Early Diffusion-Weighted MRI as a Predictor of Caspase-3 Activation After Hypoxic–Ischemic Insult in Neonatal Rodents

Michael F. Wendland, PhD; Joel Faustino, BS; Tim West, PhD; Catherine Manabat, BS; David M. Holtzman, MD; Zinaida S. Vexler, PhD

Background and Purpose—Neonatal encephalopathy in human babies is a serious condition associated with permanent neurological deficits. Diffusion-weighted MRI (DWI) is increasingly used for early diagnosis of brain injury in human babies. The relationship between the presence of DWI abnormalities and cellular injury, including apoptosis, during the neonatal period are not well understood. We asked whether the extent of injury depicted on DWI can predict the presence of caspase-3 activation, a quantitative marker of apoptotic injury, after hypoxia–ischemia (H-I) in postnatal day 7 rats.

Methods—Injury volume was determined by DWI at 2 hours, 24 hours, and 7 days after H-I and compared with histology. Caspase-3 activation and microgliosis were determined at 24 hours post-H-I.

Results—DWI-defined lesions (eg, decreased apparent diffusion coefficient) at 24 hours post-H-I correlated with a major increase in caspase-3 activity in the injured hemisphere and predicted injury. A modest but significant increase in caspase-3 activity occurred in the cortex of rats that had no apparent diffusion coefficient decrease in the injured hemisphere but had unilaterally enlarged regions of high apparent diffusion coefficient at the ipsilateral ventricle/white matter interface. Caspase-3 activity was similar in both hemispheres in pups with unchanged DWI.

Conclusions—Abnormal DWI signal at 24 hours post-H-I is predictive of caspase-3 activation and can be used as an indicator that injury involving an apoptotic-like mechanism is present. Our data also suggest that the presence of an enlarged unilateral region with high apparent diffusion coefficient at the ventricle/white matter interface without significant apparent diffusion coefficient decrease in the cortex is a sign of modest caspase-3 activation after H-I. (Stroke. 2008;39:1862-1868.)

Key Words: apoptosis ■ caspase-3 ■ diffusion-weighted MRI ■ hypoxia–ischemia ■ neonate

Serial noninvasive neuroimaging, MRI and spectroscopy, in both humans and in animal models, have shown that injury patterns depend on the severity of the insult and the age at which it occurs1 and that brain injury evolves over days and even weeks.2 The diagnostic capabilities of MRI have been used in adult stroke models for a long time, but its use in neonatal animal models has been scarce. Diffusion-weighted MRI (DWI) performed in naïve animals of different ages and the apparent diffusion coefficient (ADC) derived from DWI showed that water diffusion is substantially less restricted in the neonatal compared with the adult brain, consistent with the lack of brain myelination. Furthermore, the extent of ADC reduction after focal stroke or hypoxia–ischemia (H-I) is substantially larger in neonates3–7 than in adults.8 We and others have shown that the decrease in ADC is biphasic in the neonatal brain after focal stroke or H-I,9–11 that the first drop occurs much earlier than cell death, and that secondary decline of the ADC is associated with structural injury.9–11 Although redistribution between extracellular and intracellular water plays a major role12,13 the extent of associated metabolic stress and altered intracellular molecular interactions is not fully understood.

Compared with the adult brain after stroke or trauma, neonatal brain is much more susceptible to activation of apoptotic pathways14,15 likely due to ongoing developmental elimination of neurons by apoptosis at this stage in development. Apoptosis in the neonatal brain correlates highly with activation of the executioner caspase, caspase-3, enabling the use of caspase-3 activation as a measure of long-term injury outcome after neonatal H-I.16 Identification of a noninvasive surrogate indicator for caspase-3 activation during early injury stages, in turn, may prove to be a very useful tool in long-term studies on neuroprotection.
We asked if the extent of changes depicted on DWI can predict acute caspase-3 activation and long-term injury outcome after H-I in neonatal rats. We report here that the presence of significant ADC reduction at 24 hours post-H-I is always associated with marked caspase-3 activation and that the presence of unilaterally enlarged regions with high ADC values at the ventricular/white matter interface in the absence of ADC drop in the tissue is associated with a modest but significant caspase-3 activation.

