Regulated Intramembrane Proteolysis of the Low-Density Lipoprotein Receptor-Related Protein Mediates Ischemic Cell Death

Rohini Polavarapu,* Jie An,*[†] Chen Zhang,* and Manuel Yepes*

From the Department of Neurology and Center for Neurodegenerative Disease,* Emory University School of Medicine, Atlanta, Georgia; and the Institute of Pharmacology,[†] Shandong University School of Medicine, Jinan, China

The low-density lipoprotein receptor-related protein (LRP), a member of the low-density lipoprotein receptor gene family, mediates cellular signal transduction pathways. In this study we investigated the role of LRP in cell death. We found that incubation of mouse embryonic fibroblasts in serum-free media induces caspase-3 activation, an effect that is attenuated in LRP-deficient (LRP^{-/-}) mouse embryonic fibroblasts. Since we previously demonstrated that middle cerebral artery occlusion (MCAO) in mice induces shedding of the LRP ectodomain, we investigated here whether cerebral ischemia induces regulated intramembrane proteolysis of LRP and whether this process is related to cell death. We found that MCAO induces an increase in γ -secretase activity in the ischemic hemisphere and that treatment with the γ -secretase inhibitor L-685,458 improves the neurological outcome and results in a 50% decrease in the volume of the ischemic lesion. Furthermore, MCAO caused nuclear translocation of the intracellular domain of LRP in neurons within the area of ischemic penumbra, and this effect was attenuated in mice treated with L-685,458. Finally, inhibition of either LRP or γ -secretase attenuated cerebral ischemia-induced caspase-3 cleavage and apoptotic cell death. In summary, our results indicate that γ -secretase-mediated regulated intramembrane proteolysis of LRP results in cell death under ischemic conditions. (*Am J Pathol* 2008, 172:1355–1362; DOI: 10.2353/ajpath.2008.070975)

The low-density lipoprotein receptor-related protein (LRP) is a member of the low-density lipoprotein receptor gene family composed of a 515-kDa heavy chain noncovalently

bound to an 85-kDa light chain containing a transmembrane and a cytoplasmic domain.¹ LRP has been implicated not only in the internalization of multiple ligands,^{1–4} but also in cellular signal transduction pathways⁵ and neurotransmission.⁶ Recent work has suggested that LRP also plays a role in cell death.⁷ Likewise, we have previously demonstrated that cerebral ischemia induces the shedding of LRP's ectodomain *in vivo*,⁸ and that inhibition of this process results in attenuation of cerebral ischemia-induced nuclear factor (NF)- κ B pathway activation in astrocytes and decrease of the volume of the ischemic lesion.⁹

Regulated intramembrane proteolysis (RIP) is a highly conserved mechanism of cell signaling¹⁰ that is mediated by different families of intramembrane proteases including the presenilin-dependent γ -secretase.¹¹ RIP is initiated outside the membrane in response to ligand binding, provoking a conformational change that triggers a second intramembrane cleavage event that results in the release of an active cytoplasmic fragment. *In vitro* studies have demonstrated that like other receptors such as Notch,¹² LRP1B¹³ and the amyloid precursor protein (APP),¹⁴ LRP undergoes cleavage of its cytoplasmic site with release of the intramembranous domain,¹⁵ suggesting that RIP of LRP plays a role in cell signaling events.¹⁶

Ischemic stroke is a leading cause of disability and the second cause of mortality in the world.¹⁷ After the onset of the ischemic insult there is activation of cell signaling pathways that lead to cell death.¹⁸ A growing body of evidence indicates that apoptosis mediated by activation of a group of cysteine-aspartyl-specific proteases known as caspases¹⁹ is an important mechanism of cell death in the ischemic brain.^{20,21} Here we demonstrate that middle cerebral artery occlusion (MCAO) induces γ -secretase-dependent RIP of LRP with nuclear translocation of LRP's

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Address reprint requests to Manuel Yepes, Department of Neurology and Center for Neurodegenerative Disease, Whitehead Biomedical Research Building, 615 Michael St., Suite 505J, Atlanta, GA 30322. E-mail: myepes@emory.edu.

intracellular domain (ICD), and that inhibition of this process results in a significant attenuation of cerebral ischemia-induced caspase-3 cleavage and apoptotic cell death. In summary, we report that RIP of LRP is a novel pathway for cerebral ischemia-induced cell death and a potential target for the treatment of patients with acute ischemic stroke.

