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Hydralazine Rescues PC12 Cells From Acrolein-Mediated Death

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Acrolein, a major lipid peroxidation product, has been associated with both CNS trauma and neurodegenerative diseases. Because of its long half-life, acrolein is a potent endogenous toxin capable of killing healthy cells during the secondary injury process. Traditionally, attempts to intervene in the process of progressive cell death after the primary injury have included scavenging reactive oxygen species (so-called free radicals). The animal data supporting such an approach have generally been positive, but all human clinical trials attempting a similar outcome in human CNS injury have failed. New drugs that might reduce toxicity by scavenging the products of lipid peroxidation present a promising, and little investigated, therapeutic approach. Hydralazine, a well-known treatment for hypertension, has been reported to react with acrolein, forming hydrazone in cell-free systems. In the companion paper, we have established an acrolein-mediated cell injury model using PC12 cells in vitro. Here we test the hypothesis that the formation of hydrazone adducts with acrolein is able to reduce acrolein toxicity and spare a significant percentage of the population of PC12 cells from death. Concentrations of approximately 1 mM of this aldehyde scavenger can rescue over 80% of the population of PC12 cells. This study provides a basis for a new pharmacological treatment to reduce the effects of secondary injury in the damaged and/or diseased nervous system. In particular, we describe the need for new drugs that possess aldehyde scavenging properties but do not interfere with the regulation of blood pressure. **© 2006 Wiley-Liss, Inc.**

Key words: acrolein; secondary injury; neurotrauma; hydralazine

For over 30 years, it has been well established that many cells that are damaged but survive an initial insult die later. The nature of the initial insults may vary, ranging from oxygen/glucose deprivation to direct mechanical damage and disease. This eventual demise, mediated by biochemical mechanisms that are triggered by the insults, can feature either apoptosis or necrosis. Apoptosis is irreversible in most contexts and is widely believed to be a developmental mechanism that, in the adult, facilitates the removal of marginally healthy cells without initiating an inflammatory response. It is fortunate that this mechanism of cell death is less common than the process of necrosis after damage to the mammalian nervous system, particularly the central nervous system (CNS). The process of necrosis, in contrast, is reversible; thus therapies aimed at preventing or ameliorating necrosis are clinically practicable.

The process of necrosis is distinguished from that of apoptosis in many ways. For example in necrosis, mitochondria are not actively involved, and ATP levels are depleted. This is diametrically opposed to apoptosis (see companion paper). Many secondary injury biochemical mediators, such as glutamate, calcium, and cytokines, initiate abnormal oxygen metabolism, because mitochondrial dysfunction liberates reactive oxygen radicals, such as superoxide, hydroxyl ions, and hydrogen peroxide. These so called free radicals (or ROS) can oxidize various macromolecules, such as protein, DNA genome, and phospholipid components of the organelles and cell membrane. Lipid peroxidation (LPO) is a very destructive process, a hallmark of the secondary injury phenomenon, and feeds forward in a progressive cascade of reactions, producing numerous cell toxins within the cytoplasm. Here the oxidation of largely polyunsaturated fatty acids (PUFA) of the inner leaflet of the plasmalemma initiates the production of a series of intermediates, leading to continued degradation of the membrane and, simultaneously, the production of increasing concentrations, particularly of aldehyde cell toxins.

A crucial and relevant end point of LPO is the accumulation of these aldehydes in the cytoplasm, e.g., acrolein, 4-hydrononeal-trans-2-nonenal (HNE), and malondealdehyde (MDA). Unlike free radicals, aldehydes possess half-lives ranging from a few hours to days. Thus, it is both instructive and reasonable to consider these toxins as stable and able to pass through undamaged, normal, cell membranes. In this way, damaged cells help to poison neighboring healthy cells. Like the chemical products of

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macrophages, the aldehyde products of LPO accumulate at the site of injury and sometimes disease, producing "bystander damage," and inexorably proceed to a state in which the lesion is steadily and progressively enlarged.

