

Review

The Role of Acrolein in Spinal Cord Injury

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The most severe functional damage resulting from spinal cord injury (SCI) does not immediately follow the primary insult but occurs later through a secondary-injury mechanism. Unlike primary injury, secondary injury occurs not only at the original site but also in adjacent tissues.

A cascade of biochemical reactions mediates the delayed secondary neurologic damage. Among these reactions, oxidative stress has long been recognized as playing a critical role.¹ In fact, oxidative stress and free radical-mediated injuries have been associated with numerous diseases that few other pathologic factors can match.² They have been implicated in diseases associated with pollution, smoking, aging, and trauma.³

The mechanisms of generation and action of reactive oxygen species (ROS) and lipid peroxidation (LPO) have been the topic of intense research aimed at preventing, slowing down, or even reversing various disease processes. Evi-

dence strongly suggests that post-traumatic oxidative stress plays a critical role in the pathogenesis of SCI.^{1,4} However, despite years of effort, conventional strategies aimed at scavenging free radicals have failed to produce treatment that could effectively curtail oxidative injury. Hence, further understanding of the mechanisms of oxidative stress and identification of a novel and more effective target are needed.

In recent years, evidence has emerged that acrolein may play an important role in nervous system pathology such as trauma and chronic degenerative disease.⁵⁻¹⁰ Acrolein has been shown to be elevated following SCI,¹¹ to cause toxicity in nervous tissue,^{5,6,8,9,12}

and to have a much longer half-life than ROS.¹³ Thus, acrolein may be a key factor in perpetuating oxidative stress, and it may represent an effective target for therapeutic treatments.

This review presents evidence that acrolein toxicity occurs in nervous tissue and discusses the possible mechanism of action with emphasis on SCI. We also present preliminary data suggesting that an acrolein-trapping agent may significantly enhance viability and function.

WHAT IS ACROLEIN?

Acrolein is a highly electrophilic α , β -unsaturated aldehyde that can be generated—among many other pathways in biologic systems—as a product of free radical-mediated LPO. How metabolic pathways generate acrolein has been previously reviewed.¹³⁻¹⁵

Acrolein is the strongest electrophile among the unsaturated aldehydes.^{5,14,16} In vivo, acrolein exhibits facile reactivity with various biomolecules, including proteins, DNA, and phospholipids, and thus has the potential to disrupt the function of these molecules.^{14,15} It can react with sulfhydryl groups of cysteine, histidine, and lysine residues of proteins¹⁵ and has been shown to rapidly incorporate into proteins

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and generate carbonyl derivatives.¹⁷ Acrolein also can react with nucleophilic sites in DNA and modify DNA bases through the formation of exocyclic adducts.

What makes acrolein a particularly deadly killer is that its half-life, in a range of 7 to 10 days,¹³ is much longer than that of the much-studied oxygenfree radicals, which have a half-life of 10⁻¹² seconds. Furthermore, compared with other alkenals, such as 4-hydroxynonenal (HNE), acrolein is formed at 40 times greater concentration and is 100 times more reactive.^{14,16} Acrolein's role in oxidative stress, therefore, may be particularly damaging.

ACROLEIN TOXICITY IN NERVOUS TISSUE

Similar to its toxicity in many other cell types,^{14,15} acrolein causes structural and functional damage in nervous tissue. We previously showed that exposure of nervous tissue to acrolein causes increased membrane permeability,^{8,9} impulse conduction block,⁸ demyelination (unpublished observation), oxidative stress,^{9,12,18} and impaired mitochondrial function.^{9,12,18} Indeed, acrolein seems to act on at least 3 subcellular targets: plasma membrane, mitochondria, and myelin sheaths.