Materials and Methods

Cleaved Caspase-3 and Terminal dUTP Nick-End Labeling (TUNEL) Staining

To study the effect of LRP deficiency on cell death LRP^{-/-} (PEA-13) and LRP^{+/-} (PEA-10) mouse embryonic fibroblasts (MEFs; American Type Culture Collection, Manassas, VA) were incubated with normal serum (NS) or serum-free media (SFM). To investigate the effect of LRP inhibition on neuronal cell death primary cortical neuronal cultures were prepared from wild-type (WT) C57BL/6J mice as described elsewhere,^{22,23} and incubated with NS or SFM either alone or in combination with the receptor-associated protein (RAP) (9 μ mol/L; kindly provided by Dr. Dudley K Strickland, University of Maryland, Baltimore, MD). Twelve hours later, both MEFs and neurons were fixed and stained with either an antibody directed against active-caspase-3 (1:500; Cell Signaling Technology, Beverly, MA) or with the ApopTag Plus Fluorescein *In Situ* Apoptosis Detection Kit S7111 (Millipore, Billerica, MA) following the instructions provided by the manufacturer. To determine the number of cleaved caspase-3- and TUNEL-positive cells, images were digitized in an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) (20-fold objective) with a Zeiss AxioCam and imported into AxioVision. Images were then viewed at 150% of the original $\times 20$ images with an Image MetaMorph Software (Molecular Devices, Sunnyvale, CA). The number of cleaved caspase-3- and TUNEL-positive cells was expressed as a percentage of the total number of cells in each field. Each experiment was repeated six times. Statistical analysis was performed with a one-way analysis of variance test.

Animal Model, Neurological Examination, and Quantification of the Volume of the Ischemic Lesion

WT C57BL/6J mice were anesthetized with 4% chloral hydrate. The rectal and masseter muscle temperatures were controlled at 37°C with a homeothermic blanket. Cerebral perfusion in the distribution of the middle cerebral artery was monitored throughout the surgical procedure with a laser Doppler (Perimed Inc., North Royalton, OH), and only animals with a >80% decrease in cerebral perfusion were included in this study. The middle cerebral artery was exposed and occluded with a 10-0 suture as described.^{24,25} Immediately after MCAO, a subgroup of mice was placed on a stereotactic frame and intracortically injected with 2 μ l of either phosphate-buffered

saline (PBS) or the γ -secretase inhibitor L-685,458 (0.5 μ mol/L; Sigma-Aldrich, St. Louis, MO), or purified goat anti-LRP antibodies (85 μ g/ml), or the receptor-associated protein RAP (9 μ mol/L). RAP and anti-LRP antibodies were kindly provided by Dr. Dudley Strickland. The injections were performed at bregma, -1 mm; mediolateral, 3 mm; and dorsoventral, 3 mm for 5 minutes and the infusion rate was controlled by a microsyringe pump controller (World Precision Instruments, Sarasota, FL) attached to a syringe holder (World Precision Instruments). After the end of the infusion the needle was left in place for 5 minutes to avoid reflow. After 1, 24, and 48 hours of MCAO mice underwent a neurological assessment in a blinded manner using a five-point neurological deficit score (0, no deficit; 1, failure to extend right paw; 2, circling to the right; 3, falling to the right; and 4, unable to walk spontaneously). After the last neurological evaluation, animals were sacrificed and brains were sectioned at 1 mm thickness and stained with 2,3,5-triphenyltetrazolium chloride to measure the volume of the ischemic lesion as described.^{9,23,24,26} Eight animals were included in each experimental group. Statistical analysis was performed with one-way analysis of variance test. All procedures were approved by the Emory University Institutional Animal Care and Use Committee.

