animal and *in vitro* studies (Zhao et al., 2006). Activation of the Nrf2 pathway by both sulforaphane and Nrf2 overexpression in astrocytes was able to provide neurons with protection from non-excitotoxic glutamate toxicity (Kraft et al., 2004). A study by Lee *et al.* (Lee and Johnson, 2004) showed that neural cells from Nrf2^{-/-} mice were more highly sensitive to oxidative stress than were neurons from control animals, but when the cells were transfected with a functional Nrf2 construct, they became less prone to oxidative stress. Consistent with the results of these studies, dominant negative-Nrf2 stable cells and Nrf2-sensitized neuroblastoma cells silenced with siRNA were more susceptible to apoptosis induced by nitric oxide (Dhakshinamoorthy and Porter, 2004).

The published reports suggest that Nrf2 is important for protecting cells and multiple tissues by coordinately up-regulating ARE-related detoxification and antioxidant genes and molecules required for the defense system in each specific environment. However, to our knowledge, no one has shown directly the role of the Nrf2 gene in a transient ischemic reperfusion model or in an *in vitro* system by observing its effect in the presence of potential stressors (such as oxidative stress, NMDA, and glutamate). Therefore, we examined the ability of Nrf2 to protect mice from brain damage caused by transient middle cerebral artery (MCA) occlusion (MCAO) followed by reperfusion and investigated its role in modulating neuronal cell death following *tert*-butylhydroperoxide (*t*-BuOOH)-, glutamate-, and NMDA-induced toxicity.

Experimental procedures

Animals

This study was approved by the Institutional Animal Care and Use Committee of Johns Hopkins University. Female Nrf2^{-/-} and wildtype (WT) mice with CD1 background (20-25 g) were generated as described previously (Itoh et al., 1997; Rangasamy et al., 2005). Mice were subjected to genotyping for Nrf2 status by PCR amplification of genomic DNA extracted from the blood (Ramos-Gomez et al., 2001). Three primers were used to perform PCR amplification: 5'-TGGACGGGACTATTGAAGGCTG-3' (sense for both genotypes), 5'-CGCCTTTTCAGTAGATGGAGG-3' [antisense for wildtype (WT) mice], and 5'-GCGGATTGACCGTAATGGGATAGG-3' (antisense for LacZ). These mice were fed with an AIN-76A diet, given water *ad libitum*, and housed under controlled conditions (23 ± 2°C; 12 h light/dark periods).

Suture preparation

Filaments were made by the method of Shah *et al.* (Shah et al., 2006) and slightly modified to occlude the MCA. Under an operating microscope, a minute quantity of silicone (CutterSil Light, Heraeus Kulzer, GmbH, Hanau, Germany) and hardener (CutterSil Universal, Heraeus Kulzer, Dormagen, Germany) were combined, and 5 mm of a 7-0 Ethilon suture (Ethicon, Inc., Somerville, NJ) was coated with the mixture. The diameter of the resulting filament tip was 180-200 µm. Filaments were dried overnight and used in MCAO surgeries the next morning.

Transient occlusion of the MCA

The first cohort of mice (8 WT; 8 Nrf2^{-/-}) was subjected to 90 min transient MCA occlusion as previously reported (Shah et al., 2006). Briefly, each mouse was anesthetized with halothane (3% initial, 1% to 1.5% maintenance) in O₂ and air (80%:20%). Relative cerebral blood flow (CBF) was measured via a 0.5-mm diameter microfiber attached to the skull over the parietal cortex (6 mm lateral and 1 mm posterior of bregma) with cyanoacrylate glue (Super Glue Gel, Ross Products, Inc.) and connected to a Laser-Doppler flowmeter (DRT4, Moor Instruments Ltd, Devon, England) (Shah et al., 2006). Mice were placed in the supine position, a mid-line incision was made in the neck, and the right common carotid (CCA), external carotid (ECA), and internal carotid (ICA) arteries were isolated. After blocking the CCA, the distal ECA was cut to form a stump. While monitoring the CBF, a silicone-coated monofilament was introduced into the ECA stump and advanced up to 11 mm from the carotid artery bifurcation to block the MCA or circle of Willis. An 80% drop in CBF was considered a successful occlusion. During occlusion, mice were kept in a humidity-controlled, 32°C chamber to help maintain a body core temperature of 37°C. After 90 min of occlusion, mice were briefly re-anesthetized, the midline was reopened, and the filament was removed to establish reperfusion. After the incision was sutured, mice were again placed in the humidity- and temperature-controlled chamber for another 2 h and finally returned to their respective cages for survival up to 24 h.

