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Acrolein-Mediated Mechanisms of Neuronal Death

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It is well known that traumatic injury in the central nervous system can be viewed as a primary injury and a secondary injury. Increases in oxidative stress lead to breakdown of membrane lipids (lipid peroxidation) during secondary injury. Acrolein, an alpha,beta-unsaturated aldehyde, together with other aldehydes, increases as a result of self-propagating lipid peroxidation. Historically, most research on the pathology of secondary injury has focused on reactive oxygen species (ROS) rather than lipid peroxidation products. Little is known about the toxicology and cell death mediated by these aldehydes. In this study, we investigated and characterized certain features of cell death induced by acrolein on PC12 cells as well as cells from dorsal root ganglion (DRG) and sympathetic ganglion in vitro. In the companion paper, we evaluated a possible means to interfere with this toxicity by application of a compound that can bind to and inactivate acrolein. Here we use both light and atomic force microscopy to study cell morphology after exposure to acrolein. Administration of 100 μM acrolein caused a dramatic change in cell morphology as early as 4 hr. Cytoskeletal structures significantly deteriorated after exposure to 100 μM acrolein as demonstrated by fluorescence microscopy, whereas calpain activity increased significantly at this concentration. Cell viability assays indicated significant cell death with 100 μM acrolein by 4 hr. Caspase 3 activity and DNA fragmentation assays were performed and supported the notion that 100 μM acrolein induced PC12 cell death by the mechanism of necrosis, not apoptosis.

Key words: acrolein; endogenous toxin; secondary injury; neurotrauma; lipid peroxidation

There were two interests in our laboratories driving this investigation: 1) understanding the mechanisms of early progressive cell death and degeneration after mechanical damage to the central nervous system (CNS) and 2) developing practical ways to interfere with these events in the acute phase to spare neuronal tissue and thus behavior (see companion paper). Irreparable injury to the CNS can be considered to occur in two phases: the acute phase and the chronic phase. Although the temporal difference between these phases cannot be exactly defined, the anatomical state of the nervous system after injury more clearly defines their character. In the acute phase, considerable amounts of both gray and white matter survive the insult, and it is the goal of therapies applied during this phase to protect cells and tissues from further destruction. In the chronic phase, considerable amounts of nervous tissues have been permanently lost. Thus, the biology of CNS injury can be considered as two separate—sometimes mutually exclusive—processes of trauma relative to the experimental interventions aimed at them (Borgens, 2003).

In the 1980s, much effort was directed at interfering with the early, acute events after spinal cord damage, leading to the adoption of ''megadoses'' of the corticosteroid methylprednisolone (MP) during the first 8 hr after injury (Braken et al., 1985, 1990, 1992, 1997). This clinical use of MP has recently come under severe criticism (Short et al., 2000; Pointlillart et al., 2000).

We have revisited the acute injury, believing that there has been too little knowledge relative to the critical biochemistries underpinning secondary injury. It has long been understood that aberrant oxidative metabolism after mechanical damage to neurons leads to the formation of highly reactive oxygen species (ROS; so-called free radicals) which in turn facilitate the breakdown of membrane lipids in a positive feedback spiral of lipid peroxidation (LPO; Hall, 1993). It is the breakdown products of LPO that are likely potent endogenous toxins, which sequentially fuel this continuing and progressive destruction of parenchyma. Calcium-activated lipases also produce and liberate arachadonic acid from fatty acids; however, the three most destructive cellular poisons are aldehydes. The culprits that kill in a wholesale way both injured and nearby normal cells after CNS injury are 4-hydrononeal-trans-2-nonenal (HNE), malondealdehyde (MDA), and acrolein, yet these toxins and their mechanisms of action have received relatively little attention compared with

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ROS. Acrolein is of interest insofar as it is a well-known aldehyde often used in tissue fixatives and passes through the intact cell membrane with facility. As an endogenous toxin, this makes it especially insidious, in that high concentrations of acrolein produced by LPO can thus spread from the dying cell of origin to damage or kill adjacent cells. In the spinal cord, as in other compromised tissues, acrolein concentration increases after injury (Satoh et al., 1999; Nardini et al., 2002; Luo et al., 2005). It is unknown whether acrolein initiates cell death by apoptotic mechanisms or by cellular necrosis. If cell death occurs by the former mechanism, the challenge to "neuroprotect" such cells effectively is an immense one, insofar as there is as yet no practical means to interfere with apoptosis, leading to the rescue of dying cells. If it is the latter, several avenues of therapy exist. When these are applied in the early hours after mechanical injury, it is reasonable to suggest that effective neuroprotection may be achieved. Here we provide a characterization of acrolein-mediated cell death and determine the type of death that occurs in response to acrolein toxicity in a standardized, genetically stable cell line, PC12 cells.

MATERIALS AND METHODS

PC12 Cell Culture, Differentiation, and Acrolein

PC12 cells were grown in 5% CO_2 at 37°C. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 12.5% horse serum, 2.5% fetal bovine serum, 50 U/ml penicillin, and 5 mg/ml streptomycin. Culture medium was changed every other day, and cells were split every week. To induce neuronal differentiation, cells were seeded at 1×10^5 cells/dish in 35-mm-diameter Petri dishes, and the culture medium contained 10 ng/ml nerve growth factor (NGF). Studies began when most cells had differentiated for 4 days and exhibited extensive processes. Acrolein (10^4 μM) was made fresh in phosphate-buffered saline (PBS) as a stock solution and diluted when added to the cell culture medium.

Cytoxicity Measurement (MTT Assay)

Cells were seeded at 1×10^6 cells/well in 12-well plates for 2 hr before the application of acrolein. Cell medium was replaced by PBS right before acrolein application. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was reconstituted in PBS and added to each well 1 hr before the termination of the experiment. The experiment was terminated at 1 hr, 4 hr, and 12 hr after the application of acrolein. After incubation, a volume of MTT solubilization solution equal to that of the original medium was added to each well to dissolve the remaining formazan crystals. Spectrophotometric (SLT; Spectra) absorbance was measured at 550 nm, and the background absorbance at 660 nm was subtracted from these values.