**Methods**

**Animal Care and Surgical Procedure**

All animal research was approved by the University of California–San Francisco Institutional Animal Care and Use Committee and was performed in accordance with the Guide for the Care and Use of Laboratory Animals (US Department of Health and Human Services, Publication Number 85–23, 1985). Female Sprague-Dawley rats with a 6-day-old litter were housed in a temperature-/flight-controlled environment and given food and water ad libitum. Postnatal day 7 rats were exposed to right common carotid artery ligation followed by hypoxia (8% O2/N2) for 2.5 hours, H-I, as previously described.14

**MRI**

Pups were imaged 3 times at 2 hours, 24 hours, and 7 days after H-I for injury volume measurements or either at 2 or 3 hours after H-I for measurements of caspase-3-dependent and independent spectrin cleavage at 5 hours or at 24 hours after H-I for measurements of caspase-3 activity at the end of the imaging session. Images were acquired in anesthetized rats using a Bruker 2T system as described.10 Multislice T2 and DWI (b factor = 1030 seconds/mm² applied along the Z-axis) spin echo images were acquired with TR/TE = 2500/60 ms, field of view = 32 mm, matrix = 128 x 128 (in-plane pixel dimension of 0.25 mm), and 8 contiguous slices, 1 mm thick, 4 signal averages, 1-hour acquisition time. Signal-to-noise ratio for brain tissue was 28 to 40 on T2-weighted images. Images were analyzed using commercially available software (MRvision; The MR Vision Co., Winchester, Mass). ADC maps were calculated from T2-weighted and DWI for each animal using the equation ADC = -ln(SI(DW)/SI(T2))/b, where SI is pixel signal intensity of the indicated image.10 On the ADC map, the size of apparent injury was determined by counting the number of pixels in the hemisphere ipsilateral to common carotid artery ligation with a threshold signal ratio of 0.19 (n = 10). Ventricle was calculated as the difference in pixel count of the ipsilateral tissue volume to the volume of the contralateral tissue.

**Histological Analysis**

Brain volume was determined 7 days after H-I in paraffin-embedded coronal sections (20 μm thick cut at 400-μm intervals) obtained from paraformaldehyde perfusion-fixed animals that underwent multiple MRI sessions. Volumetric measurements were obtained from 8 cresyl violet-stained sections using Neurolucida (Microbrightfield Software Systems) as previously reported.18 Data are expressed as a ratio of the ipsilateral tissue volume to the volume of the contralateral tissue.

**Immunofluorescence**

Sections were deparaffinized and immunostained as described.19 Rabbit antirat cleaved caspase-3 (Cell Signaling; 1:150), rabbit antirat bcl-2 (Wako; 1:200), mouse antirat glial fibrillary acidic protein (Serotec; 1:50) antibodies, and secondary antibodies raised in the appropriate target species and conjugated to Cy2 or Cy3 dyes (1:100) were used. Slides were incubated in bis-benzamide (0.001%). Slides were imaged with a Zeiss Axiovert 100 fluorescent microscope equipped with OpenLab Software (Improvement).

**Results**

**Progression of Diffusion-Weighted MRI-Identifiable Injury After Hypoxia–Ischemia in Postnatal Day 7 Rats**

To determine whether DWI can be used as a surrogate measure of histological outcome after H-I 10 rats were imaged at 2 hours, 24 hours, and 7 days after H-I; the volume of abnormal DWI was compared with the volume of the histological lesion after euthanasia at 7 days (Figure 1A). At 2 hours postreoxygenation, DWI lesions were evident in 2 pups (circles). By 24 hours, DWI lesions were seen in 7 animals and all 7 showed both MRI- and histologically confirmed lesions 7 days after H-I (squares). Of 3 rats with no DWI lesion at 2 or 24 hours after H-I, injury evolved in only one rat (triangles). Animals with DWI abnormalities early after H-I had overall larger lesions. In contrast, no evidence of histological lesion was observed in 2 pups with lack of DWI lesions 7 days after H-I. Calpain-mediated spectrin cleavage, which is indicative of excitotoxic injury,17 was seen at 5 and 24 hours post-H-I in ipsilateral tissue with or without lesions on DWI 2 to 3 hours after H-I (Figure 1B). Caspase-3-mediated spectrin cleavage was minimal at 5 hours and profound at 24 hours post-H-I. Therefore, DWI underestimates long-term injury when acquired 2 hours post-H-I, whereas it delineates the evolving injury when acquired at 24 hours post-H-I. These data also indicate that DWI changes temporally correlates with the peak in caspase-3 activation shown in our prior studies.17