Definition of Areas of Interest and Laser Confocal Microscopy Studies

The areas of interest (AOI) were previously described by magnetic resonance imaging parameters.^{8,27} In brief, two AOI (AOI-1 and AOI-2) were chosen in the area surrounding the necrotic core (ischemic penumbra), whereas AOI-3 was located within the necrotic core. For the immunohistochemical studies, 20 frozen brain sections 10 μ m each were obtained 24 hours after MCAO and co-stained with the following antibodies: the neuronal marker β -tubulin (1:500; Covance, Princeton, NJ), a monoclonal antibody directed against the 13 amino acids of the C terminus of LRP (1:500; a kind gift from Dr. Dudley K. Strickland), and the nuclear marker 4,6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA). Goat anti-rabbit secondary antibodies conjugated to Alexa 488 (Invitrogen) and donkey anti-mouse antibodies conjugated to Rhodamine Red-X (Jackson ImmunoResearch, West Grove, PA) were used as secondary antibodies. As controls, a separate set of coverslips were incubated with an IgG isotype control or with the secondary antibody only. The determination of nuclear translocation of LRP was performed with a laser confocal microscope (Carl Zeiss). The number of neurons with nuclear translocation of LRP and the percentage of the area of overlap between DAPI and LRP in each cell was determined in each AOI using Metamorph Imaging System software (Universal Imaging Corp., West Chester, PA) as previously described.²⁸ Each field ($\times 40$ magnification) contained at least 20 cells and single cells were selected by manually tracing cell outlines. To subtract background, the threshold of each channel was set at the

value obtained for background. To set the threshold, the average pixel intensity +1 SD was measured for each image. The data are presented as the mean of six obser-

ventions and analyzed with one-way analysis of variance test. For the studies of caspase-3 activation a subset of brains was stained with β -tubulin (Covance), DAPI, and

Figure 1. Effect of LRP deficiency on cell death. **A:** Representative Western blot analysis of cleaved caspase-3 in PEA-10 and PEA-13 MEFs 12 hours after incubation with NS or SFM. S represents PEA-10 (LRP^{+/+}) MEFs treated with staurosporine (0.5 μ mol/L). Actin expression levels were assayed as a control for protein loading. Each observation was repeated four times. **B:** Quantification of the mean density of the band of Western blot analyses for cleaved caspase-3 from **A** ($n = 5$, $*P < 0.05$). **C:** Mean percentage of cleaved caspase-3-positive cells in PEA-10 (LRP^{+/+}, white bars) and PEA-13 (LRP^{-/-}, black bars) MEFs after 12 hours of incubation with either NS or SFM. Error bars depict SEM ($n = 6$, $*P = 0.042$) when compared to LRP^{-/-} cells incubated with SFM. **D:** Representative micrograph of PEA-10 (LRP^{+/+}) and PEA-13 (LRP^{-/-}) MEFs incubated under NS and SFM and stained with an antibody against cleaved caspase-3. Blue is DAPI and green is cleaved-caspase-3. **E:** Mean percentage of TUNEL-positive cells in PEA-10 (LRP^{+/+}, white bars) and PEA-13 (LRP^{-/-}, black bars) MEFs after 12 hours of incubation with either NS or SFM. Error bars depict SEM ($n = 6$, $*P = 0.013$) when compared to LRP^{-/-} cells incubated with SFM. **F:** Representative micrograph of TUNEL staining in PEA-10 and PEA-13 MEFs incubated under NS and SFM. Blue is DAPI and green is TUNEL-positive cells. Original magnifications, $\times 40$.

cleaved caspase-3 (1:500, Cell Signaling Technology). Each observation was repeated six times.