Microtubule Immunostaining and Confocal Microscopy

PC12 cells were fully differentiated in the presence of NGF on glass coverslips when placed into a 35-mm-diameter Petri dish. Individual experiments were stopped at 10 min, 20 min, 30 min, 1 hr, and 4 hr after acrolein had been added to each dish. The coverslips with cells attached were switched to fixative buffer [0.1% gluteraldehyde v/v, 2% paraformaldhyde v/v, 80 mM PIPES, 5 mM EGTA, 1 mM $MgCl^2$, 0.5% Triton-100 v/v] for 7 min and the coverslips washed twice with PBS (pH 7.4), 5 min each time. After that, the dishes were incubated with methanol at –20°C for 2 min and rehydrated in PBS for 5 min at 37°C. PC12 cells were incubated with 160 μl of PBS containing a monoclonal rat antitubulin IgG (1:200) for 45 min in a humidified chamber at 37°C, followed by two 5-min washes with PBS. Then, the coverslips were incubated with 16 μl of PBS containing a fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG secondary antibody (1:100) for 45 min in a humidified chamber at 37°C, followed by two 2-min washes with PBS. The coverslips were mounted in mounting media (0.5% p-phenylenediamine in 20 mM Tris, pH 8.8, 90% glycerol) and sealed with nail polish. Confocal images were acquired with an MRC-1024 (Bio-Rad, Hemel Hempstead, United Kingdom) on a Diaphot 300 (Nikon, Tokyo, Japan) inverted microscope with a 60× 1.4-NA lens. The 488-nm line of the krypton-argon laser (Ion Laser Technology, Salt Lake City, UT) was used to excite fluorescein-5-isothiocyanate-conjugated goat anti-rat IgG secondary antibody, and the emission was collected by using a 515-nm longpass filter.

Morphological Examination Via Atomic Force Microscopy

Atomic force microscopy (AFM) was performed on dorsal root ganglia (DRG) and sympathetic ganglia dissected from 7–8-day-old embryos by conventional methods (Smith, 1998). The cells were plated on a substrate of polyornithine and laminin and maintained in 2% neuron growth medium (Higgins and Banker, 1998) in 5% CO_2 at 37°C for 1–4 days. Details of the sample preparation can be found in a previous publication (McNally and Borgens, 2004).

AFM imaging was performed on two different instruments, a PSIA XE-120 mounted on a Nikon TS-2000 inverted optical scope and a Digital Instruments Dimension 3100 using both contact and tapping modes. All imaging was performed on live cells in neural media within 4 hr of removing them from the incubator, at which time the cells were still healthy and well attached to the substrate. The XE-120 was fitted with an open liquid cell and a silicon nitride substrate (Digital Instruments DNP-20). The same substrate was used on the Dimension system with an associated fluid cell (Digital Instruments DTFML). The cantilever for contact mode was V-shaped, 200 μm in length, and 23 μm wide, yielding a spring constant of 0.06 N/m. For tapping mode, the cantilever used was V-shaped, 200 μm in length, and 41 μm wide, yielding a spring constant of 0.12 N/m. The tip radius of curvature for both cantilevers was nominally 20 nm. The 256 × 256 data point image fields (20 μm^2) were obtained at 5 Hz per line, requiring approximately 10 min to complete the entire image. Live cell images were collected prior to the induction of acrolein to ensure good surface attachment and image quality. After at least two complete images, acrolein was added to the sample dish to bring the final acrolein con-

centration to 100 μM. Both height and amplitude modes were used to image the surface topography. Quantitative measurements were derived form the height mode images in WSxM software from Nanotec Electronica (www.nanotec.es/download.htm).

Calpain Activity Assay

PC12 cells were incubated with 130 μM of the membrane-permeable peptide substrate Suc-LLVY-AMC for 1 hr at 37°C (Bronk and Gores, 1993). Then, cell pellets were collected by centrifugation and resuspended in PBS and transferred to 1-ml quartz cuvettes. Acrolein was added to each cuvette at concentrations of 1, 10, and 100 μM in three separate experiments. The fluorescence emitted from free AMC that was cleaved from nonfluorescent Suc-LLVY-AMC by calpain was monitored dynamically by a Perkin-Elmer L955 fluorometer at 360 nm excitation and 480 nm emission for 1–2 hr.

DNA Ladder Assay

The DNA ladder assay was performed with an apoptotic DNA ladder kit purchased from Roche (Indianapolis, IN). Briefly, cells were seeded at 1×10^6 cells/well in 12-well plates overnight before the addition of acrolein. Cell cultures were terminated 1 hr, 4 hr, and 12 hr after the application of acrolein, and cells were washed from the well with PBS and centrifuged to obtain pellets. Cells were lysed with a binding buffer, followed by several series of washing and centrifugation. Finally, DNA was eluted with elution buffer. Loading buffer (10×; 1% sodium dodecyl sulfate, 0.25% bromophenol blue, 30% glycerol) was mixed with DNA samples at a 1:9 ratio. Samples were loaded onto a 1% agarose gel containing 0.5 μg/ml ethidium bromide. Electrophoresis was carried out for 1.5 hr. One-hundred-base-pair molecular weight markers were used, and DNA was visualized by ultraviolet light and photographed by handheld camera (Fisher Biotech photodocumentation system).

Caspase 3 Activity Assay

Caspase 3 activity was measured by using a caspase 3 apoptosis detection kit from Santa Cruz Biotechnology (Santa Cruz, CA). Cell cultures were stopped 1 hr, 4 hr, and 12 hr following the application of acrolein. PC12 cells were detached from the well and collected by centrifugation. Cells were then lysed by incubation in a lysis buffer for 10 min on ice. The caspase 3-specific substrate DEVD-AFC was added to cell lysates and incubated for 1 hr at 37°C. The level of fluorescence of free AFC released from the digestion of DEVD-AFC by caspase 3 was measured with FlouroCount (Packard Instrument Company) at 360 nm excitation and 530 nm emission.

Statistical Analysis

Data from the MTT and caspase 3 activity assays are given as mean ± SD. Student's *t*-tests were used to determine the significance between acrolein-treated and control groups. $P < 0.05$ was considered significant. All experiments were repeated a minimum of two times.

RESULTS

Choosing an Effective Concentration of Acrolein

We began this study by searching for a concentration of acrolein in PBS buffer that would produce consistent PC12 cell death within an observation period of 12 hr. The destructive effects of acrolein were characterized first by 1) loss and/or retraction of cell processes, followed by 2) loss of attachment(s) to the substrate, 3) rounding in shape, and 4) often complete detachment from the substrate, producing "floaters." Determining an effective concentration of acrolein was required before characterizing its toxicity (or the possibility of rescue by application of acrolein-trapping agent; see companion paper). We had originally planned to make careful counts of cells in vitro to accomplish this; however, this procedure was unnecessary. A concentration of 1 μM acrolein added to the culture medium was unable to produce any obvious destruction or clear changes in PC12 cells (1×10^6 cells per dish, 12 hr after the addition of acrolein; see Materials and Methods). The next serial increase of 10 μM was too variable and unpredictable in its effect on cell viability to be useful.