Acrolein-mediated plasma membrane disruptions In ex vivo spinal cord preparation, exposure to 1 μ M acrolein for 4 hours results in increased permeability to ethidium bromide (molecular weight: 400 daltons).⁹ When the preparation was exposed to acrolein for a longer time and/or exposed to higher concentrations, larger molecules such as horseradish peroxidase (HRP, 44 kd) and lactate dehydrogenase (LDH, 144 kd) leaked through the breached cell mem-

brane.⁹ Because membrane damage is critical to cellular deterioration,¹⁹⁻²¹ membrane disruption may be a mechanism through which acrolein contributes to functional loss in neurologic disease and injury in which ROS have been implicated.

Several characteristics of acrolein-mediated membrane damage are worth discussing. First, plasma membrane disruption induced by acrolein is a progressive process that includes a delay in the first evidence of damage ranging from minutes to hours. Conversely, the most severe plasma membrane leakage occurs immediately after physical trauma.²²⁻²⁵ Membrane breaches attributed to primary injury in vitro reseal in a time-dependent manner after trauma, as demonstrated by a continued reduction of permeability to different molecular markers.²²⁻²⁵ The delayed time course and progressive nature of acrolein-mediated membrane damage suggest that acrolein may play an important role in secondary, rather than primary, injury following mechanical trauma. Furthermore, because acrolein can inflict membrane disruption, membrane breakdown may be produced not only by direct physical insult but also by exposure to toxins over time.

The finding that acrolein at micromolar levels can induce significant membrane damage in healthy uninjured tissue suggests that acrolein may be responsible for the delayed membrane damage to uninjured nerve tissue adjacent to the site of injury. This may help explain the phenomenon of "diffusive axonal injury," postulated by Povlishock and colleagues to characterize the widespread axonal deterioration outside the impact zone.²⁶⁻²⁸ We hypothesize that acro-

lein may act as a messenger toxin that inflicts secondary and diffusive membrane damage after physical trauma.²⁹

We recently detected a significant elevation of acrolein and, more important, we detected concomitant membrane damage at a location more than 10 mm from the original site of trauma in a guinea-pig spinal cord compression-injury model.^{11,29} In addition, we showed that membrane damage is accompanied by functional loss. Specifically, we demonstrated that such membrane damage (detectable by HRP) could lead to loss of compound action potential conduction as well as loss of resting membrane potential.⁸

To further support a possible role of acrolein in SCI, we showed that acrolein is significantly more toxic than HNE, which has already been implicated in spinal cord trauma.^{30,31} HNE applied for 4 hours at a concentration of 200 μ M did not cause significant HRP labeling, while acrolein at the same concentration inflicted significant membrane disruption as early as 15 minutes after application.⁹

We believe that the concentrations of acrolein used in our in vitro studies occur in vivo. The level of acrolein in the sera of healthy persons was measured at as high as 50 μ M³² and was measured at as high as 80 μ M in respiratory tract lining fluids in cigarette smokers.³³ Furthermore, the 1 μ M acrolein used in our study⁹ equals approximately 2 nmol acrolein/mg protein (calculated based on the average weight and protein content of spinal cord segments suspended in 20 mL of 1 μ M acrolein solution). The level of acrolein in the brain tissue of patients with Alzheimer disease (AD) reaches 2.5 \pm 0.9 nmol/mg protein in the amygdala

and 5.0 ± 1.6 nmol/mg protein in the parahippocampal gyrus.⁶ The concentration of acrolein-protein adducts in normal human plasma is 30 to 50 μM ^{32,34}; in patients with renal failure, this concentration can be as high as 180 μM .³⁴

We also believe that the toxicity of acrolein in functional injuries may be more severe than our estimates because exposure is probably longer than 4 hours—the longest incubation time used in our *in vitro* studies.⁹ This is suggested by the prolonged elevation of ROS and LPO seen in various diseases.^{1,30,31}

We demonstrated that the acrolein level was increased 1 to 7 days after traumatic SCI.¹¹ In addition to traumatic injury, the temporal increase of acrolein in chronic neurodegenerative diseases such as AD is likely to be even longer.⁶ Because of the time dependence of acrolein toxicity, it is reasonable to assume that the concentration threshold of acrolein toxicity *in vivo* may be lower than 1 μM .

