Reperfusion Rather than Ischemia Drives the Formation of Ubiquitin Aggregates After Middle Cerebral Artery Occlusion

Karin Hochrainer, PhD*; Katherine Jackman, PhD*; Josef Anrather, VMD; Costantino Iadecola, MD

Background and Purpose—Cerebral ischemia leads to accumulation of ubiquitinated protein aggregates. However, the factors triggering ubiquitination and their impact on the outcome of cerebral ischemia remain poorly understood. Here we investigate the relationship between ubiquitin aggregation and duration of ischemia/reperfusion, infarct volume, and proteasomal activity in a mouse model of focal ischemia.

Methods—Free ubiquitin and ubiquitin aggregate levels were examined by Western blotting in the mouse neocortex and striatum after different periods of ischemia/reperfusion and permanent ischemia induced by middle cerebral artery occlusion. Infarct volumes were measured in thionin-stained brain sections. Proteasome activity was studied by fluorometric peptidase activity assay.

Results—Following transient ischemia, ubiquitin aggregates were detected in the ipsilateral neocortex and, to a lesser extent, striatum only after induction of reperfusion. In permanent ischemia, no ubiquitin aggregates were found. Shorter ischemic periods producing no or minimal tissue damage (10–15 minutes) resulted in ubiquitin aggregate levels similar to those produced by ischemia resulting in substantial infarction (30 minutes). Proteasomal impairment was greatest in ischemia without reperfusion, in which no ubiquitin aggregates were detected.

Conclusions—The data demonstrate that reperfusion rather than ischemia leads to the appearance of ubiquitinated aggregates, which form in the absence of major tissue damage and are not correlated with decreased proteasomal peptidase activity. Ubiquitin aggregates may form in potentially viable brain tissue, which may be later recruited into infarction by factors independent of ubiquitination. (Stroke. 2012;43:2229-2235.)

Key Words: middle cerebral artery occlusion ■ reperfusion ■ ubiquitination ■ protein aggregation ■ stroke

Ischemic stroke, occurring as a result of occlusion of major cerebral arteries, is a devastating disease, representing a leading cause of death and disability worldwide.1 Despite recent advances, there are major gaps in our understanding of the pathogenesis of cerebral ischemic injury, and therapeutic options for stroke patients remain limited.2 The accumulation of ubiquitin-containing protein aggregates following ischemia is a relatively unexplored research area that may provide new mechanistic insights into ischemic brain damage. However, the factors leading to deposition of these aggregates and their consequences for stroke outcome are largely unknown and, as such, require additional elucidation.

The ubiquitin-proteasome system represents the main cellular pathway for protein degradation,3 contributing to important cellular processes, including transcriptional regulation4 and apoptosis.5 To be recognized by the 26S proteasome, a large multicatalytic protease complex, proteins must be tagged with polyubiquitin chains via the sequential action of 3 distinct enzymes.6 Free ubiquitin molecules are activated and bound by an E1-activating enzyme in an adenosine triphosphate (ATP)-dependent manner, before they are transferred via E2-conjugating and E3-ligating enzymes to target proteins. The ubiquitinated substrate is then bound by the proteasome, where it is, in the presence of ATP, unfolded and degraded.7 Concomitantly, polyubiquitin chains are removed by deubiquitinating enzymes and free ubiquitin is released to promote a new cycle of degradation.8 The ubiquitin-proteasome system becomes particularly important under conditions of stress or injury, where it is essential for maintaining cellular homeostasis by removing damaged, oxidized, and misfolded proteins.9 Disruption of this process by certain pathological stimuli causes the formation of ubiquitin-containing protein aggregates, commonly found in neurodegenerative diseases.10 It remains unclear, however, whether accumulation of aggregates is directly linked to cytotoxicity or it represents a protective mechanism.11

Ubiquitinated protein aggregation related to proteasomal dysfunction has also been observed following cardiac12,13 and...
cerebral ischemia. However, the factors triggering ubiquitin aggregate formation have not been defined, and it is unclear whether ubiquitin aggregates are detrimental or beneficial to tissue outcome. To gain a better insight into the formation of ubiquitin aggregates in the ischemic brain, we investigated the relationships between aggregate accumulation, duration of ischemia/reperfusion, and resultant tissue damage in a mouse model of focal cerebral ischemia. To assess the involvement of the proteasome in aggregate formation, we monitored proteasomal peptidase activity during ischemic periods and after onset of reperfusion.