However, the entire population of PC12 cells was consistently killed, usually in less than 12 hr, with the application of 100 μM acrolein. Figure 1 shows the effects of serial concentrations of acrolein compared with control cells (PBS added rather than acrolein), underscoring the effectiveness of the higher concentration tested that produced consistent cell death.

Mitochondrial Function in Acrolein-Exposed Cell Populations

Other, more informative indices of the gross change in cell populations exposed to different concentrations of acrolein were used to characterize and quantify "cell death." One expected failure would be respiratory function/oxidative metabolism. To monitor this the MTT assay was used. Figure 2 shows the results of the MTT assay as indexed by spectrophotometric absorbance (550–660 nm). Note that cultures treated with 1 and 10 μM acrolein, as well as the control group, did not show a significant reduction of MTT activity at any of the three assay times (1, 4, 12 hr). Indeed, a statistically significant increase in oxidative metabolism occurred at 4 hr after 1 μM acrolein exposure and at 12 hr at 10 μM acrolein exposure. Compare these responses at all assay times with the complete collapse of mitochondrial functioning after exposure to 100 μM acrolein by 4 and 12 hr (Fig. 2).

Destruction of the Cytoskeleton: Light Microscopy

The morphological changes in cells shown in Figure 1 should also be accompanied by a destruction of the cytoskeleton. We studied several members of the cytoskeleton family but report only results evaluating microtubules, because these data are representative of all. We tested calpain activity as well (an increase in this enzyme is required for cytoskeleton disassembly). The morphological changes in microtubular structure (with a fluorescence-conjugated

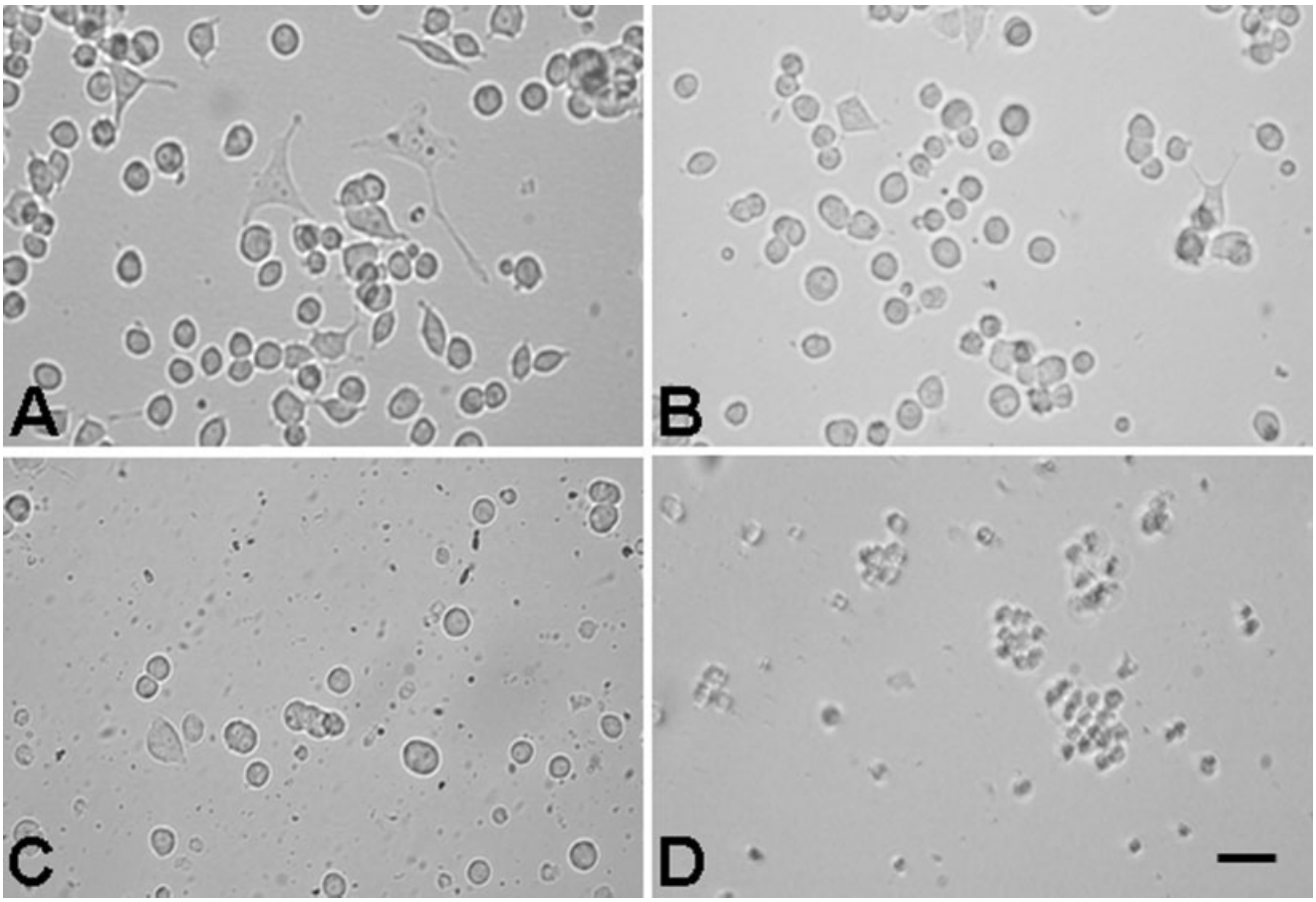


Fig. 1. General morphology of PC12 cells in vitro and their death after acrolein exposure. **A:** Untreated PC12 cells. Note that only a few stellate cells have spread out and formed neurites and extended filapodia; others have become spindle shaped, but the majority remain isodiametric. **B:** A similar density of PC12 cells, 12 hr after application of PBS containing 1 μm acrolein. Qualitatively, there is little change in the morphology of these cells and no apparent cell death. **C:** Similarly to A and B, 10 μM acrolein was dissolved in the PBS solution. Note a striking dying off of cells, cell debris, and the lack of nonuniform morphology such as stellate or spindle-shaped cells. **D:** Similarly to A–C, cells were treated with 100 μM acrolein. By 12 hr, the entire population of PC12 cells was dead and degraded. Scale bar = 30 μm.

antibody to microtubules) was our means to image these changes in response to acrolein.