Here we explore a potential rescue strategy, attempting to scavenge the aldehydes themselves accumulating in the cytosol. Hydralazine, an antihypertension drug, has been found to possess a very novel property, aldehyde trapping. Carrying nucleophilic nitrogen, hydralazine has been shown to form an adduct called *hydrazone* when in contact with acrolein. Compared with other aldehyde-trapping agents, hydralazine has the highest efficiency (Burcham et al., 2000). In the current study, we used an acrolein-mediated cell death/cell injury model in cultured PC12 cells to examine whether the adducting of hydralazine with acrolein is sufficient to reduce the acrolein cytotoxicity. We report that application of the acrolein scavenger hydralazine clearly and convincingly spares the lives of acrolein-injured cells.

MATERIALS AND METHODS

Cell Culture, Experimental Treatment, and Materials

Many of the culture procedures used here are detailed in the companion report, and we direct the interested reader to those methods. Briefly, PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, La Jolla, CA) supplemented with 12.5% horse serum, 2.5% fetal bovine serum, 50 U/ml penicillin, and 5 mg/ml streptomycin. The incubator was set at 5% CO_2 at 37°C. Culture media were changed every other day, and cells were split every week. Cells were switched from DMEM-supplemented culture medium to Hank's balanced salt solution (HBSS) before they were treated with acrolein/HNE/MDA. Acrolein (10^4 μM; Sigma, St. Louis, MO), HNE (10^4 μM; Cayman Chemical Co., Ann Arbor, MI), and MDA (10^4 μM; Alpha Diagnostics, San Antonio, TX) were prepared fresh in phosphate-buffered saline (PBS) as stock solutions and diluted to the specific concentrations upon use. Hydralazine was dissolved at 30 mM, 45 mM, and 100 mM in double-distilled water as stock solutions. Hydralazine application was typically delayed for about 15 min after the application of aldehydes.

Cell Morphology and Light Scatter Flow Cytometry

For morphological study, cells were seeded at 1×10^6 cells/dish in a 60-mm-diameter petri dish for 2 hr before each experiment was started, at which time the culture medium was also replaced with HBSS. Photomicrographs of selected regions of the culture were taken within several minutes with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI) on a Nikon Diaphot 300 inverted microscope with a 20× objective (Nikon USA). Acrolein and hydralazine were applied thereafter. Four hours later, another set of images was taken for comparison.

For the flow cytometry study, 1×10^6 cells/ml were transferred into 5-ml plastic tubes. Light scatter flow cytometry analysis was performed on a Cytomics FC500 cytometer (Beckman-Coulter, Miami, FL). Electronic gating was used to eliminate subcellular debris. For each sample, 5×10^5 events/cells were counted. Only forward scatter and side scatter data were collected by using CXP 2.0 software (Beckman-Coulter).

Trypan Blue Cell Viability Assay

Trypan blue is a vital dye that is imbibed by cells after their membranes are damaged. Normally, undamaged cells exclude trypan blue, because the chromopore is negatively charged and cannot enter the cell in the absence of breaches to the membrane. All the cells excluding the dye were considered viable, whereas labeled cells were considered otherwise dying or dead. A cell suspension (0.5 ml, 1×10^6 cells/ml in HBSS) was mixed thoroughly with 0.5 ml of 0.4% trypan blue for 2 min at room temperature. With a micropipette, 10 μl of the mixture was withdrawn to fill a hemocytometer on each side. The total number of cells and viable cells were counted under the light microscope. Percentage viability was calculated as

$$\%\ \text{Viability} = \frac{\textit{Viable cells}}{\textit{Total cells}} \times 100.$$

The percentage viability was averaged by duplicate readings from both sides of the hemocytometer. Each experiment was repeated four times.