Methods

Mice
All procedures were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College. Experiments were performed in male C57Bl/6J mice (age 6–8 weeks; mean weight, 22.9 g; n = 124).

Middle Cerebral Artery Occlusion Model of Focal Ischemia
Transient focal cerebral ischemia was induced using the intraluminal filament model of middle cerebral artery occlusion (MCAO) as described previously. Briefly, a heat-blunted 6-0 nylon suture was inserted into the right external carotid artery and was advanced along the internal carotid artery until it obstructed the MCA. The right common carotid artery was simultaneously ligated. The occluding filament remained in position for the ischemic period and was subsequently retracted, inducing reperfusion. The filament was not retracted in mice subjected to permanent ischemia. Cerebral blood flow was measured using transcranial laser Doppler flowmetry (Periflux System 5010), and only mice with >85% flow reduction during the ischemic period and >80% increase within the first 10 minutes of reperfusion were included in this study. For sham MCAO, vessels were visualized and cleared of connective tissue, but no additional manipulations were made. Where indicated, the proteasome inhibitor Bortezomb (600 ng/μL; BioVision) or vehicle (0.75% acetic acid in 1X phosphate-buffered saline) was administered intracerebroventricularly 30 minutes before induction of cerebral ischemia.

Measurement of Infarct Volume
Brains were removed 24 hours after reperfusion and infarct volume assessed as described elsewhere. Thirty-μm sections were obtained at 600-μm intervals throughout the infarcted territories of the brain and were stained with cresyl violet. Infarct volume, corrected for swelling, was measured using image analysis software (MCID, Imaging Research, Inc.).

Isolation of Soluble Ubiquitin and Ubiquitin Aggregate Fractions from Mouse Brain Tissue
Brains were removed and sectioned using a brain matrix. Two-mm slices were taken within the MCA territory (approx. +1.2 to −0.8 mm bregma) ipsilateral and contralateral to the occluded artery. The entire cortex and striatum within the brain slices were dissected using a surgical scalpel and were homogenized in a Dounce homogenizer in buffer without detergent (15 mmol/L Tris-HCl pH 7.6, 250 mmol/L sucrose, 1 mmol/L MgCl₂, 2.5 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L Na₂VO₃, 5 mmol/L NaF, 20 mmol/L phenylphosphate, 20 mmol/L N-ethylmaleimide, 1 mmol/L dithiothreitol, 1X protease inhibitors [Roche Applied Sciences]). Homogenates were centrifuged at 10,000g for 10 minutes at 4°C to obtain crude cytosolic fractions containing free ubiquitin, which were additionally purified by centrifugation at 100,000g for 1 hour. Pellets were resuspended, sonicated (20% amplitude for 10 seconds at 4°C; Branson Sonifier), and after addition of 2% Triton X-100 and 150 mmol/L KCl, were rotated for 60 minutes at 4°C. Lysates were centrifuged at 100,000g for 10 minutes at 4°C to obtain insoluble pellet fractions containing ubiquitin aggregates; these were suspended in SDS-sample buffer and sonicated. Protein concentrations of cytosolic fractions were determined by Bradford protein assay (Bio-Rad).