In Figure 3, the ordinate is an indirect measure of calpain activity in cells exposed to 380-nm excitation wavelength and read at a fluorescence wavelength of 460 nm (see Materials and Methods). The abscissa is time in minutes. This data set represents one experiment from eight and reveals characteristics identical to all. The change in intracellular calpain was revealed in this assay as a change in the fluorescence intensity of AMC from Suc-LLVY-AMC, a specific substrate of calpain. Note that, as expected, calpain activity was not apparent in unpoisoned cells (negative values of fluorescence are due to the subtraction of background fluorescence during normalization). Figure 3 shows a clear and sustained exponential increase in intracellular calpain after exposure to 100 μM acrolein but not to 1 or 10 μM acrolein. Not shown is a brief (<1 hr) and variable initial boost in calpain activity in response to the application of 1 and 10 μM acrolein; however, this fell off significantly prior to the termination of the experiment.

In Figure 4, confocal microscopy shows the responses of microtubules to 100 μM acrolein over time. Microtubules were studied by using a monoclonal antibody in differentiated PC12 cells. Note, in Figure 4A, the filamentous arrays of tubular components in the soma extending into the processes of the stellate cells; the faint outline of individual microtubules subtending this architecture can be seen. The reduction of cell size and an initial ''blebbing'' of processes (the formation of fiber varicosities) were obvious after only 10 min of acrolein exposure (Fig. 4B). Note, in Figure 4C (upper right), the cytoskeleton retracted centripetally, the general picnotic appearance, the progressive loss of neurites, and the rounding of the cells into an isodiametric form. Note also that, by 1 and 4 hr after acrolein treatment, microtubules have been dissassembled, degraded, and dissolved and that, by 4 hr (Fig. 4D), little cytoplasm was left in these cells.

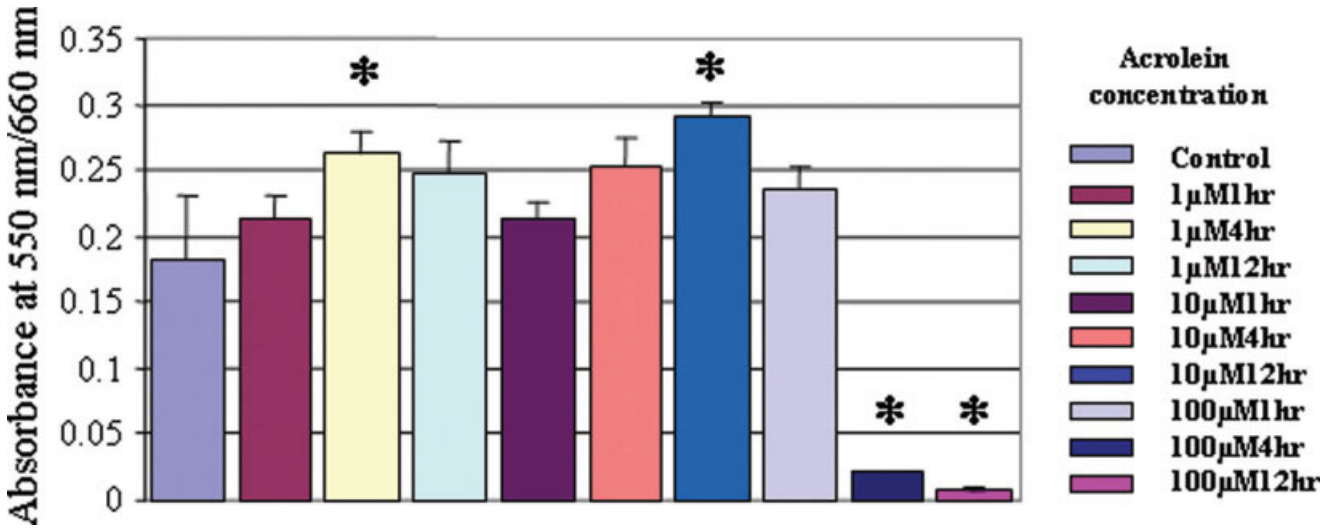


Fig. 2. Mitochondrial functioning assessed by the MTT assay. Oxidative metabolism was followed by the MTT assay at different times after application of acrolein. The absorbance of each sample is given, which reflects the product of the MTT reaction (see Materials and Methods). Note that a small, but statistically significant, increase in mitochondrial function after exposure to acrolein was evident at 1 and 10 μM at 4 and 12 hr, respectively. This result is not unexpected, given the typical increase in metabolism often observed after challenge with a nonlethal dose of toxin. Most importantly, note the complete collapse of oxidative metabolism at 100 μM acrolein. $*P < 0.05$.