**Diffusion-Weighted MRI-Identifiable Lesions and Increase in Caspase-3 Activity 24 Hours After Hypoxia–Ischemia**

A separate group of rats was imaged 24 hours post-H-I and then euthanized and assayed for caspase-3 activation. Three patterns of injury were evident by DWI (Figure 2): (1) hyperintense DWI signal and corresponding ADC drop was observed in the ipsilateral hemisphere (group 1, top); (2) normal ADC signal in the H-I cortex but an enlarged region with high ADC in the lateral ventricle in the vicinity of the white matter in the ipsilateral hemisphere (group 2, middle); and (3) unchanged ADC values in the H-I hemisphere and...
symmetrical appearance of regions in the vicinity to the ventricles (group 3, bottom). ADC values in the contralateral hemisphere were not different from values in control animals.

In group 1, the ADC values in the ipsilateral regions were significantly reduced \(0.60 \pm 0.16 \times 10^{-3}\) mm\(^2\)/s compared with the contralateral cortex \(1.04 \pm 0.09 \times 10^{-3}\) mm\(^2\)/s, \(n=9\), \(P=0.004\). All pups in this group showed markedly increased caspase-3 activity in the ipsilateral cortex compared with contralateral regions \((P=0.001;\ Figure 3A–B)\). The size and distribution of regions with abnormal ADC values varied considerably within the group, 27.6\(\pm\)16.8\% of the ipsilateral hemisphere, with no significant correlation between caspase-3 activity and either size of injury \(R^2=0.58,\ P=0.17\) or ipsilateral ADC value \(R^2=0.44,\ P=0.32\). 

Calpain-mediated spectrin cleavage was seen in all animals (Figure 3C). Only modest hyperintensity was seen on T2-
weighted images in the injured cortex, precluding confident delineation of injury size.

In group 2, no ADC reduction was seen in the tissue, but a 2.32/0.48-fold increase in the number of pixels with high ADC (2.0/0.15 × 10⁻³ mm²/s, n=6) was observed at the ipsilateral lateral ventricle/white matter interface suggesting the presence of both fluid and tissue in these voxels. Pups in group 2 exhibited modest but clear and statistically significantly increased caspase-3 activity in the ipsilateral versus contralateral cortex (P=0.004; Figure 3A–B). Calpain-mediated spectrin cleavage was lower; modest caspase-3 activation produced only subtle cleavage of spectrin (Figure 3C).

In group 3, the ratio of the number of pixels with high ADC was 1.15/0.23 for ipsilateral/contralateral hemispheres in the remaining 6 pups post-H-I, not statistically different from that in the 4 control pups. Calpain-mediated spectrin cleavage (Figure 3C) and similar levels of caspase-3 activity were observed in both hemispheres (P=0.34; Figure 3B).

Distribution of Cells With Active Caspase-3 Differs After Severe and Modest Injury
The spatial pattern of caspase-3 activation was investigated using immunofluorescence. Essentially no cells with active caspase-3 were seen in the hemisphere contralateral to common carotid artery ligation (Figure 4A–C, n=3) or in either hemisphere in rats with no DWI changes (not shown). In group 2, few cells with cleaved caspase-3 were seen in the cortex (Figure 4D–I, n=3) as well as in the hippocampus and caudate (not shown). In contrast, substantial caspase-3 act-
Modest Caspase-3 Activation Is Associated With Local Inflammation