Western Blot Analysis for Detection of Ubiquitin in Subcellular Fractions
Ten μg of cytosolic and 5 μg of Triton X-100/KCl-insoluble pellet fractions were separated on 16% Tris-Tricine and 7% Tris-Glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis, respectively. Proteins were transferred to polyvinylidene difluoride membranes (Millipore), which were incubated with anti-ubiquitin (clone Ubi-1; Invitrogen) or anti-glyceraldehyde-3-phosphate dehydrogenase antibodies (clone 6C5; Millipore) and subsequently with a horseradish-peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Protein bands were visualized with enhanced chemiluminescence Plus Detection Reagent (GE Healthcare) using a Kodak Molecular Imaging Station. Optical band densities were measured, values were normalized to glyceraldehyde-3-phosphate dehydrogenase, and relative levels of ubiquitin aggregates as well as free ubiquitin were compared with sham-operated or vehicle-treated controls.

Fluorometric Measurement of Proteasome Activity
Neocortical samples were homogenized in 20 mmol/L Tris-HCl pH 7.5, 1 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 1X protease inhibitors [Roche Applied Sciences], centrifuged at 10,000g for 10 minutes at 4°C, and protein concentration of supernatants was determined. Activity of 20S chymotryptic-like proteasomes was evaluated by incubation of 50 μg protein lysate with 2 mmol/L synthetic peptide Succinyl-Leu-Leu-Val-Tyr-7-Amino-4-methylcoumarin (Boston Biochem) at 37°C. Release of AMC from the substrate was measured with using a 380/460 nm filter set on a F-2500 fluorescence spectrophotometer (Hitachi). Functionality of the assay was ensured by successful total inhibition of proteasomal activity in 2 naïve controls after administration of 600 ng Bortezomb in vitro.

Statistical Analysis
Data are represented as mean±SEM. Comparisons between 2 groups were statistically evaluated by the Student t test. Comparisons among multiple groups were performed with 1-way ANOVA followed by Bonferroni post hoc test. Differences were considered significant at P<0.05.

Results
Reperfusion is Essential for the Formation of Ubiquitin Aggregates
First, we sought to establish the time course of ubiquitin aggregate formation and free ubiquitin levels in the neocortex after transient focal ischemia. Mice euthanized immediately after ischemia without reperfusion had only slightly higher levels of ubiquitin aggregates than did sham-operated animals (Figure 1A, B). In contrast, ischemia followed by reperfusion led to significant ubiquitin aggregate formation, which was maximal at 1 and 3 hours, and gradually declined at 6, 9, 12, and 24 hours after reperfusion. The increase in ubiquitin aggregates was accompanied by a depletion of free ubiquitin, which gradually recovered as aggregates diminished (Figure 1A, B). Ubiquitin aggregates were observed in the neocortex ipsilateral to, but not contralateral to, the occluded MCA (Figure 1C, D). Levels of ubiquitin aggregates reached a maximum at 1 hour reperfusion, and were not detected in the absence of reperfusion. To identify the earliest time point at which
aggregates were formed, we examined ubiquitin aggregate levels following shorter reperfusion periods. Ubiquitinated aggregates were detected as early as 5 minutes after the onset of reperfusion, and reached near maximum by 15 minutes (Figure 2).

Given that the appearance of ubiquitin aggregates in the cortex was highly dependent on the re-establishment of blood flow, we investigated whether ubiquitin aggregates were less in the center of the ischemic territory (ischemic core), corresponding to the striatum in this model, where reperfusion is more limited. Consistent with this prediction, we detected a minimal increase in aggregates in the ipsilateral striatum after 1, 3, and 6 hours reperfusion and no detectable aggregates at 24 hours reperfusion (Figure 3). In contrast, in the neocortex, abundant ubiquitin aggregation was observed at all time points studied. Free ubiquitin was present in striatal tissue throughout and even increased at 24 hours reperfusion (Figure 3), suggesting that lower levels of ubiquitin aggregates in the ischemic core may not be related to enhanced cell death in this region.

We then investigated whether aggregate formation could be induced by longer periods of ischemia. One, 3, 6, and 24 hours of permanent ischemia without reperfusion failed to induce significant increases in ubiquitin aggregation, whereas free ubiquitin was readily detected at all time points (Figure 4). These results indicate that reperfusion is absolutely required for postischemic ubiquitin aggregate formation.