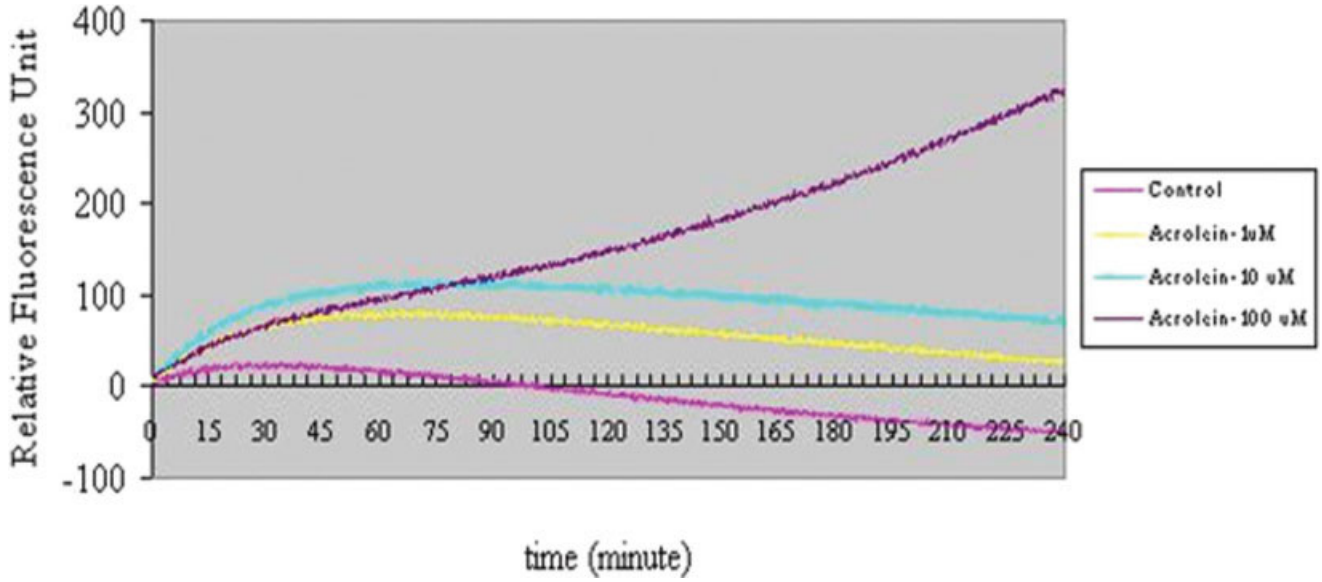


Fig. 3. Calpain activities in PC12 cells after acrolein exposure. Typically, calpain activity secondary to both 1 and 10 μM acrolein exposure varied little from that detected in uninjured cells, whereas only 100 μM acrolein exposure produced an exponential increase in calpain activity detected by this method. The ordinate is in minutes; the abscissa is units of fluorescence in nm. PC12 cells were incubated with the calpain-specific substrate Suc-LLVY-AMC. The fluorescence derived from release of the AMC moiety was measured by fluorimetry at 380 nm excitation and 460 nm emission.

Destruction of the Cytoskeleton: AFM Analysis

AFM imaging of live cells has been performed in tapping and contact mode for periods of 4 hr with no detrimental effect to the cell. With the introduction of acrolein into the culture media, changes in the morphology of the growth cone and cell bodies are seen within 30 min, whereas complete retraction of the extending neurites was seen within 1 hr, and release of cell bodies from the surface was seen after 2 hr. Figure 5 shows the growth cone regions of two DRG cells, one without acrolein (top row) and one treated with acrolein (bottom row). Figure 5a–c depicts a growth cone region continuously scanned in tap-

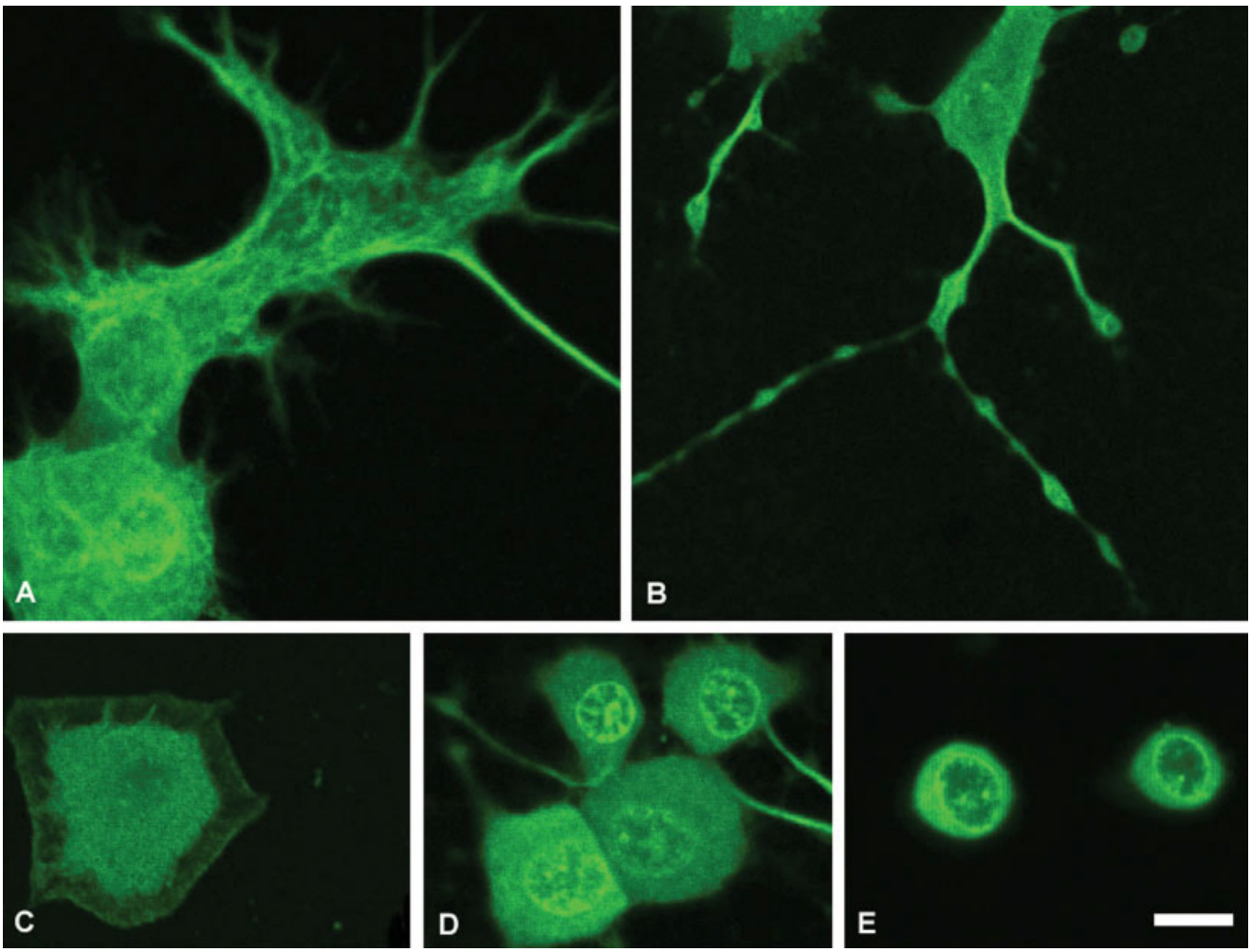


Fig. 4. Changes in microtubule structure after acrolein treatment under confocal light microscopy. **A:** A well-differentiated PC12 cell is shown; note the arrays of individual microtubules extending throughout the cytosol. **B:** Changes in cell processes 10 min following the addition of 100 μM acrolein. Note the complete loss of microtubular fine structure from the cell body and processes, and the blebbing of neurites. In **C**, a formerly differentiated PC12 cell after acrolein exposure at 30 min, and others at 1 hr (**D**) and at 4 hr (**E**) later. Note the transformation to shrunken, picnotic, and spherical cells. Scale bar = 10 μm.