In addition, although acrolein was tested as a single factor for its toxicity, many other injuries occur simultaneously in the setting of SCI. These so-called secondary-injury factors probably work synergistically to produce more severe injury than that produced by any single factor. For example, ischemia can exacerbate acrolein-mediated membrane damage.^{35,36} The mechanism of such exacerbation may involve the increased generation of ROS, decreased production of adenosine triphosphate (ATP), and intracellular accumulation of toxic levels of calcium resulting from ischemia.^{35,37,38} Because ischemia usually accompanies traumatic brain injury and SCIs, the detrimental effect of acrolein in functional injury is likely to be

more severe than that seen in studies in which injuries were not associated with a shortage of oxygen or glucose.^{8,9,12,18}

The mechanism at work in acrolein-mediated membrane damage is not fully understood but is probably, at least in part, mediated by ROS. A significant increase in ROS, LPO, and protein carbonyl levels, as well as depletion of endogenous glutathione (GSH), occurs during acrolein exposure.^{9,12} In addition, the application of antioxidants *in vitro* and *ex vivo* can significantly lessen acrolein-mediated membrane damage.⁹

Of all the antioxidants used, glutathione diethyl ester, which can be easily converted to GSH after entering the cell, is the most effective antioxidant for countering acrolein-mediated membrane damage.⁹ This probably is attributed to (1) the ability of GSH to detoxify acrolein by direct binding, and (2) the ability of GSH to scavenge hydrogen peroxide.¹⁵ Other antioxidants, including catalase, formate, and manganese(III)tetrakis(1-methyl-4-pyridyl)porphyrin, can reduce the acrolein-mediated damage by at least 50%.⁹

Acrolein-mediated mitochondrial dysfunction Mitochondria are one of the most important cellular sources of ROS production and are particularly susceptible to oxidative stress.^{39,40} Specifically, the mitochondrial respiratory chain represents a major source of ROS production. During normal cellular metabolism, an estimated 1% to 2% of the electrons that flow into the electron transport chain catalyze the incomplete reduction of oxygen to superoxide radical.⁴¹ The generation of ROS significantly increases when the function of the electron transport chain is compromised.^{39,40}

Acrolein has been shown to impair the function of the respiratory chain in mitochondria isolated from heart and brain tissue.^{12,42,43} Therefore, acrolein probably exacerbates oxidative stress in part by inhibiting mitochondria in injured neuronal tissue. Consistent with this hypothesis, our findings show that acrolein stimulated significant ROS production in isolated brain mitochondria.¹² We have further shown that acrolein-induced mitochondrial oxidative stress is caused by increased production of ROS and the decrease of antioxidants, specifically GSH.¹²

GSH is one of the most important antioxidants in brain mitochondria. Specifically, GSH plays a critical role in the detoxification of hydrogen peroxide because brain mitochondria lack catalase.⁴⁴ Accordingly, acrolein rapidly binds to and depletes intracellular GSH.¹⁵

Another relevant finding is that impairment of adenine nucleotide translocase (ANT) activity accompanies acrolein-induced oxidative stress and inhibition of electron transport. ANT deficit can lead to inhibition of the mitochondrial electron transport function, which in turn has 2 direct effects on oxidative stress: (1) it promotes generation of ROS and (2) it lowers the resistance of mitochondria to prooxidants.⁴⁵ Therefore, we believe that ANT inactivation is an important contributing factor to overall increases in ROS following acrolein exposure.

The mechanism of ROS elevation caused by ANT inactivation is not well established. However, several hypotheses already have been suggested. Wallace^{46,47} postulated that deficits in ANT reduce matrix adenosine diphosphate (ADP) and limit matrix ADP-dependent proton translocata-

tion through F1-F0 ATPase. The reduction in proton transport to the mitochondrial matrix yields hyperpolarization of the mitochondrial membrane, which further limits electron transfer through the respiratory chain. Since acrolein has been shown to be ineffective at inhibiting the activities of the respiratory complexes I-V,⁴³ ANT may be the major, if not the sole, target of acrolein action on the electron transport chain in mitochondria.