The Accumulation of Ubiquitin Aggregates Does not Correlate With Tissue Damage

Next, we sought to correlate the accumulation of ubiquitin aggregates with the development of ischemic brain injury. To this end, we analyzed ubiquitin aggregate levels in the neocortex after increasing occlusion times followed by 1 hour reperfusion. Infarct volumes were determined in separate mice subjected to the same ischemia/reperfusion protocol. Both 5 and 10 minutes of ischemia were sufficient to trigger ubiquitin aggregation in the ipsilateral cortex, but failed to induce detectable tissue damage in thionin-stained sections (Figure 5A, B). Comparable ubiquitin aggregate levels were observed with 15 and 30 minutes of ischemia, despite the much smaller damage obtained with 15 versus 30 minutes ischemia (Figure 5A, B). To investigate the possibility that ubiquitin aggregate levels were transiently elevated in animals subjected to sublethal ischemic periods, we measured ubiquitin levels in detergent-insoluble fractions after 10 minutes of transient ischemia followed by extended reperfusion times. The elevation in ubiquitin aggregates induced by 10 minutes of ischemia remained stable at 1, 3, or 6 hours reperfusion (Figure 5C). These findings strongly suggest that
the formation and stability of ubiquitin-containing aggregates following focal ischemia are not related to the magnitude of brain injury.

Proteosomal Peptidase Inhibition is Greater During Ischemia than in the Reperfusion Period

The formation of ubiquitin aggregates after ischemia could be the result of proteasomal dysfunction, which inhibits clearance of polyubiquitinated proteins. Accordingly, proteasomal activity should be unaffected during ischemia without reperfusion, which does not result in ubiquitin aggregation. To test this hypothesis, we compared proteasome activity in lysates obtained from cortical tissue before and after onset of reperfusion. We observed a significant reduction in proteasomal activity after 30 minutes, and an even greater inhibition after 3 hours of ischemia without reperfusion (Figure 6A, left panel). Induction of reperfusion, however, led to a partial recovery of proteasomal activity 3 hours later (Figure 6A, left panel). Although not significant, a similar trend was observed in the contralateral cortex (Figure 6A, right panel). In addition, administration of the proteasomal inhibitor Bortezomib failed to promote ubiquitin aggregate formation in ischemia without reperfusion (Figure 6B, C). The ability of Bortezomib to block proteasomal peptidase activity in the cortex was verified in sham animals (Figure 6D). These observations collectively indicate that postischemic formation of ubiquitin aggregates is not caused by proteasomal inhibition.

Discussion

One of the major findings of this study is that accumulation of ubiquitinated aggregates after focal cerebral ischemia is related to reperfusion, occurring promptly after blood flow reintroduction mostly in the cerebral cortex, an area of more intense reperfusion. Considering that re-establishment of blood flow after an ischemic insult is critical for survival of the brain, it is tempting to speculate that the presence of ubiquitin aggregates may indicate a greater potential for tissue viability after ischemia/reperfusion. In support of this idea, our data indicate that absence of aggregates in the postischemic tissue may in fact be an index of more severe ischemic injury. For example, in transient MCAO, we detected lower levels of ubiquitin aggregates in the striatum, which corresponds to the ischemic core, than in the cortex, in which the ischemia is less severe and the potential for survival is greater. Furthermore, we found no ubiquitin aggregates in the neocortex after permanent MCAO, which consistently induces a greater degree of brain injury than does transient occlusion. Therefore, the presence of ubiquitin aggregates in the ischemic brain may represent a biomarker for potentially salvageable tissue.
We also found that the formation of ubiquitin aggregates following cerebral ischemia is not linked to cerebral infarction, indicating that ubiquitin aggregates do not constitute a threat to the viability of the tissue. Our findings are in contrast to those of a previous study suggesting that the increase in ubiquitin conjugates is dependent on increased ischemic damage. However, in that study, a gerbil model of transient global ischemia was used, making a comparison with our study difficult because of differences in the degree of ischemia, location of the damage, and mechanism of injury.