ping mode. Changes in the morphology can be noted; the surrounding filopodia are changing in size and direction, and the height and shape of the neurite is varying between scans. This is expected, insofar as the neurons are living and thus growing and altering their shape. Overall, the growth cone has not changed significantly; in fact, this neurite was imaged for 3 hr without retracting or losing contact with the substrate. Figure 5d–f shows a neurite undergoing changes after exposure to acrolein. Figure 5d was obtained prior to introduction of acrolein into the culture media. This view shows a healthy neurite well attached to the substrate. The neurite length was greater than 20 μm, with an average height of 612.6 nm. The area covered by the neurite was 175.7 μm^2, and its total volume above the substrate was 42.3 μm^3. Figure 5e was obtained 25 min after acrolein was added. The neurite had retracted to a length of 17.2 μm and was reduced in height to 508.8 nm. The area covered was 176.6 μm^2, with a total volume of 53.7 μm^3. In addition, the image provides less detail compared with that in Figure 5d, which implies that the cell membrane had become more pliable and less resistant to the AFM tip. This is again seen in Figure 5f, where the neurite had retracted to a length of 14.68 μm and a height of 475.9 nm. In this image, the neurite is thicker (or swollen) giving an area of 175.3 μm^2 and a total volume of 63.2 μm^3. In general, neural cells that had been exposed to acrolein retracted their neurites by 10% in the first 30 min and completely retracted them by 60 min. All cell bodies were released from the surface within 2 hr.

Is Acrolein-Mediated Cell Death Produced by Apoptosis?

Apoptosis is relatively well characterized by several features: chromatin condensation, an increase in caspase 3 activity in cytosol, and DNA fragmentation by endonucle-

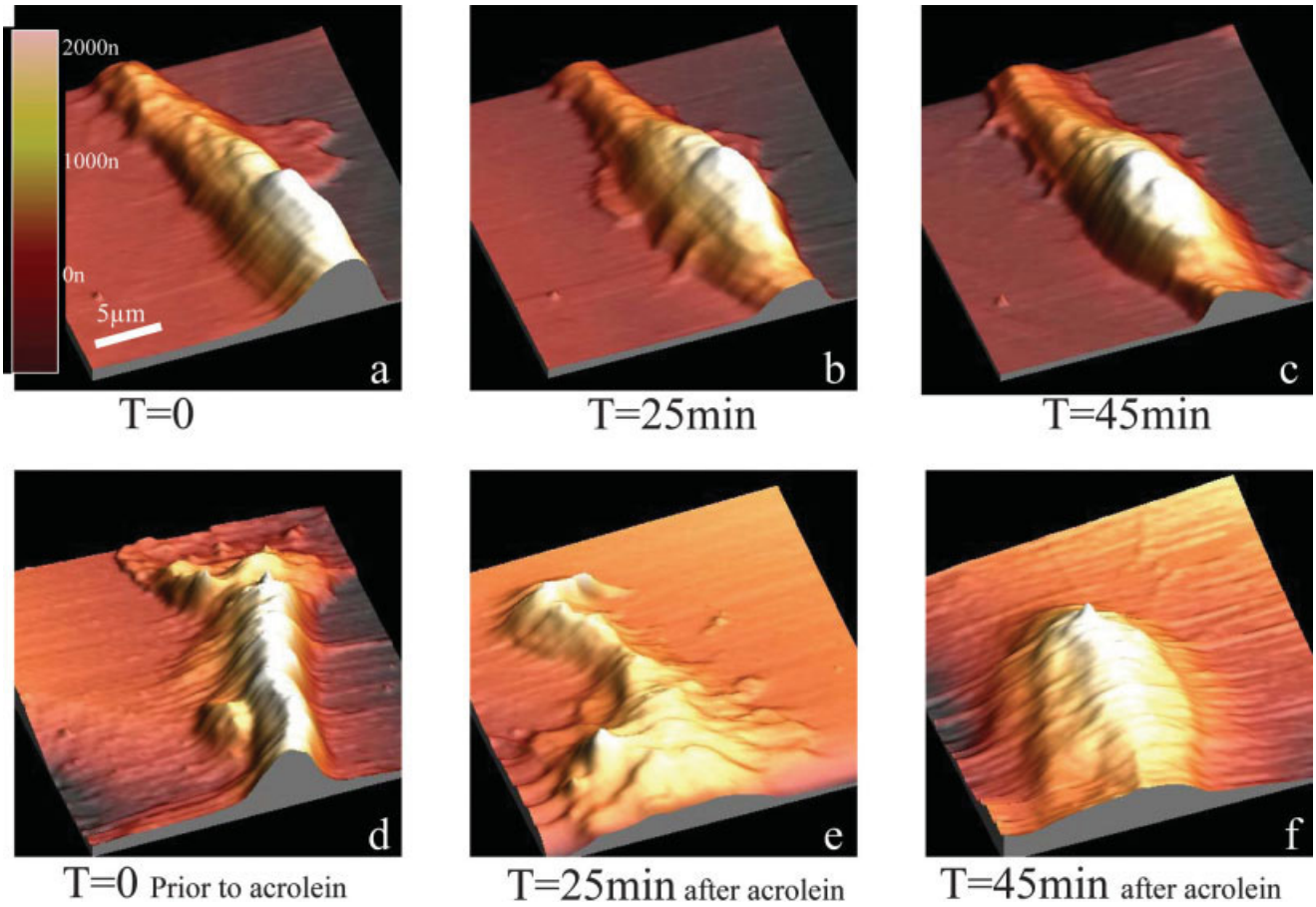


Fig. 5. Changes in neurite structure after acrolein treatment: AFM. Tips of DRG neurites imaged with atomic force microscopy. **a–c:** A healthy growth cone region. Note subtle changes in the neurite size and the surrounding filapodia. **d–f:** Significant changes in a neurite exposed to 100 μM acrolein. The neurite reduces its height and swells in diameter as it retracts. Scale bar and color bar in a apply to all panels.