The mechanisms by which acrolein inhibits ANT, the most abundant single protein within the inner mitochondrial membrane,⁴⁸ are not known. Acrolein may modify ANT directly. Furthermore, in its cysteine residues, ANT contains sulfhydryl moieties.^{48,49} These moieties are a likely target of acrolein modification.¹⁵

Mitochondrial calcium influx is a well-known mechanism by which mitochondria induce ROS formation. Elevated mitochondrial calcium concentration may inhibit electron transport and oxidative phosphorylation, as well as activate key enzymes responsible for ROS generation, leading to the increased production of ROS.^{38,50,51} However, we found that acrolein did not cause significant calcium influx at 1 and 10 μM concentrations, although significant ROS generation was observed at these concentrations.¹² Moreover, calcium chelator ethylenediaminetetraacetic acid did not prevent the acrolein-induced generation of ROS.¹² These results suggest that calcium plays a relatively less important role in acrolein-induced mitochondrial generation of ROS. This is especially relevant at lower concentrations of acrolein, which may be more clinically relevant than higher concentrations.

Another common factor for inducing mitochondrial oxidative stress is mitochondrial membrane permeability transition.⁵² The permeability transition occurs through the opening of a transmembrane pore in the inner mitochondrial membrane. This process collapses ion gradients across the inner mitochondrial membrane, leading to mitochondrial depolarization, reduction of oxidative phosphorylation, and generation of ROS.⁵² However, in isolated guinea pig spinal cord, acrolein actually had a mild inhibitory effect on calcium-induced permeability transition.¹² This is consistent with other reports showing that some products of LPO, such as HNE and aldehydes, are potent inhibitors of mitochondrial permeability transition under certain conditions.^{53,54}

Several enzymes within the mitochondria are involved in the degradation or detoxification of acrolein.⁵⁵ However, this degradation process is not completely harmless. For example, xanthine oxidase may produce superoxide while metabolizing acrolein.⁵⁶ Such mechanisms also may play a role in the overall acrolein-mediated ROS elevation. However, the data from our laboratory indicate that xanthine oxidase-dependent ROS contributes minimally to the overall ROS level induced by acrolein. We have shown that the inhibition of this enzyme by allopurinol, an inhibitor of xanthine oxidase, did not significantly prevent acrolein-induced ROS generation.¹² Studies^{12,42,43,57} suggest that CNS mitochondria may be more vulnerable to acrolein than are mitochondria from other organs such as the liver and heart.

Acrolein-induced acute demyelination Using a newly developed technique, coherent anti-Stokes

Raman scattering microscopy,⁵⁸ we recently obtained data demonstrating that acrolein can inflict acute demyelination in isolated guinea pig spinal cord white matter (unpublished observation). Specifically, acrolein at a concentration of 200 to 500 μM can inflict myelin damage as early as 25 minutes after exposure. Histologic abnormalities include loosening of, and vesicle formation around, the myelin sheath.

These changes could be attributed to the direct attack of the myelin or could be caused indirectly through acrolein-mediated oligodendrocyte cytotoxicity. This damage may be mediated at least in part through ROS. Regardless of the mechanism, acrolein-mediated damage would be expected to produce functional deficits such as axonal conduction block. Indeed, we have shown that 200 μM of acrolein can cause significant conduction deficits that start 30 minutes after initial exposure and progressively worsen.⁸

DETECTION OF ACROLEIN AFTER SCI

Despite overwhelming evidence of acrolein toxicity in vitro, little is known about concentrations of acrolein in the nervous system. Technical difficulties in detecting acrolein, which is a volatile and small molecule, contribute to this lack of knowledge. Using high-performance liquid chromatography on postmortem tissues, Lovell and colleagues compiled data suggesting that acrolein levels are significantly higher in the brains of patients with AD than in those of age-matched controls.⁶ Uchida and colleagues^{59,60} recently developed a sensitive measurement tool that uses enzyme-linked immunosorbent assay to detect acrolein-pro-

tein adduct following acute injury.