Next we determined whether microglial activation occurs in animals from groups 1 and 2. In the contralateral cortex, Iba1-immunoreactive microglial cells remained quiescent based on unchanged morphology compared with controls, eg, small cell bodies and multiple long web-looking processes (Figures 5A, D, and G). Larger cell bodies and shorter processes observed within the white matter tracts in the hypoxic hemisphere (Figures 5A and E) were similar to those in naïve animals. In group 2, a range of morphologies, from quiescent cells to small patches of round cells (Figure 5H), was seen in the H-I cortex, consistent with the presence of patches of caspase-3 positive cells (Figure 4). The overall increased number of Iba1-positive cells with larger bodies and retracted processes was also seen in white matter of the H-I hemisphere. In group 1, round Iba1-positive cells were in abundance in the H-I hemisphere, spatially corresponding to injury (Figures 5C, F, I, and J). Glial fibrillary acidic protein-immunoreactive cells were observed in the contralateral cortex, particularly around vessels, and in the white matter (Figures 5A, D, and G). In group 2, the density of glial fibrillary acidic protein-positive cells tended to increase in the H-I white matter and some reactive astrocytosis was seen in the H-I cortex in association with the presence of caspase-3 and Iba1-positive cells (Figures 5B and E). In group 1, glial fibrillary acidic protein-positive cells were seen in the periphery rather than in the center of injured cortex.

Discussion

We show that the presence of a decrease in ADC at 24 hours post-H-I is always associated with a marked increase in caspase-3 activity as detected by biochemical, functional, and immunostaining assays. We also show modest, but significantly increased, caspase-3 activation in brains without reduced ADC in the tissue but with a unilaterally enlarged region with high ADC at the ventricular white matter junction at 24 hours post-H-I. These data suggest that DWI acquired 24 hours post-H-I indicates that if DWI abnormalities are present, at a minimum, caspase-3-mediated apoptotic cell death is present.

In neonatal rodents, although pathways independent of caspase-3 are important contributors to H-I injury,20–22 caspase-3 activation plays a central role in the pathophysiology of H-I. Caspase-3 activation starts increasing 6 to 12 hours after the injury and peaks between 24 to 36 hours after H-I.14,15 Studies of neonatal rats and mice with perturbed apoptotic pathways have estimated that apoptosis is responsible for up to 50% of the tissue loss that occurs at 7 days after H-I.17,23 but H-I injury varies in individual animals as shown previously by others and in this article. This raises the question of whether modest or absent injury results from a subtle acute injury or from injury resolution over time. We show that this distinction, which is important for long-term studies of neuroprotective or repair-enhancing agents, can be
obtained from noninvasive estimates of caspase-3 activation during early stages of H-I.

We show that the timing of DWI acquisition plays a critical role in the ability to predict long-term injury. DWI performed shortly after reoxygenation consistently underestimates histological outcome 7 days later and fails to predict the presence of excitotoxic injury or caspase-3 activation 5 to 24 hours post-H-I. In contrast, a DWI lesion in the tissue at 24 hours is associated with a major increase in caspase-3 activity at the time of imaging and translates to a histological lesion at 7 days in 9 of 10 rats. Early ischemia-induced ADC decrease is caused, in part, by redistribution of water between the extracellular and intracellular space.13,24 The reduction in ADC in adult rodents during H-I is not necessarily associated with a metabolic energy failure,9 potentially permitting apoptosis (an energy-dependent process) to occur. DWI hyperintensity is followed by renormalization of DWI signal shortly after reoxygenation as previously reported by several,12,13,24 but not all, laboratories.11 Our data on calpain activation at 5 hours post-H-I show that excitotoxic injury in the injured hemisphere is not predicted by DWI acquired 2 to 3 hours earlier. Although limitations in DWI injury detection sensitivity may contribute to the lack of injury prediction, physiological factors associated with partial-to-complete renormalization of DWI are of importance, including variable degree of post-H-I cerebral blood flow restoration and time needed for cellular injury to substantiate.