Measurement of relative CBF and blood gases

A second cohort of mice (5 WT; 5 Nrf2^{-/-}) underwent an identical stroke protocol, but CBF was measured continuously, before and during the occlusion and for 1 h of reperfusion. Blood samples were collected through a PE-10 femoral artery catheter (Intramedic; BD Diagnostic Systems, Sparks, MD) 30 min before MCAO, 1 h after initiation of MCAO, and 1 h after reperfusion. The blood was evaluated for pH, PaO₂, and PaCO₂ via blood gas analysis (Rapidlab 248; Chiron Diagnostic Corporation, Norwood, MA).

Evaluation of neurological deficits

Mice were tested for sensorimotor performance via a 5-point neurological severity scale at 1 h after MCAO, 1 h after reperfusion and 24 h after occlusion (Shah et al., 2006). Briefly, neurological deficits were graded by the following scale: 0, no deficit; 1, forelimb weakness; 2, circling to affected side; 3, inability to bear weight on the effected side; 4, no spontaneous motor activity.

Infarct volume

After 24 h of reperfusion, mice were anesthetized, and their brains were removed and cut into 2-mm coronal sections that were stained with 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma Co, St. Louis, MO). Brain slices were scanned individually, and the unstained area was analyzed by a video image analyzing system (SigmaScan pro 5, Systat, Inc., Point Richmond, CA). Infarct volume was calculated as the percentage of infarct area to the total hemispheric area for each slice.

Primary cultures of neuronal cells

Cortical neuronal cells were isolated from 17-day-old embryos of timed-pregnant mice and cultured in serum-free conditions. Neurons were plated onto poly-D-lysine-coated 24-well dishes at a density of 0.5×10^6 cells/well in HEPES-buffered, high glucose Neurobasal medium with B27 supplement (Invitrogen, Carlsbad, CA), and cultured at 37°C in a 95% air/5% CO₂ humidified atmosphere. As previously described (Echeverria et al., 2005), all experiments were performed after 14 days *in vitro*, using cortical cell cultures that contained 94–95% neuronal cells and 5–6% glia, as determined by immunoreactivity analysis with the neuronal marker

MAP2 (Sigma) and the glial cell markers, GFAP (astrocytes; Sigma) and CD11b (microglia; Serotec, Raleigh, NC).

MTT assay

Cultured cortical neurons were exposed to various drugs for 24 h and then assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) colorimetric assay, an indicator of the mitochondrial activity of living cells. After 2 h incubation at 37°C with 0.5 mg/ml of MTT, living cells containing MTT formazan crystals were solubilized in a solution of anhydrous isopropanol, 0.1 N HCl, and 0.1% Triton X-100. The optical density was measured at 570 nm. All experiments were repeated with at least three separate batches of cultures.

Caspase assay

After cells were incubated for 8 h at 37°C in the presence of the appropriate agents, homogeneous caspase-3/7 reagent (Promega, Madison, WI) was added and incubated at room temperature for 30 min, as described previously (Li et al., 2004b).

