Inhibition of Mitochondrial P53 Abolishes the Detrimental Effects of Social Isolation on Ischemic Brain Injury

Venugopal Reddy Venna, PhD; Rajkumar Verma, PhD; Lena M. O’Keefe, MS; Yan Xu, BS; Joshua Crapser, BS; Brett Friedler, MS; Louise D. McCullough, MD, PhD

Background and Purpose—Social isolation (SI) increases stroke incidence and delays poststroke recovery. Women may be at greater risk from the negative consequences of SI, but few studies have examined both sexes in experimental models, and none have evaluated the effects of isolation initiated after stroke. The effects of poststroke SI in men and women were examined, and the role of mitochondrial P53 was evaluated.

Methods—C57Bl6 mice were pair-housed (PH; male and ovariectomized female) for 2 weeks, subjected to stroke and then assigned to a housing condition (isolated or PH). The effects of housing on infarct volume and recovery were examined. Changes in Bcl-2 and mitochondrial p53 were assessed by Western blot. A mitochondrial p53 inhibitor (pifithrin-μ) was given to mice of both sexes.

Results—Compared with pair-housed mice, poststroke SI significantly increased infarct size in both sexes; SI mice also had worse neurological deficits. The detrimental effects of SI paralleled increases in mitochondrial p53 levels. Pharmacological inhibition of mitochondrial p53 using pifithrin-μ abolished the detrimental effects of SI and reduced cell death.

Conclusions—Poststroke SI results in increased ischemic injury in both sexes. The effect of housing on infarct was more pronounced in women. Targeting the mitochondrial P53 pathway could minimize the detrimental effects of isolation after stroke. (Stroke. 2014;45:00-00.)

Key Words: apoptosis ■ ischemia ■ reperfusion injury ■ sex-specific ■ stroke

Social isolation (SI) enhances morbidity and mortality from a multitude of health conditions, including stroke.1 It is increasingly recognized that sex differences exist in the pathogenesis, presentation, management, and outcome from stroke.2,3 Large gaps still remain in our understanding of the mechanism underlying these sex disparities.4 Early reports suggested that women were less likely than men to receive appropriate care after an acute stroke, but these sex gaps are closing.5 Despite equivalent care, women continue to have poorer functional outcomes compared with age-matched men.6 Importantly, women are 3.5 times more likely than men to be widowed and living alone at the time of their stroke, and this lack of social support and the higher prevalence of depression could be the important contributing factors to the poorer recovery in women.2,4 Attesting to the importance of social factors on stroke outcome, studies have successfully modeled these detrimental effects in animals.6,8 However, most of these experimental studies have been performed exclusively in men who were isolated before stroke. No studies have examined the effects of poststroke isolation in women.

The tumor protein p53 is known to be activated in response to stress and ischemic insults.10–12 Recent studies have demonstrated that mitochondrial association of p53 is an important regulator of mitochondrial membrane pore opening, leading to the release of proapoptotic signaling molecules, including cytochrome c and apoptosis-inducing factor,10,11,13 which are known to exhibit sex differences.14 We hypothesized that sex differences in SI-enhanced stroke injury are mediated by mitochondrial p53.
P53 Inhibitor Treatment

Pifithrin-μ (Calbiochem, CA) was dissolved in dimethylsulfoxide and diluted to 4% in saline (2 mg/kg) and a final volume of 100 μL/10 g body weight of drug or vehicle (4% dimethylsulfoxide in saline) was injected intraperitoneally to randomized mice at 3, 24, and 48 hours after stroke. Pifithrin-μ is a selective and specific mitochondrial inhibitor as determined previously in vivo and in vitro. Binding studies have shown that pifithrin-μ binds to p53. The dose used was determined by pilot experiments and from the literature\(^1\); a low dose was selected to avoid nonselective infarct reduction in all groups with a higher dose. This dose did not completely abolish p53 levels to sham levels but reduced it to levels comparable with PH stroke mice.

Neurological Deficit Scores, Infarct Analysis, and Survival Rates

After 72 hours of reperfusion, the neurological deficit scores (NDSs) were recorded by a blinded investigator as in Ref.\(^7\). Infarcts were quantified from 2,3,5-triphenyltetrazolium chloride–stained coronal sections as detailed previously.\(^6,7\) Mice that died during reperfusion were included in mortality rates, and if paired, the partner was not used for analysis.