Another key finding of the present study is that inhibition of proteasomal activity alone does not appear to account for the accumulation of ubiquitinated aggregates. It is widely assumed that the enrichment of ubiquitinated aggregates after cerebral ischemia is a direct result of proteasomal impairment. Accordingly, several studies have reported decreased proteasomal peptidase activity during reperfusion. Although we did observe reduced proteasomal activity during reperfusion, we found that proteasomal dysfunction was greater during permanent ischemia, despite lack of aggregate accumulation. Our observation that inhibition of the ubiquitin-proteasome system is greater during ischemia than during reperfusion seems conceivable because ubiquitination and degradation of proteins by the proteasome require ATP. With lower ATP concentrations during the ischemic period, it is expected that fewer proteins become conjugated to ubiquitin, possibly explaining the lack of ubiquitin aggregates during ischemia. Permanent ischemia leads to a severe and prolonged ATP deficiency, whereas in transient ischemia, ATP synthesis recovers to normal levels after 1 hour reperfusion. The fact that ubiquitin aggregates accumulate at a time when proteasomal activity is restored indicates that ubiquitin aggregate formation is not just the result of an ATP-dependent inhibition of protein degradation.

It was also suggested that proteasomal inhibition during ischemia might occur as a result of oxidative damage to proteasomal subunits. Although it is well established that peptidases associated with the proteasome are irreparably damaged by oxidizing radicals, we have previously shown that, in this model, free radical production after focal ischemia reaches a maximum only after 2 to 4 hours of reperfusion. This observation would suggest a more severe proteasomal impairment during reperfusion than during ischemia, which is the opposite of our findings. Interestingly, we observed a trend for reduced proteasomal peptidase activity in the contralateral cortex following ischemia. This effect is likely caused by changes in perfusion and cerebral metabolism of the contralateral hemisphere in response to focal ischemia, and it may suggest a high sensitivity of the proteasome even to modest alterations in blood flow.

Focal cerebral ischemia also enhances conjugation of additional ubiquitin-like proteins, including small ubiquitin-like modifier (SUMO) and interferon-stimulated gene 15 (ISG15). Consistent with our findings for ubiquitin, levels of SUMO conjugation were elevated as early as 30 minutes after reperfusion; the increase in SUMOylation was similarly restricted to the cortex and not detected in the striatum, and both short and long periods of ischemia resulted in the same amount of SUMO conjugates. The link between SUMO and ubiquitin conjugation after ischemia was recently emphasized by the finding that oxygen/glucose deprivation-induced SUMO conjugation serves as a prerequisite for ubiquitination. Increased conjugation of SUMO and ISG15 was found to be neuroprotective in the setting of cerebral ischemia, as suppression of both SUMOylation and ISGylation resulted in...
exacerbated neuronal damage and infarction.\textsuperscript{30,32} Given that ubiquitin and SUMO share similar conjugation dynamics, it seems plausible that ubiquitin aggregation might also participate in neuroprotection. Indeed, neurons were shown to be highly immunoreactive for ubiquitin after ischemia.\textsuperscript{16,33–35} However, a caveat with these studies is that antibodies used in immunohistochemistry do not clearly differentiate between free ubiquitin and ubiquitin aggregates. Nevertheless, support for a protective role of ubiquitin comes from the observation that ubiquitin aggregation\textsuperscript{16} and SUMO conjugation\textsuperscript{37} are both massively increased in multiple tissues, including brain, of animals undergoing hibernation torpor, a powerful example of ischemic tolerance. However, the presence of comparable levels of ubiquitin aggregates shortly after reperfusion in tissue destined either to die or survive suggests the participation of other downstream factors that determine the outcome of the ischemic insult.

Taken together, our findings highlight a critical role of cerebral reperfusion in the formation of ubiquitin aggregates, which may reflect a greater potential for tissue survival in the immediate posts ischemic period. However, this speculation needs to be substantiated by additional studies in which the molecular components present in ubiquitin aggregates are identified and pathways leading to their formation are fully characterized.

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Disclosures

None.

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