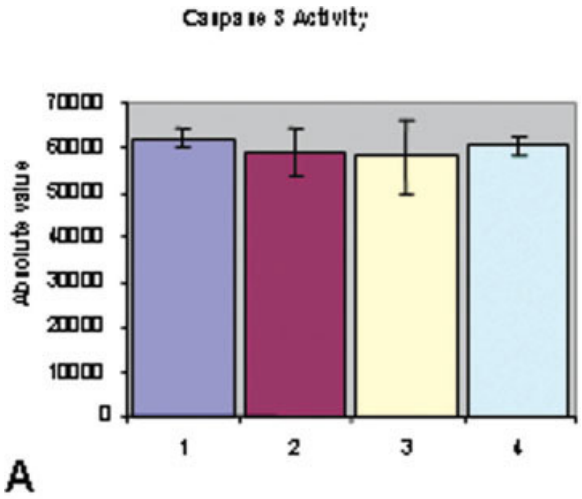
ase into nucleosomal size fragments revealed by the DNA ladder assay. We first observed that exposure to any concentration of acrolein (1, 10, or 100 μM) did not produce an up-regulation of caspase 3 activity (Fig. 6). Caspase 3 activity was indexed indirectly by the fluorescence intensity of AFC, a degradation product of a caspase 3-specific substrate, DEVD-AFC. The agarose gel shown in Figure 7 is typical of three independent experiments. Note the typical DNA patterns (the ''ladder'' characteristic) in lane 1, the apoptotic control. Lanes 2–7 containing the DNA samples tested with 1 and 10 μM acrolein exposures, as well as the control group (lane 11), do not exhibit laddering, whereas lanes 8–10 (exposure to 100 μM acrolein) reveal loss of DNA. Note the absence of a laddering pattern (characteristic of apoptotic-mediated destruction as in lane 1). Lane 12 provided molecular weight markers.

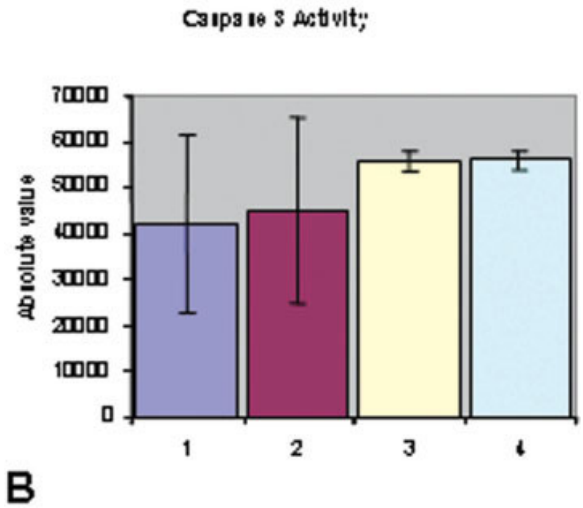
DISCUSSION

The results of this study are based on exposing PC12 cells to 100 μM acrolein that is lethal to all the cells within 12 hr in vitro. We chose this concentration after serial dilution experiments such that the evaluation was not weakened by inconsistent or variable responses of cells to this endogenous toxin in the various biochemical and morphological tests. Over two decades of study of CNS trauma, we have often chosen primary cultures of various types of CNS cells, such as DRG, sympathetic ganglion neurons, and type I astrocytes. Here, we opted to use a genetically stable cell line, a well-known neuronal model, the PC12 cell, to ensure further consistency in the taking of, and evaluation of, biochemical data. DRG cells and sympathetic ganglion cells were used during AFM imaging.

Characteristics of Acrolein Toxicity

Given the conditions used in these experiments, we have learned that 1) acrolein is a potent toxin that can destroy entire populations of PC12 cells in less than 12 hr; 2) its toxicity is associated with gross morphological changes that can be observed by using a typical brightfield microscope as well as with the atomic force microscope starting minutes after exposure; 3) the collapse of oxidative metabolism by mitochondria is significant after acrolein





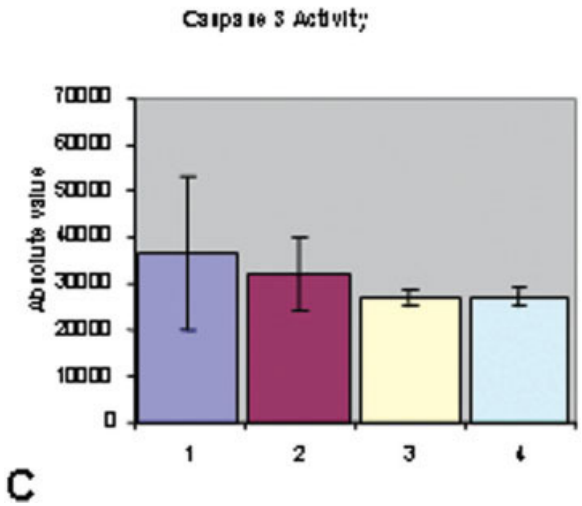


Fig. 6. Caspase 3 activity after acrolein exposure. For each of the three sets of data, the ordinate is value of absorbance, and the abscissa is time after exposure to acrolein. Absorbance values are derived from fluorescence of the degradation product of a specific caspase 3 substrate (see Materials and Methods). For all three sets of data, the control group (1) is displayed first and then 1 hr (2), 4 hr (3), and 12 hr (4) exposure. Cells were exposed to 1 μM acrolein (**A**), 10 μM acrolein (**B**), and 100 μM acrolein (**C**). Comparison of the means (error bars = standard deviation) revealed that there was no significant difference between any of the groups at any time point.

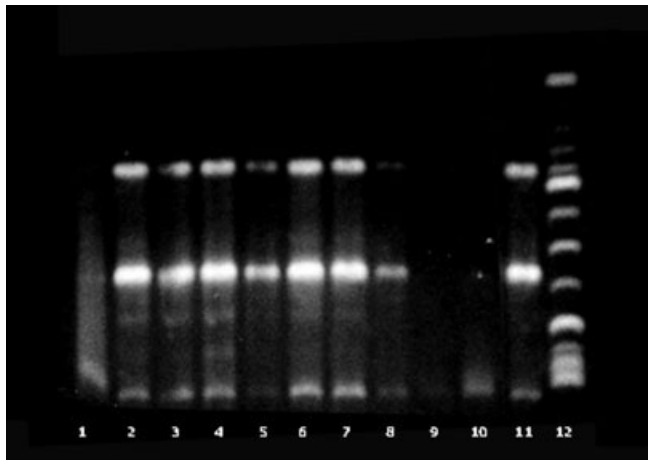


Fig. 7. DNA ladder assay. This photograph of an agarose gel shows the results of a typical DNA ladder test for apoptosis. Lane 1 is an apoptotic control. Lanes 2–4 were loaded with samples from 1 μM acrolein treatment for 1, 4, and 12 hr, respectively. Lanes 5–7 were loaded with samples from 10 μM acrolein treatment for 1, 4, and 12 hr respectively. Lane 8–10 were loaded with samples from 100 μM acrolein treatment for 1, 4, and 12 hr, respectively. Lane 11 was a control sample without exposure to acrolein. Lane 12 was loaded with molecular weight markers. Lane 1 was an apoptotic control revealing the characteristic features of DNA fragmentation resulting from apoptosis (a DNA ''ladder''). Note that, for all concentrations and for all durations of exposure, a typical ladder pattern indicative of apoptosis was absent. The relative lack of DNA in lanes 8–10 is characteristic of the loss of these cells by acute poisoning; however, a ladder pattern was still absent.