Open Field Analysis

Spontaneous locomotor activity was performed during the light phase of the circadian cycle, between 9:00 AM and 12:00 PM under normal fluorescent room lights. For testing, mice were acclimatized to the room conditions and were individually placed in the open field chamber (15″×15″) equipped with 16 infrared beam emitting LEDs on each side for a duration of 20 minutes. The total number of beam breaks was automatically collected by a computer-operated PAS Open Field system (San Diego Instruments, San Diego, CA).\(^6\) The open field chambers were cleaned after each individual test session using 70% ethanol. All animals were assessed by a blinded investigator.

Western Blots

An additional cohort of mice was euthanized at 24 hours after stroke for protein analysis. Brains were homogenized, and a portion of lysate was used for whole cell lysate analysis. The mitochondrial fraction was obtained as described previously.\(^4\) Protein levels were assessed for mitochondrial P53 (1:200; abcam) and Bcl-2 (1:500; cell signaling) using actin and cytochrome c oxidase (COX IV; 1:2000; abcam) as loading controls. Densitometry was performed with ImageJ software.

Statistics

Data are presented as mean±SEM except for NDS, which was presented as median (interquartile range). IBM SPSS Ver.20 was used to perform Student’s t test or analysis of variance with Tukey post hoc correction to determine significance and interactions. P<0.05 was considered statistically significant. Investigators performing infarct size analysis and NDS were blinded to treatment conditions.

Results

Infarct Analysis

Poststroke SI significantly exacerbated infarct size compared with PH animals both in males (Figure 1A; P<0.05; t test) and females (Figure 1B; P<0.001; t test). Equivalent blood flow reduction was seen by laser Doppler in both sexes and in both housing conditions. A significant 3-way interaction between SI, infarct size, and sex was also found F(1,44)=4.2, P<0.05, suggesting that the detrimental effect of housing on infarct is more pronounced in ovariectomized females.

NDS and Mortality

The detrimental effect of SI was also reflected in the NDS. SI males had significant worsening of their NDS (Figure 1C; P<0.05) as did females (Figure 1D; P<0.05) compared with PH. A higher mortality was also seen in SI male (29%) and female (36%) mice compared with PH male (7%) and female (0%) mice.

Protein Analysis

Analysis of variance for mitochondrial P53 protein levels revealed a significant effect of stroke in males (Figure 2B;
Poststroke Isolation and P53

Bcl-2 levels were significantly elevated after stroke in both housing conditions, but this increase was less in SI mice (Figure 2B and 2D; \(P<0.05\)) and in SI females \((P<0.05)\) compared with PH mice. Moreover, a significant stroke×housing interaction was seen in both sexes \((P<0.05)\), suggesting that stroke-induced increases in p53 and Bcl-2 expression was significantly altered in SI mice versus PH mice.

Pifithrin-\(\mu\) Treatment Reverses SI Effects

Pifithrin-\(\mu\) (2 mg/kg) abolished the detrimental effects of SI on infarct (Figure 3A) but had no neuroprotective effect in PH mice. Two-way analysis of variance yielded a significant effect of housing \((F(1,42)=17.1, P<0.05)\) and a significant effect of drug \((F(1,42)=18.7, P<0.05)\), and a significant interaction between housing and drug \((F(1,42)=10.9, P=0.002)\) in males (Figure 3A) and also in females (Figure 3B). The beneficial effect of P53 inhibition was also reflected in the restoration of spontaneous locomotor activity after stroke in SI males. Two-way analysis of variance yielded a significant effect of housing, \(F(1,42)=4.49, P<0.05\), and a significant effect of drug, \(F(1,42)=4.38, P<0.05\), and a significant interaction between housing and drug, \(F(1,42)=5.88, P<0.05\). These findings indicate that drug abolished the detrimental effects of isolation.

Discussion

In this study, we found that poststroke SI significantly worsens stroke damage in both sexes, and this effect can be abolished by inhibition of mitochondrial P53 activation. SI before stroke worsens outcomes in experimental models, as well as in clinical populations, in both sexes.\(^{2,6,8,9}\) However, much less is known about the effects of SI initiated after injury. As most patients who are isolated do not come to medical attention until after the injury occurs, the ability to manipulate post-stroke housing environments has broader translational significance to functional recovery. Stroke is a sexually dimorphic disease. Several molecular pathways have been identified as important contributors to cell death after ischemic injury, and these pathways, although not completely distinct, are differentially regulated by sex. Women are more sensitive to caspase-dependent cell death, whereas caspase-independent or poly[ADP-ribose] polymerase-1–mediated cell death predominates in men.\(^{4,14}\) Both these cell death signaling pathways converge on mitochondrial permeability transition pore dysfunction.\(^{10,11}\) This work suggests that mitochondrial stress underlies some of the detrimental effects of SI. Other mechanisms may also contribute such enhancement of inflammation or late effects on cerebral blood flow.