Western blot analysis

Cortical neurons were incubated in medium containing B27 minus antioxidant (B27-AO™, Sigma) 2 h before each experiment because this medium does not contain antioxidants that could interfere with the analysis of free-radical damage to neurons. The neurons were exposed to 60 μ M *t*-BuOOH (a free-radical donor), 300 μ M glutamate, or 100 μ M NMDA for 6 h. Experiments were terminated by applying sample buffer. Equivalent amounts of protein per sample were resolved via SDS-PAGE electrophoresis on 10% gels. After electrophoretic transfer, the nitrocellulose membrane was blocked for 1 h at room temperature with 5% dried milk. Then the membrane was incubated overnight at 4°C with anti-actin (1:200; Sigma) or anti-Nrf2 primary antibody (1:200; Santa Cruz, Santa Cruz, CA), an affinity-purified, rabbit, polyclonal antibody raised against a peptide mapping to the C-terminus of human Nrf2 (see product citations) (Bloom and Jaiswal, 2003; Furukawa and Xiong, 2005). With Western blotting, this antibody has been shown to detect Nrf2 of mouse, rat, and human origin at a molecular mass of 57 kDa. After being washed, the membranes were incubated with the appropriate secondary antibody (1:5000, Zymed, San Francisco, CA) for 1 h at 22°C. Immunocomplexes were visualized using enhanced chemiluminescence detection (Amersham, Piscataway, NJ). Western blotting experiments were repeated at least three separate times, and the protein bands were analyzed by Image J software provided by the NIH (Ahmad et al., 2006). The densitometric values were normalized with respect to the values of actin immunoreactivity to correct for any loading and transfer differences between samples.

Isolation of cytosolic/nuclear fractions

Primary mouse cortical neurons were scraped from culture dishes, resuspended in cold Buffer A [10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)], and kept on ice for 10 min. Then, 25 μ l of 10% v/v Nonidet P40 was added to the cell suspension. Samples were then centrifuged at 12,000 g for 5 min at 4°C. The resultant supernatant was removed as the cytosolic fraction. Pellets were resuspended in 80 μ l of Buffer B [20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF] and kept on ice for 20 min for high salt extraction. After a final 2-min centrifugation at 4°C, the supernatant, which contained the nuclear fraction, was collected and stored at -70°C. Samples were analyzed on 10% polyacrylamide gels as described as above.

Statistical analysis

Analysis of variance (ANOVA) with Student's *t*-test was used to compare groups. Data are represented as mean $\pm$ standard error of the mean (S.E.M.). Significance was set at $P < 0.05$.

Results

MCA occlusion and reperfusion

Measurement of infarct volume by TTC staining revealed that the percent of brain damaged by ischemia (corrected for brain edema) was significantly larger in the Nrf2^{-/-} mice ($30.8 \pm 6.1\%$) than in the WT mice ($17.0 \pm 5.1\%$; $P < 0.01$; Fig. 1). Additionally, neurological deficits were significantly greater in the Nrf2^{-/-} mice (3.1 ± 0.3) than in the WT mice (2.5 ± 0.2) 24 h after ischemia, $P < 0.04$ (Fig. 2), whereas at 1 h after MCAO and 1 h after reperfusion, no differences were observed between the two strains. In the second cohort, in which WT and Nrf2^{-/-} mice underwent an identical stroke protocol and were monitored for CBF continuously, no significant differences in CBF were observed at any time point during MCAO or within the first hour of reperfusion. CBF in the MCA territory was reduced to the same level during occlusion in WT and Nrf2^{-/-} mice ($13.5 \pm 2.0\%$ and $11.9 \pm 1.8\%$ of baseline, respectively; Fig. 3). Finally, blood drawn from these mice 30 min before MCAO, 1 h after MCAO, and 1 h after reperfusion revealed that blood gases were within the physiological range before and during surgery and were not different between the groups (Table 1).

tert-BuOOH, glutamate, and NMDA-mediated effects on Nrf2

Mouse cultured cortical neurons were exposed to *t*-BuOOH, glutamate, or NMDA to determine their effects on Nrf2 location in the nuclear and cytosolic fractions. Western blotting revealed a basal level of Nrf2 in the nucleus of cultured neuronal cells before exposure to the chemical stressors. *Tert*-BuOOH induced time-dependent changes in Nrf2 presence in the nuclear fraction. Protein expression was elevated at 30 min and continued to increase through the full time course (360 min; Fig. 4). Furthermore, in the cytosolic fraction, Nrf2 remained at baseline levels for 15 min, but decreased to below baseline after 30 min. In contrast, under our experimental conditions, NMDA and glutamate were unable to stimulate Nrf2 translocation, affecting neither the nuclear nor the cytosolic levels (Fig. 4). The expression levels of actin (used as a positive control) were unaffected by any of the treatments. Figure 4 shows the mean chemiluminescence ratio of Nrf2 to actin for each sample.