The underlying reasons for secondary decline of the ADC recently shown after H-I or focal stroke in neonatal rodents13,24,25 are likely to be similar to that in stroke models in adult rodents.26 The drop of ADC parallels cerebral blood flow restoration and altered expression of a broad range of proteins, including proteins indicating the presence of metabolic stress (HSP7226), inflammation,27 ongoing necrosis (calpain and clusterin20,21), and apoptosis (caspase-3,7,17,21). Some reports suggest that secondary ischemia-induced energy failure is the major underlying mechanism for subsequent secondary ADC reduction,28 whereas others show that decreased energy metabolism relates to severity and spatial distribution of initial injury.13 Glial swelling has been suggested to contribute to the late decline of the ADC signal,22 but glial scar formation evolves over weeks.

Apparently, secondary ADC reduction depends on multiple mechanisms that ultimately contribute to both necrosis and apoptosis, which can both be severe at 24 hours after H-I. Necrosis in the neonatal brain may be a potent trigger of apoptosis through release of toxic molecules that endanger neighboring neurons directly, by activating microglial cells and priming endothelium, and by eliminating the target of neuronal projections. In the adult, ADC reduction is most profound in the ischemic core, a region with predominant necrosis after focal ischemia–reperfusion. In contrast, in postnatal day 7 rats, an age when apoptosis and intermittent (“continuum”) cell death are most prominent,29 caspase-3 activity is profoundly increased early after reperfusion in regions with relatively modest early reduction of the ADC, whereas a more delayed but marked caspase-3 activation is observed in the ischemic core.7 Although caspase-3 activation is likely not the only source of ADC drop in H-I tissue, the important finding of this article is that ADC decreases 24 hours post-H-I are always associated with major caspase-3 activation. Lack of correlation between the magnitude of caspase-3 activation in animals with DWI lesions and lesion size or the extent of ADC reduction can be affected by measurements of caspase-3 activity in whole tissue homogenates obtained from regions with heterogeneous injury severity as seen both on DWI and sections immunostained for cleaved caspase-3. Partial volume error in a relatively thick DWI coronal plane can also affect correlation between 2 parameters.

Importantly, our data show that the presence of asymmetry on DWI at the ventricular/white matter interface in the absence of ADC drop within the tissue is a useful diagnostic tool for identifying cases with subtle early injury, cases with increased apoptosis without obvious necrotic lesions. The mechanisms for asymmetry are yet to be understood. The high ADC threshold used to quantify asymmetry at the lateral ventricle interface suggests an enlarged compartment of fluid. This may be due to enlarged ventricular space, tissue loss, or an intrinsic change in tissue water content in the white matter. Loss of tissue, cortical or white matter, could result in enlarged ventricle size with the consequent high ADC values, but tissue loss evolves over days, making it unlikely that tissue loss accounts for the high ADC seen at 24 hours after H-I. Another factor affecting ADC values is the partial volume error due to presence of the ventricular space and adjacent brain tissue in image voxels. In this case, reduction rather than increase of ADC in white matter would be expected acutely as seen in the cortex. Largely unchanged T2-weighted signal reduces the likelihood of vasogenic edema in the white matter. Finally, the presence of both excitotoxic component and local inflammation may affect ventricular size or fluid within the tissue in this region. Our data on gradual microglial activation both in the white matter tracts and in the cortex may be important in interpreting DWI data. Although MRI findings consistent with increased fluid in the ventricle should correlate with increased size of the ventricles by histological analysis, histological changes were not clearly identified. However, tissue paraffin embedding and processing cause nonlinear tissue shrinkage, potentially precluding visible changes in ventricular space in the histological specimens. There have been no studies using a neonatal H-I model looking at the relationship between the effect of systemic and local inflammation and the accumulation of inflammatory mediators in the cerebrospinal fluid, but based on the demonstrated modulatory effects of inflammation on the developing brain (exposure of a neonatal brain to low concentration of lipopolysaccharide30), it is conceivable that increased modest cytokine release, both locally and systemically, trigger caspase-3 activation when necrosis is minimal.

In summary, although the underlying mechanisms of ADC reduction are yet to be fully understood, these data provide the basis for using DWI at 24 hours postinjury as a surrogate measure for the presence of apoptotic-like cell death and caspase-3 activation in the neonate after H-I.
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Disclosures
None.

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