Lactate Dehydrogenase as an Assay for Cell Membrane Integrity

The lactate dehydrogenase (LDH) assay is used to measure relative amount of cytoplasmic LDH released into the medium as an indicator of membrane permeability. The amount of LDH in the cell and the medium was measured fluorometrically as a function of the reduction of NAD by LDH to form a tetrazolium dye. The amount of product can be measured spectrophotometrically at 492 nm (SLT, Spectra). The background absorbance was measured at 660 nm and subtracted from the reading at 492 nm.

$$\text{LDH}\ (\%) = \frac{A_{medium(492\ nm-660\ nm)}}{A_{total(492\ nm-660\ nm)}} \times 100, \qquad (1)$$

where A = absorbance of the resulting compound.

$$\textit{Change in LDH release}\ (\%) = \frac{T - C}{C} \times 100, \qquad (2)$$

where T and C = percentage LDH release (Eq. 1) in experimental (T) and control (C) groups individually.

Cells were seeded in 12-well plates at a density of 1×10^6 cells/well in HBSS, with one plate dedicated for total LDH and the other plate dedicated for LDH release into the medium. Total LDH was measured when the cells were lysed by 0.1% (w/v) Triton X-100.

MTT Assay

Cells were seeded in 12-well plates at 1×10^6 cells/well in HBSS. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazo-

lium bromide (MTT) was reconstituted in PBS and added to each well 1 hr before the termination of the experiment. After incubation, an equal volume of MTT solubilization solution was added to each well to dissolve the remaining formazan crystals. The resulting absorbance was measured spectrophotometrically (SLT, Spectra) at 550 nm, and the background absorbance at 660 nm was subtracted from these values. For each experiment, the final MTT measurement for each sample was expressed as the percentage of control sample (no treatment).

Glutathione Assay

The glutathione assay kit was purchased from Chemicon (Temecula, CA) to detect intracellular levels of reduced glutathione (GSH). GSH detoxifies ROS by glutathione peroxidase and xenobiotics by glutathione transferase. Monochlorobimane (MCB), a glutathione-specific dye was added to the cell lysate, binding of MCB to GSH produces blue fluorescence detected by a FLx800 Multi-Detection Microplate Reader (Winooski, VT) at 460 nm when excited at 360 nm. The reaction is catalyzed by glutathione S-transferase. Each sample was averaged by duplicate readings and results were expressed as a percentage of control sample (no treatment). Each experiment was repeated four times.

ATP Assay

The ATP bioluminescence assay kit was purchased from Molecular Probes (Eugene, OR). This assay is based on the fact that ATP is needed for the production of light from D-luciferin catalyzed by firefly luciferase. Cells at 2×10^6 were used for each sample and centrifuged at the end of the experiment. The cell pellets were lysed in lysis buffer and broken down by a sonicator at two bursts of 10 sec each. The supernatant was separated by centrifugation and then boiled at 98°C for 4 min to denature proteins. A mixture of soluble lysate and standard reaction solution was then ready for the detection of luminescence in 96-well plates by a FLx800 Multi-Detection Microplate Reader. Each sample was averaged by two duplicate readings and expressed as the percentage of control sample (no treatment).

Statistical Analysis

Data from trypan blue, LDH, MTT, GSH, and ATP assays are given as the mean ± SD. One-way ANOVA was used to determine the significance between treated and control groups or among treated group. $P < 0.05$ was considered significant. For all figures, the experiments were repeated three or four times.

RESULTS

Changes in PC12 Cell Morphology After Acrolein and Hydralazine Application

Evaluating cell morphology after acrolein application has been described in detail in the the companion paper. Briefly, PC12 cells were seeded in 60-mm Petri dishes at a density of 1×10^6 cells/dish for 2 hr before the experiment started. A considerable portion of these cells developed filapodia or even long neurites. Most of this cell population was well attached to the substrate. Cells without any treatment are shown 4 hr later in Figure 1A. There was no morphological change detected in these cells. However, PC12 cell morphology (Fig. 1B) changed dramatically 4 hr after exposure to 75 μM acrolein. The degenerate appearance of these cells is characterized by swelling, loss of filapodia and absorption or degeneration of neurites, and detachment from the substrate. In contrast to Figure 1B, destruction of cells has been avoided, as apparent in Figure 1C,D, 4 hr after the application of 450 μM and 1 mM hydralazine, respectively. Compared with the untreated cells, cells treated with either 450 μM or 1 mM hydralazine produced few cell processes.