Effect of the Nrf2-inducer *tert*-butylhydroquinone (*t*-BHQ) on cell death induced by *t*-BuOOH, NMDA, and glutamate

The MTT assay was used to assess the ability of the Nrf2-inducer *tert*-butylhydroquinone (*t*-BHQ) to modulate the neurotoxicity of *t*-BuOOH, NMDA, or glutamate in primary cultured neurons. Application of *t*-BuOOH (60 μ M), NMDA (100 μ M), and glutamate (300 μ M) each significantly decreased the number of viable neurons after 24 h, compared with the number of untreated control neurons (Fig. 5A). This decrease was abolished by 20 μ M *t*-BHQ. Furthermore, *t*-BHQ alone had no effect on neuronal viability.

To further corroborate the protection observed by *t*-BHQ treatment, we examined the activity of caspase-3, described as a terminal effector of the apoptotic-like cell death pathway. We found that *t*-BuOOH, NMDA, and glutamate each induced an increase in caspase-3 activity, compared with untreated control cells (Fig. 5B). The control groups had only a basal level of caspase 3 activity. Similar to *t*-BuOOH, NMDA and glutamate caused an increase in caspase 3 activity. In contrast, *t*-BHQ had no effect on basal levels of caspase-3 activity but was able to prevent the increase evoked by all three stressors (Fig. 5B).

Discussion

In this study, the MCAO and reperfusion model was selected particularly because it is known to induce a transient focal ischemic cascade that uniquely includes a substantial surge of free radical damage. This model allows us to investigate the role of Nrf2 specifically during oxidative stress/ischemia-mediated brain insult. Interestingly, we showed that Nrf2^{-/-} mice are significantly more prone to ischemic brain injury and neurological deficits than their counterpart WT mice. Although we demonstrated the involvement of Nrf2 in brain protection with the MCAO/reperfusion model, it was still unclear what mechanisms could be involved. Therefore we sought to determine whether the reduction in infarct size mediated by Nrf2 was a result of neuronal protection. To that end, we examined the Nrf2 properties in mouse primary cortical neuronal cultures exposed to different types of stressors. The data suggest that Nrf2 translocation mediated by oxidative stress-induced injury is protective in cultured neurons, and that levels of Nrf2 found in the nucleus increase in response to *t*-BuOOH-mediated oxidative stress but not in response to NMDA- or glutamate-mediated excitotoxicity.

The transient MCAO silicone-coated filament model was selected because it produces consistent infarctions in the areas of striatum and cortex, as already shown in our previous studies with different knockout mice (Shah et al., 2006), and confirmed by the >80% drop in CBF in both Nrf2^{-/-} and WT mice. We observed clear differences in stroke outcome between the Nrf2^{-/-} and WT mice as early as 24 h after reperfusion, whereas Shih *et al.* (Shih et al., 2005) reported differences only after 7 days. One reason for that disparity is that, in contrast to our model, which includes a reperfusion phase characterized by a surge in ROS, they used a permanent occlusion model, which is less likely to produce the high levels of ROS. The disparity between these results may also be attributed to differences between the ischemic models used because the permanent focal ischemia of the distal MCA used in the study by Shih *et al.* produces an infarct that is mostly limited to the cortex. The authors previously raised concerns regarding a high incidence of inconsistent strokes, and for that reason they selected the permanent model rather than the intraluminal model. We avoided these problems by using the silicone-coated filament model (Shah et al., 2006), which has significantly overcome some of the previous reported limitations.

Evidence in the literature indicates that the transcription factor Nrf2 is protective in *in vitro* and *in vivo* studies. Activation of Nrf2 upregulates defensive antioxidant mechanisms in neurons and glia in rat cortex through upregulation of the antioxidant response and ARE-driven genes (Lee et al., 2003; Shih et al., 2003). ARE-regulated genes, such as heme oxygenase-1, I-ferritin, and glutathione peroxidase, maintain redox homeostasis and influence the inflammatory response. Similar pathways involved in detoxifying ROS have been shown to play a crucial role in regulation of other transcription factors, i.e., hypoxia-inducible transcription factor-1 α and cAMP-responsive element binding protein, which are in turn regulated by redox-sensitive transcription factors, NF- κ B and AP-1 (Schreck et al., 1992; Bedogni et al., 2003; Gorlach et al., 2003). Therefore, it is likely that deletion of the Nrf2 gene renders animals more susceptible to various types of stress exposure, including hypoxia, mainly because of failure to induce phase II enzymes.