γ -Secretase Assay

γ -Secretase activity was measured in sham mice and 10, 30, 60, and 360 minutes after MCAO and treatment with either vehicle (control) or L-685,458 (Sigma-Aldrich) as described elsewhere.²⁹ Briefly, the ischemic and non-ischemic hemispheres were homogenized in a buffer containing 20 mmol/L HEPES, 50 mmol/L KCl, 2 mmol/L ethylenediaminetetraacetic acid, and protease inhibitor cocktail, and centrifuged at $800 \times g$ for 10 minutes to remove nuclei. The supernatant was then collected, centrifuged at $100,000 \times g$ for 1 hour and the pellet was washed, centrifuged for 30 minutes at $100,000 \times g$, and resuspended in the homogenizing buffer. The membranes were homogenized in buffer with 1% CHAPSO and centrifuged at $100,000 \times g$ for 1 hour. The supernatant was incubated overnight with 8 μ mol/L NMA(2-N-methylaminobenzoyl)-Gly-Gly-Val-Val-Ile-Ala-Thr-Val-Lys[D-NP(dinitro-phenyl)]-D-Arg-D-Arg-D-Arg-NH₂ (Calbiochem, San Diego, CA), an internally quenched fluorogenic peptide containing the amino acid sequence of the C terminus of the APP that is cleaved by γ -secretase. This generates cleavage sites resulting in enhanced fluorescence,³⁰ that was measured at excitation 350 nm and emission 440 nm. Each observation was repeated eight times and results were analyzed with one-way analysis of variance test.

Western Blot Analysis

Polyclonal antibodies to total caspase-3 and cleaved caspase-3 were purchased from Cell Signaling Technology. Monoclonal antibodies to β -actin were obtained from Sigma-Aldrich. WT mice underwent MCAO followed by the intracerebral injection of either PBS or RAP or LRP-blocking antibodies or L-685,458. Brains were extracted 24 hours after MCAO for analysis of caspase-3 activation. WT and LRP^{-/-} MEFs were plated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. After reaching 80% confluence cells were maintained either on complete medium or in SFM for 12 hours. Tissue and cells were processed and gels were loaded as described elsewhere.^{9,31} In each case, the density of the bands was measured with the Image Analyzer System (National Institutes of Health, Bethesda, MD). Each observation was repeated four times.

Results

Effect of LRP Deficiency on Caspase-3 Activation

To study the role of LRP on cell death, we performed Western blot analysis and immunohistochemical studies for active (cleaved) caspase-3 in LRP^{+/-} (PEA-10) and LRP^{-/-} (PEA-13) MEFs and in WT neurons incubated for

12 hours with NS or SFM alone or in combination with the receptor-associated protein (RAP). We found that incubation with SFM increased the expression of active caspase-3 in PEA-10 MEFs and that this effect was significantly attenuated in LRP^{-/-} MEFs (Figure 1, A and B). Indeed, the percentage of MEFs immunopositive for cleaved caspase-3 after 12 hours of incubation in SFM increased from $5.1 \pm 1.4\%$ to $15.5 \pm 4.14\%$ in PEA-10 cells ($n = 6$, $P = 0.032$) and from $2.92 \pm 0.73\%$ to $7.03 \pm 1.41\%$ in PEA-13 MEFs ($n = 6$, $P = 0.079$; Figure 1C) ($*P < 0.05$ when WT cells incubated with SFM were compared with either PEA-13 cells incubated with SFM, or PEA-10 and PEA-13 cells incubated with NS). Likewise, the percentage of cleaved caspase-3-positive WT neurons increased from $3.4 \pm 0.59\%$ after incubation with NS to $27.58 \pm 4.8\%$ in neurons incubated with SFM, and decreased to $8.65 \pm 2.2\%$ in neurons incubated with SFM and RAP (Figure 2A; $n = 6$, $*P < 0.05$ when neurons incubated with SFM are compared with either cells incubated with NS or neurons incubated with NS and RAP).