In this work, we found that SI significantly increased cell death after ischemic stroke in both sexes, although women were more susceptible to the detrimental effects of isolation.

\(P<0.05\) and females (Figure 2D) both by stroke \((P<0.05)\) and by housing \((P<0.05)\). Bcl-2 levels were significantly elevated after stroke in both housing conditions, but this increase was less in SI mice (Figure 2B and 2D; \(P<0.05\)) and in SI females \((P<0.05)\) compared with PH mice. Moreover, a significant stroke×housing interaction was seen in both sexes \((P<0.05)\), suggesting that stroke-induced increases in p53 and Bcl-2 expression was significantly altered in SI mice versus PH mice.

Pifithrin-\(\mu\) Treatment Reverses SI Effects

Pifithrin-\(\mu\) (2 mg/kg) abolished the detrimental effects of SI on infarct (Figure 3A) but had no neuroprotective effect in PH mice. Two-way analysis of variance yielded a significant effect of housing \((F(1,42)=17.1, P<0.05)\) and a significant effect of drug \((F(1,42)=18.7, P<0.05)\), and a significant interaction between housing and drug \((F(1,42)=10.9, P=0.002)\) in males (Figure 3A) and also in females (Figure 3B). The beneficial effect of P53 inhibition was also reflected in the restoration of spontaneous locomotor activity after stroke in SI males. Two-way analysis of variance yielded a significant effect of housing, \(F(1,42)=4.49, P<0.05\), and a significant effect of drug, \(F(1,42)=4.38, P<0.05\), and a significant interaction between housing and drug, \(F(1,42)=5.88, P<0.05\). These findings indicate that drug abolished the detrimental effects of isolation.

Discussion

In this study, we found that poststroke SI significantly worsens stroke damage in both sexes, and this effect can be abolished by inhibition of mitochondrial P53 activation. SI before stroke worsens outcomes in experimental models, as well as in clinical populations, in both sexes.\(^{2,6,8,9}\) However, much less is known about the effects of SI initiated after injury. As most patients who are isolated do not come to medical attention until after the injury occurs, the ability to manipulate post-stroke housing environments has broader translational significance to functional recovery. Stroke is a sexually dimorphic disease. Several molecular pathways have been identified as important contributors to cell death after ischemic injury, and these pathways, although not completely distinct, are differentially regulated by sex. Women are more sensitive to caspase-dependent cell death, whereas caspase-independent or poly[ADP-ribose] polymerase-1–mediated cell death predominates in men.\(^{4,14}\) Both these cell death signaling pathways converge on mitochondrial permeability transition pore dysfunction.\(^{10,11}\) This work suggests that mitochondrial stress underlies some of the detrimental effects of SI. Other mechanisms may also contribute such enhancement of inflammation or late effects on cerebral blood flow.

In this work, we found that SI significantly increased cell death after ischemic stroke in both sexes, although women were more susceptible to the detrimental effects of isolation.
Although statistically significant, this interaction between isolation, infarct, and sex requires further study to determine its physiological relevance. Both sexes responded to a p53 inhibitor, with a reversal in isolation-induced brain injury. Future studies are needed to specifically examine poststroke depressive phenotypes and cognitive deficits after SI in both sexes, using chronic end points and aged animals. As these deficits often lead to nursing home placement in stroke survivors, especially elderly women, the effect of social factors on stroke recovery deserves investigation.

Conclusions
Our findings suggest that mitochondrial association of p53 is an important underlying mechanism for SI-enhanced ischemic injury in both sexes. These findings demonstrate that poststroke SI has detrimental effects on ischemic injury. Consistent with clinical studies, men seem to have a greater susceptibility to the negative effects of isolation.

Sources of Funding
This work was supported by National Institutes of Health R01 NS050505 and NS055215 (to L.D. McCullough) and American Heart Association grant 11POST7430045 (to V.R. Venna).

Disclosures
None.

References
Inhibition of Mitochondrial P53 Abolishes the Detrimental Effects of Social Isolation on Ischemic Brain Injury
Venugopal Reddy Venna, Rajkumar Verma, Lena M. O'Keefe, Yan Xu, Joshua Crapser, Brett Friedler and Louise D. McCullough

Stroke. published online September 9, 2014;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/early/2014/09/09/STROKEAHA.114.006553

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/