The main purpose of the *in vitro* work here was to investigate to what extent Nrf2 plays a role in the survival mechanisms of neurons subjected to either excitotoxic or oxidative stressors. Glutamate- and NMDA-mediated excitotoxicity is accepted as a major mechanism of ischemic neuronal damage (Szatkowski and Attwell, 1994). The influx of calcium through glutamate-activated NMDA receptors is thought to relate to neuronal death after ischemia, particularly because glutamate is released in excess from glutamatergic nerve terminals during brain ischemia. Studies suggest that *t*-BuOOH can enhance mitochondrial Ca²⁺ uptake, leading to increases in matrix Ca²⁺, ROS formation, and cell death (Byrne et al., 1999). Oxidative stress,

or electrophilic agents that mimic oxidative stress (Nrf2 inducers), can modify key sulfhydryl group interactions in the Keap-Nrf2 complex, allowing disassociation and nuclear translocation of Nrf2 (Itoh et al., 1999; Dinkova-Kostova et al., 2002). It is probable that glutamate and NMDA do not similarly trigger this Keap-Nrf2 pathway, and in particular, may not produce peroxidation to the extent that *t*-BuOOH does.

As evidenced by Western blot analysis, *t*-BuOOH, but neither glutamate nor NMDA, was able to induce Nrf2 translocation from the cytoplasm to the nucleus. Although Nrf2 was protective against the cell death produced by all three agents *in vitro*, the mechanism by which glutamate and NMDA damage cells (excitotoxicity) is likely to be different than that mediated by *t*-BuOOH (oxidative stress). It has been reported that glutamate and NMDA also can generate cellular oxidative damage (Dawson and Dawson, 1996; Love, 1999; Parfenova et al., 2005), but if the neurotoxicity of glutamate and NMDA were due to oxidative stress in our experiments, we would also expect to see an increase of Nrf2 transactivation. Without the ability to enhance Nrf2 transactivation, glutamate- and NMDA-induced insults might not trigger cell defense mechanisms, at least not those mediated by Nrf2. In future studies, it would be interesting to compare the outcomes of glutamate or NMDA-induced insults in Nrf2^{-/-} mice with those of our current results.

Shih et al. (Shih et al., 2003) showed that exogenous *t*-BHQ can block neuronal cell death induced by glutamate-induced oxidative toxicity, but here we showed that it could protect cells from damage induced by excitotoxicity (NMDA or glutamate) as well as by free radicals (*t*-BuOOH) *in vitro*. It should be noted; however, that Shih et al. used glutamate to evaluate oxidative toxicity (via depletion of glutathione levels) in primary neurons 1 to 4 days *in vitro*, rather than as an NMDA-receptor-mediated excitotoxin. In contrast, we used the cortical neurons after 10 to 14 days *in vitro*, a time at which the primary neurons may have been more mature and therefore expressing functional glutamate channels. Hence, in our studies, glutamate may have played a role as an excitotoxin, not only a glutathione depleting agent.

Our data indicate that the induction of Nrf2 translocation is able to reduce cell death caused not only by excitotoxicity but also by oxidative stress. It appears that Nrf2 might trigger more than one protective mechanism combating these stresses. However, both types of injury could trigger various paths leading to cell death, as illustrated by the ability of Nrf2 to reduce caspase activity induced by each type of stressor. Therefore, the apoptotic pathway is a probable pathway for further investigation.

In conclusion, we have shown that transient ischemia followed by reperfusion induced more extensive brain damage in Nrf2^{-/-} mice than in WT mice. Furthermore, an Nrf2 inducer, *t*-BHQ, was able to reverse neuronal cell death induced by *t*-BuOOH, glutamate, and NMDA. In general, there is mounting evidence that investigation of genes involved in xenobiotic detoxification and oxidative defensive systems could provide insight and pharmacologic perspective in the fight against ischemia-induced brain injury. Therefore, we believe that our results could have direct relevance for drug-targeting paradigms and may prove beneficial for the treatment of stroke.