Effect of LRP Deficiency on Apoptotic Cell Death

To characterize further the role of LRP on cell death, we quantified the number of TUNEL-positive cells in PEA-10 and PEA-13 MEFs after 12 hours of exposure to NS or SFM. We found that incubation with SFM increased the percentage of TUNEL-positive cells from $2.55 \pm 1.02\%$ to $29.8 \pm 4.2\%$ in PEA-10 MEFs ($n = 6$, $P < 0.05$), and from $4.1 \pm 1.5\%$ to $10.9 \pm 4.2\%$ in PEA-13 cells ($n = 6$, $P = 0.16$). $*P = 0.013$ when PEA-10 MEFs incubated with SFM were compared to PEA-13 cells incubated with SFM (Figure 1, E and F). Likewise, the percentage of TUNEL-positive WT neurons increased from $2.3 \pm 0.39\%$ after incubation with NS to $13.31 \pm 1.77\%$ in neurons incubated with SFM, and decreased to $5.35 \pm 0.68\%$ in neurons incubated with SFM and RAP (Figure 2B; $n = 6$, $*P < 0.05$ when neurons incubated with SFM are compared with either cells incubated with NS or neurons incubated with NS and RAP).

Figure 2. Effect of LRP inhibition on apoptotic cell death in neurons. **A** and **B**: Mean percentage of cleaved caspase-3-positive (**A**) and TUNEL-positive (**B**) WT neurons after 12 hours of incubation with either NS (white bar) or SFM either alone (black bar) or in combination with RAP (SFM plus RAP, gray bar). Error bars depict SEM ($n = 6$ to 9 , $*P < 0.05$) when compared to either neurons incubated with NS or cells incubated with SFM and RAP.

Figure 3. Effect of cerebral ischemia on γ -secretase activity. Quantitative analysis of γ -secretase activity in sham animals (black bar) or in the ischemic hemisphere of either vehicle control-treated (white bar) or L-685,458-treated (gray bar) mice 30 minutes after MCAO ($n = 8$). Lines denote SEM. * $P < 0.05$ compared to sham and L-685,458-treated mice.

Effect of Cerebral Ischemia on γ -Secretase Activity

To study the effect of cerebral ischemia on γ -secretase activity, we incubated brain extracts from WT mice 0 to 6 hours after MCAO with a fluorogenic peptide substrate containing the C-terminal amino acid sequence of APP that is cleaved by γ -secretase. We found that compared to sham-operated animals the onset of the ischemic insult induced a transient increase in γ -secretase activity 30 minutes after MCAO (1.8 ± 0.17 -fold increase compared to sham animals, $n = 8$; * $P < 0.05$) that was attenuated after treatment with the γ -secretase inhibitor L-685,458 (Figure 3).

γ -Secretase Inhibition Improves the Neurological Outcome after MCAO

To investigate the role of γ -secretase in the ischemic brain, WT mice underwent MCAO and treatment with the γ -secretase inhibitor L-685,458 followed 1, 24, and 48 hours later by a five-point neurological examination and measurement of the volume of the ischemic lesion. We found that compared to control mice, inhibition of γ -secretase resulted in a faster improvement in neurological function (Figure 4A), and in a 49% decrease in the volume of the ischemic lesion ($n = 8$, * $P < 0.05$; Figure 4B).

Figure 4. Effect of γ -secretase inhibition after MCAO. **A:** Neurological examination 6, 24, and 48 hours after MCAO and treatment with either vehicle (controls, **white diamonds**) or L-685,458 (**black triangles**). **Black circles** correspond to sham animals ($n = 8$). * $P < 0.05$ compared to vehicle-treated mice. Lines denote SEM. **B:** Mean volume of the ischemic lesion in the same cohort of animals in **A** 48 hours after MCAO. Black bar corresponds to vehicle-treated mice. White bar represents L-685,458-treated mice. $n = 8$. * $P < 0.05$.

Cerebral Ischemia Induces RIP of LRP

To determine the effect of cerebral ischemia on LRP's RIP and to investigate the role of γ -secretase in this process, we used laser confocal microscopy to quantify the number of neurons with nuclear translocation of LRP-ICD 24 hours after MCAO and treatment with either vehicle or L-685,458. We found that the number of neurons with nuclear translocation of LRP-ICD increased from 6 ± 1 cells in sham animals to 120 ± 12 in vehicle-treated mice, and that this effect is significantly attenuated by treatment with the γ -secretase inhibitor L-685,458 (30 ± 8 cells, $n = 6$, * $P < 0.05$; Figure 5, A–C). Additionally, the average percentage co-localization of LRP-ICD and DAPI in neurons with nuclear translocation of LRP-ICD was $2.0 \pm 0.3\%$ in sham animals, $52 \pm 15\%$ in vehicle-treated mice, and $12 \pm 3\%$ after treatment with L-685,458 ($n = 6$, * $P < 0.05$; Figure 5D).