Light Scatter Properties of PC12 Cells

The light scatter properties of PC12 cells in suspension were evaluated by using forward and side scatter. The intensity of light in the forward direction is an indicator of cell size, whereas that scattered to the side (90° angle to the incident beam) is an index of cell shape and quantity of granular structures within the cell. Data have been displayed as pseudocolor dot plots (Fig. 2A–C), where red represents the high density and blue represents the low density of cells. As can be observed in Figure 2A, non-treated cells possessed a one-tailed distribution, whereas t, the "tail," in Figure 2B disappeared after exposure to 75 μM acrolein for 4 hr. The results from light scatter evaluation paralleled the morphological changes observed in PC12 cells following acrolein application. Hydralazine treatment (Fig. 2C) maintains the one-tailed distribution characteristic of the control groups.

Neuroprotection by Hydralazine: Results of the Trypan Blue, LDH, and MTT Assays

As shown previously, exposing PC12 cells to various concentrations of acrolein (75 or 100 μM) induces total cell death in culture by 8–12 hr. In fact, most of the population is dead only 4 hr after application of the aldehyde (companion paper). Hydralazine was able to protect PC12 cells from acrolein toxicity as determined by trypan blue, LDH, and MTT assays. In a normal population (Fig. 3), only about 14% of cells imbibed trypan blue. The percentage increased to 98.1% when exposed to 75 μM acrolein. A delayed (15 min) application of hydralazine reduced death/damage to the cells as demonstrated by a significant decrease in the percentage of cells stained with trypan blue from 98.1% to 69.8% ($P < 0.001$, n = 4). The percent of trypan blue-positive cells was inversely proportional to the concentration of hydralazine present in the culture medium.

Similar results were obtained in the LDH assay. LDH leaves cells after acrolein exposure, leading to an increased percentage of LDH in the medium. The change in LDH release is expressed as the discrepancy between the percentage LDH present in the medium in the experimental groups and that in the control group. In the acrolein-treated groups (Fig. 4), the increase in LDH release was ~36.7% ($P < 0.001$, n = 4). Nevertheless, in both groups treated with hydralazine (450 μM and 1 mM), the relative increases (3.9% and 3.3%, respectively) were not significantly different from the control group.