exposure; 4) the key enzyme responsible for microtubule degradation (calpain) is up-regulated after acrolein exposure; and 5) the progressive process of destruction of the cytoarchitecture is coincident with a loss/retraction of cell processes and picnosis and a reduction in the size of the cell coincident with a rounding of its shape (a final stage of cell death), and also a loss of cell attachment to the substrate was associated with acrolein toxicity. Furthermore, we have determined that PC12 cell death from acrolein exposure under the conditions of these experiments likely is due to a process of necrosis and likely is not due to apoptotic mechanisms. This interpretation is based on: 1) the failure of an enzymatic marker for a dominant form of apoptosis in the CNS, caspase 3, to be up-regulated at any time after exposure or at any concentration of acrolein tested and 2) the failure to detect the characteristic ''laddering'' pattern of DNA associated with apoptosis.

Caveats

In addition to the use of a transformed cell type as a neuronal model, it is reasonable to suggest that these observations might be indicative of cell responses to only very high concentrations of acrolein. Put another way, at the concentrations likely encountered in vivo (Nardini et al., 2002; Satoh et al., 1999), the responses might be different. The level of acrolein in the serum of a normal human was estimated to reach 50 μM (Satoh et al., 1999). Moreover, acrolein is estimated to reach 80 μM in respiratory tract lining fluids as a result of smoke (Nardini et al., 2002). Although it is likely that the 100 μM acrolein used in this study is relevant to in vivo concentrations, we do not claim this to be the lowest concentration threshold initiating cell death. Exposure to even lower concentrations of acrolein for much longer times, such as days (as likely occurs in vivo), is still likely to induce widespread cell death. Furthermore, it would be expected that acrolein concentration in vivo would climb over time after injury, insofar as lipid peroxidation is self-propagating, and dead and dying cells would liberate acrolein from the cytosol into the interstitial milieu.

Particularly worth considering is the possibility of a subtle initiation of apoptosis at very low concentrations of endogenous toxin exposure, whereas the much higher concentrations act directly and uniformly to kill cells rapidly by necrosis. The results of these experiments do not easily allow such an interpretation, however. Indeed, we have provided data characterizing caspase 3 activities and DNA fragmentation in response to serial dilution of 100 μM acrolein, and these data contain no hint of apoptosis at any concentration. Therefore, we believe that the major toxic products of lipid peroxidation (acrolein and its close analogs MDA and HNE) kill cells via cellular necrosis. This finding is fortuitous, suggesting hope for an acute therapy that might target LPO as a realistic approach to intervention. If cell death were mediated by apoptosis (particularly at low concentrations encountered early in the injury process), no known means would be clinically practicable for interfering or blocking this process.

Thoughts on the History and Future of Interventions Targeting Secondary Injury in the CNS

These data suggest shifting attention from free radical production to the products initiated by ROS as a worthwhile avenue of experimentation. Students of neurotrauma have always known that injury to the nervous system produces a continuing and progressive destruction of tissue, similar in principle to degenerative disease, in that physiological and morphological losses grow worse with time. Only in the last quarter century has the nature of this process been revealed and named ''secondary injury.'' The ''primary'' injury is of course the initial insult to cells and tissues, such as a mechanical one. The early studies of Demopoulos and colleagues (1982) emphasized the production of highly reactive oxygen species, so-called free radicals, that are increased in concentration in the cytosol because of a shift to pathological oxidative metabolism, primarily in mitochondria. The production of potent oxidizing agents (such as O_2^- and ·OH) is also known to be a self-reinforcing process. Moreover, free radicals are produced within the cell, leading to even higher concentrations of ROS through the peroxidation of the membrane (glycolipids and fatty acids). Furthermore, this increase was worrisome given the precipitous fall in endogenous antioxidants secondary to spinal cord injury. Thus, the first practical, acute, intervention in the secondary injury process in injured human spinal cords was predicated on the potent scavenging of free radicals. Chief among the numerous compounds tested were glucocorticoids such as methylprednisolone (MP) and other nonglucocorticoid antioxidants such as the 21 amino steroids. It is sometimes overlooked that ROS production was the main element of secondary injury to be attacked by administration of megadoses of steroids and not based on the more commonly appreciated ability to reduce tissue swelling. Indeed, if the common responses to glucocorticoid administration in other CNS ischemic injury paradigms were thoroughly considered (in that they are often contraindicated; Sapolsky and Pusinelli, 1985), it is reasonable to suggest that megadoses of MP might not have been attempted at all in human spinal cord injury (SCI).

There are compelling new reasons why ROS production should not be the main target to be attacked by a hypothetical acute intervention but rather the toxins produced by LPO. First, the half-life of ROS is 10^{-12} sec, compared with acrolein, whose half-life in vivo is measured in days (Ghilarducci and Tjeerdema, 1995). Second, although it is unquestioned that ROS is part of the pernicious cycle of destructive chemistry initiated by various CNS insults, there is little evidence suggesting that ROS themselves are acutely, or immediately, toxic to cells. Evidence abounds, however, that aldehydes such as acrolein, MDA, and HNE are indeed dangerous cellular poisons, individually highly toxic to cells at several levels of interaction. Acrolein has long been appreciated as a potent cross-linker of proteins and a common ingredient in cell and tissue fixatives. HNE is also toxic to cells, but, among the products of LPO, acrolein has been emphasized because its extracellular concentration may reach 40 times that of HNE and its reactivity may approach 100-fold (Esterbauer et al., 1991). These aldehydes also cross cell membranes easily and are thus toxins that undoubtedly play a role in ''bystander'' damage in SCI, and likely other ischemic and hemorrhagic episodes in brain.

Finally, with respect to SCI, it is the damage to white matter and the resulting loss of conduction that produces catastrophic behavioral loss. Loss of gray matter plays only a minor role in the extreme behavioral deficits associated with SCI, diametrically opposite to the important contribution of neuronal cell loss in brain injury and stroke (Borgens, 2003). White matter is curiously resistant to a significantly reduced oxygen tension and glucose deprivation (Shi et al., 2002; Peasley and Shi, 2003), so it is both convenient and instructive to consider the alkene products of lipid peroxidation to be the most serious

agents of cell and tissue loss during the process of secondary injury. In the companion paper, we evaluated whether an effective acrolein-trapping agent, hydralazine, might offer protection from acrolein-mediated cell death in the PC12 cell model.

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