Role of RIP of LRP on Cerebral Ischemia-Induced Cell Death

To study whether LRP's RIP has an effect on cerebral ischemia-induced cell death, we performed immunohistochemical studies and Western blot analysis for cleaved caspase-3, 6 hours after MCAO and treatment with either the receptor-associated protein (RAP) or anti-LRP antibodies or L-685,458. Our results indicate that inhibition of either LRP with RAP or anti-LRP antibodies, or blockade of γ -secretase-dependent LRP's intramembrane proteolysis with L-685,458 results in a significant decrease in MCAO-induced caspase-3 cleavage (Figure 6).

Discussion

The low-density LRP is a member of the low-density lipoprotein receptor gene family that mediates the internalization of apoE-enriched lipoprotein particles,³ α -2-macroglobulin-protease complexes⁴ and several other ligands including plasminogen activators, proteinase-inhibitor complexes, clotting factors, and the APP.¹ In the central nervous system LRP is found in neurons and perivascular astrocytes.³² In neurons LRP mediates events such as long-term potentiation⁶ and calcium influx

Figure 5. γ -Secretase mediates cerebral ischemia-induced nuclear translocation of LRP-ICD. **A** and **B:** **a–i:** Representative pictures of laser confocal microscopy analysis of LRP-ICD expression in the area of ischemic penumbra (AOI-2) in sham animals (**a–c**) or 6 hours after MCAO and treatment with either vehicle (control, **d–f**), or the γ -secretase inhibitor L-685,458 (**g–i**). **Arrows** in **e** depict examples of cells with nuclear translocation of LRP-ICD. Green is β -tubulin, red is LRP-ICD, and blue is DAPI. $n = 6$. **j–l:** The expression of LRP extracellular domain (R2629 antibody) in brain sections contiguous to those presented in **d–f**. Green is β -tubulin, red is LRP-extracellular domain, and blue is DAPI. **C:** Quantification of neurons exhibiting nuclear translocation of LRP-ICD in the three AOI in sham animals (white bar), or 6 hours after MCAO in mice treated with vehicle (black bar) or L-685,458 (gray bar) immediately after the onset of the ischemic insult. Error bars describe SEM ($n = 6$). * $P < 0.05$ compared to sham and L-685,458-treated mice. **D:** Average co-localization of LRP-ICD and DAPI in neurons exhibiting nuclear translocation of LRP-ICD in the three AOI in sham animals and 6 hours after MCAO and treatment with either vehicle (black bar) or L-685,458 (gray bar) ($n = 6$). * $P < 0.05$ compared to controls and L-685,458-treated mice. Error bars describe SEM. Original magnifications: $\times 40$ (**A**); $\times 100$ (**B**).

via NMDA receptors.³³ In astrocytes, the interaction between tissue-type plasminogen activator (tPA) and LRP increases the permeability of the neurovascular unit.^{8,25} LRP is also expressed in smooth muscle cells, and specific deletion of the LRP gene from vascular smooth muscle cells on a background of low-density lipoprotein receptor deficiency causes smooth muscle cell proliferation,

increased susceptibility to cholesterol-induced atherosclerosis, and aneurysm formation.³⁴

A link between LRP and cell death has been recently identified. In fact, it has been reported that genetic deficiency of LRP is associated with an increase in tumor necrosis factor- α -induced caspase-3 cleavage.⁷ In contrast, our results indicate that both the expression of

cleaved caspase-3 and the percentage of TUNEL-positive cells after incubation with SFM is attenuated by genetic deficiency of LRP. This apparent contradiction sug-

gests that the role of LRP on cell death may depend of the mechanism of injury. Caspase-mediated apoptosis is a mechanism of ischemic cell death in the murine and human brain.²⁰ Therefore, to better characterize the role of LRP on cell death *in vivo* we used an animal model of focal cerebral ischemia.