Using this technique, we demonstrated that the level of acrolein is indeed significantly elevated after spinal cord compression injury in a guinea pig model.¹¹ Acrolein elevation was demonstrated as early as 4 hours, peaked at day 1, and persisted at least 7 days after injury. We also performed immunohistochemical staining, using the same antibody to visualize the accumulation of acrolein-modified protein. Similar to the results of Western blotting, the acrolein–keyhole limpet hemocyanin (KLH) immunoreactivity was significantly increased in spinal cord tissue after compression injury. The signal of acrolein-KLH immunoreactivity in the injured spinal cord tissue was significantly stronger in both gray and white matter area compared with controls.¹¹

Interestingly, the elevation in acrolein level was not limited to the injury site but spread to several cranial and caudal vertebral segments.¹¹ As mentioned earlier, the diffuse elevation of acrolein spatially coincides with membrane disruption. Specifically, significantly elevated levels of acrolein have been measured not only at the original compression site, but also at 10 mm from the injury site as well as 24 hours post-injury.¹¹ Severe axonal membrane damage also was present in the same injury model but was not apparent until 3 days after injury.²⁹ These data are consistent with the hypothesis that diffuse elevation of acrolein precedes and leads to membrane disruption and subsequent cell death in functional spinal cord trauma. Additional findings,^{30,31} along with the results from our laboratory, reveal that secondary oxidative stress is not limited to the original impact zone but is more widely

distributed.

ACROLEIN AS A THERAPEUTIC TARGET

Because acrolein may be a key factor in perpetuating oxidative stress-related damages, it may constitute an effective target for therapeutic treatments. Drugs that can reduce acrolein toxicity present a promising therapeutic approach. Hydralazine, a commercially available antihypertension drug, has been reported to bind and neutralize acrolein in a cell-free system.⁶¹⁻⁶³ Using a PC-12 cell model, we found that hydralazine significantly attenuated acrolein-mediated membrane damage, disruption of mitochondrial function, depletion of intracellular GSH, and ATP exhaustion (unpublished observations).

The protective effects probably are mediated by the ability of hydralazine, a strong nucleophile, to react with acrolein. We also have noted that the neuroprotective effects of hydralazine are not specific to acrolein. In fact, hydralazine improved mitochondrial functions in HNE and malondialdehyde-treated cell populations as well. This finding is especially promising, since it is well known that LPO yields multiple toxic aldehydes in addition to acrolein. Experiments examining the ability of hydralazine to protect cells from acrolein toxicity in *ex vivo* and *in vivo* preparations are currently under way in our laboratories. Hydralazine and its related compounds are expected to be effective at reducing oxidative stress by trapping acrolein.

CONCLUSION

Strong evidence indicates that acrolein plays a critical role in oxidative stress because of its long

half-life, ability to generate free radicals, potent cytotoxicity, and elevation in certain conditions associated with oxidative stress. It also has synergistic effects with other common secondary injuries in which oxidative stress is known to exist. One particularly compelling characteristic of acrolein is its half-life, which is 100 quadrillion times longer (10^{17}) than that of transient ROS.¹³ This also could explain why years of research in targeting transient ROS using free radical scavengers has yielded no effective treatment. Hence, acrolein constitutes a more logical target for effective therapeutic intervention to reduce oxidative stress.

Mammalian cells have a built-in antioxidant system to combat oxidative stress in most situations.³ However, in acute or chronic injury, the endogenous antioxidant system is usually overwhelmed by the constant production of free radicals.^{37,38,64-67} Acrolein could be responsible not only for stimulating the generation of free radicals but also for depleting endogenous antioxidants such as GSH. Therefore, scavenging acrolein may impede the vicious cycle that perpetuates oxidative stress. Once established, such therapeutic strategies would not only benefit patients with SCI but also patients with other diseases associated with oxidative stress, such as Parkinson disease, AD, and perhaps even cancer.

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