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List of Abbreviations

ARE	antioxidant response element
CBF	cerebral blood flow
DTT	dithiothreitol
Keap1	Kelch-like ECH-associated protein 1
MCAO	

	Middle cerebral artery occlusion
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMDA	<i>N</i> -methyl-D-aspartic acid
Nrf2	nuclear factor erythroid 2-related factor 2
PMSF	phenylmethylsulfonyl fluoride
ROS	reactive oxygen species
<i>t</i>-BHQ	<i>tert</i> -butylhydroquinone
<i>t</i>-BuOOH	<i>tert</i> -butyl hydroperoxide
TTC	2, 3, 5-triphenyltetrazolium chloride

Fig 1.

Comparison of the infarct volumes of WT and Nrf2^{-/-} mice after middle cerebral artery occlusion (MCAO). Representative photographs show infarcted brains from WT and Nrf2^{-/-} mice (n = 8/group), subjected to 90-min MCAO and 24-h reperfusion. Scale bar represents 1 mm. The histogram represents corrected infarct volume, which was significantly larger in the Nrf2^{-/-} mice (30.8 ± 6.1%) than in the WT mice (17.0 ± 5.1%); **P* < 0.01.

Fig 2. Neurological scores of mice 1, 2, and 24 h after the initiation of ischemia. Neurological dysfunction was significantly greater in the Nrf2^{-/-} mice (3.1 ± 0.3) than in the WT mice (2.5 ± 0.2) 24 h after ischemia; MCAO, midd ; * $P < 0.04$.

Fig 3.

Measurement of cerebral blood flow (CBF). Relative CBF was evaluated in WT and Nrf2^{-/-} mice (n = 5/group) with laser-Doppler flowmetry. Mice underwent 90 min MCAO, and 1 h reperfusion. CBF was monitored from 15 min before MCAO through 1 h of reperfusion. No significant differences in CBF were observed between WT and Nrf2^{-/-} mice at any time during the experiment.

Fig 4.

Effect of *t*-BuOOH, glutamate, and NMDA on protein expression and nuclear accumulation of Nrf2. Primary cortical neurons were incubated for the times shown (min) with serum-free B27 minus antioxidant supplement media alone or that containing *t*-BuOOH (60 μ M), NMDA (100 μ M), or glutamate (300 μ M). Nuclear and cytoplasmic samples were analyzed by Western blotting with antibodies to Nrf2 and actin. The actin expression level was unchanged. The histograms show the ratio of chemiluminescence emitted from the Nrf2 to that of actin. Values shown are means $\pm$ S.E.M. for three independent blots. * $P < 0.001$ vs control.

Fig 5.

Neuronal protection of *t*-BHQ. Neurons were grown for 24 h in culture medium alone (control), or in the presence of *t*-BuOOH (60 μ M), NMDA (100 μ M), or glutamate (300 μ M) with or without *t*-BHQ (20 μ M). (A) Neuronal viability was assessed by MTT assay, and the absorbance at 570 nm is shown (expressed as percent of control). * P <0.001 vs control; # P <0.05 vs *t*-BuOOH, NMDA, or glutamate, respectively. (B) Caspase-3 activity was determined and shown as the amount of fluorescent substrate formed * P <0.001 vs control; # P <0.05 vs *t*-BuOOH, NMDA, or glutamate, respectively.

Table 1

Blood gas measurements before, during, and after MCAO

Parameter	WT			Nrf2 ^{-/-}		
	1 h before MCAO	1 h after MCAO	1 h after reperfusion	1 h before MCAO	1 h after MCAO	1 h after reperfusion
pH	7.39 ± 0.01	7.39 ± 0.02	7.40 ± 0.04	7.40 ± 0.02	7.30 ± 0.04	7.40 ± 0.03
P _a CO ₂	44.0 ± 1.7	44.2 ± 1.9	44.2 ± 1.9	46.0 ± 2.3	45.2 ± 2.6	45.2 ± 2.0
P _a O ₂	122 ± 6	127 ± 5	128 ± 6	128 ± 4	128 ± 4	128 ± 6

Data are given as mean ± S.E.M.; MCAO, middle cerebral artery occlusion