There is a growing body of evidence indicating that LRP plays a role in the pathophysiology of cerebral ischemia.^{8,25,27,35,36} Indeed, we have demonstrated that after the onset of the ischemic insult LRP expression increases in the area of ischemic penumbra, and that inhibition of LRP with either RAP or anti-LRP antibodies decreases the volume of the ischemic lesion after MCAO.^{8,25,27}

RIP is a highly conserved mechanism of cell signaling mediated by intramembrane proteases that cleave substrates within their transmembrane domains (TMDs).¹¹ RIP requires two cleavage events. In the first, ligand binding results in ectodomain shedding of the transmembrane protein. This generates a membrane-bound protein that in a second step is cleaved within the TMD releasing the ICD.¹³ *In vitro* studies have indicated that LRP's RIP is preceded by shedding of the receptor's ectodomain that may increase substrate availability for the enzymes that are required for the cleavage of the intramembraneous or cytosolic sites.^{15,37} We have previously demonstrated that MCAO induces shedding of LRP's ectodomain in the ischemic hemisphere⁸ suggesting that LRP's RIP plays a role in the pathophysiology of cerebral ischemia. This hypothesis is supported by our observation that MCAO induces nuclear translocation of LRP-ICD in the area of ischemic penumbra.

Three families of proteases regulate RIP: presenilin-type aspartyl proteases, zinc metalloproteases of the site-2 protease (SP2) family, and rhomboid serine proteases.¹⁰ γ -Secretase is an aspartyl protease that catalyzes proteolysis within the TMD. Although the activity of this protease does not need a specific amino acid target sequence within the TMD, it requires ectodomain shedding to produce membrane-bound C-terminal substrate fragments.³⁸ It has been demonstrated that presenilin-1 is essential for γ -secretase activity,¹¹ and that presenilin-1 mutations increase the vulnerability of neurons to ischemic injury.³⁹ Our results indicate that MCAO induces a rapid increase in γ -secretase activity in the ischemic hemisphere, and that treatment with an inhibitor of γ -secretase attenuates cerebral ischemia-induced nuclear translocation of LRP-ICD. The importance of this process is supported by the finding that inhibition of either γ -secretase or LRP attenuates the cleavage of caspase-3 in the ischemic hemisphere, improves the neurological examination, and decreases the volume of the ischemic lesion after MCAO. Together, our data indicates that cerebral ischemia induces a γ -secretase-mediated RIP of LRP, and that this process has a deleterious effect on the ischemic tissue.

Based on our results we propose a model in which ischemic cell death is the result of a cell-signaling event initiated by the shedding of LRP's ectodomain.⁸ This is followed by γ -secretase-mediated cleavage of LRP's TMD and nuclear translocation of LRP-ICD. Together, our results indicate that RIP of LRP is a novel pathway for

Figure 6. Effect of inhibition of either LRP or γ -secretase on cerebral ischemia-induced cell death. **A:** Representative picture of immunohistochemical analysis of cleaved caspase-3 in AOI-2 in WT animals 6 hours after MCAO and treatment with either vehicle (control, **a** and **b**) or L-685,458 (**c** and **d**). Red is cleaved caspase-3, green is β -tubulin; blue is DAPI. **B:** Representative Western blot analysis of cleaved caspase-3 6 hours after MCAO in animals treated with either PBS or RAP, or anti-LRP antibodies, or L-685,458. Actin expression levels were assayed as a control for protein loading. Each observation was repeated four times. **C:** Quantification of the mean density of the band of Western blot analyses for cleaved caspase-3 from **B** ($n = 4$). * $P < 0.05$. Original magnifications, $\times 40$.

cerebral ischemia-induced cell death and a potential target for the treatment of patients with acute ischemic stroke.

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