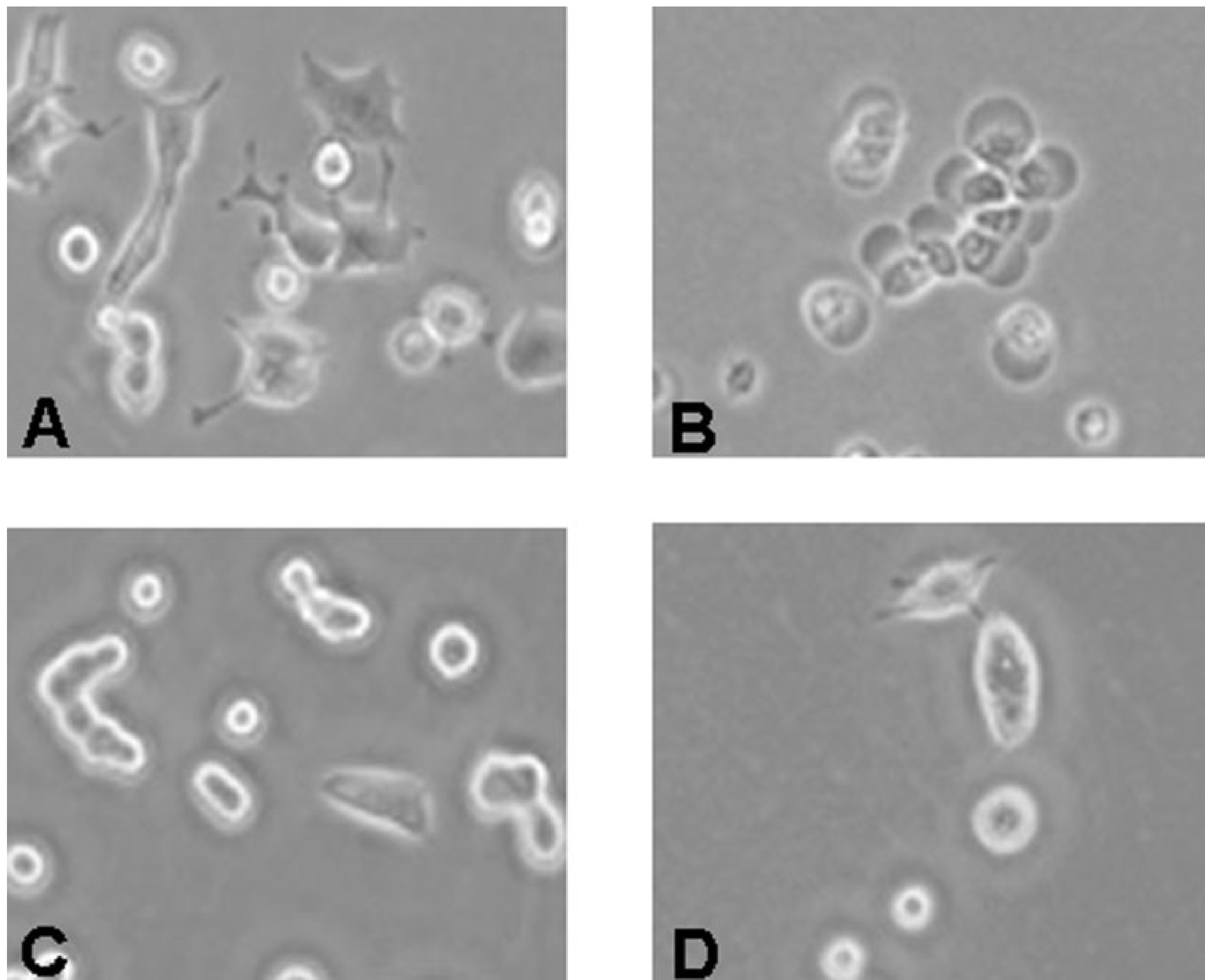

Fig. 1. General morphologies of PC12 cells in vitro and their death induced by 75 μM acrolein and protection by 450 μM hydralazine. **A–D** were taken 4 hr after the application of acrolein. Cells were plated at a density of 1×10^6/dish for each group. A: Untreated PC12 cells. Most of the cells were well attached to the substrate and exhibited isodiametric morphology. As PC12 differentiated, they became irregular. Differentiated cells had spread out and formed filapodia or long neurites. In B, PC12 cells were exposed to 75 μM acrolein. PC12 cells had detached from the culture dishes and were floating at the time when images were taken. Cells were swollen, with a balloon-like appearance, and lost all their filopodias and processes. In C and D, PC12 cells were exposed to 75 μM acrolein, followed by 450 μM or 1 mM hydralazine, respectively. PC12 cells remained relatively normal in appearance. Few cells that have been rescued have reformed filapodias and processes characteristic of the untreated PC12 cells by the end of the experiment (4 hr).

The MTT assay is a common measure of cell viability and oxidative function (Fig. 5). Cell viability was expressed as the ratio of MTT absorbance in the experimental groups to that of the control group. In PC12 cells that were treated with 75 μM acrolein, cell viability as a function of the MTT test was reduced to 1.2% ($P < 0.001$, n = 6) by 12 hr after exposure. However, this loss of mitochondrial function was rescued by varying degrees, dependent on the concentration of hydralazine present in the medium. This clear inverse relationship between concentration of hydralazine and cell viability as defined in the MTT assay was evident in all groups except for the 450 μM hydralazine groups (81.2% respectively, between which these values were not significantly different; $P > 0.05$). Within the concentration range (1 μM to 1 mM) of hydralazine tested, cell viability at 12 hr as a function of the MTT assay was significantly lower than that in the control group ($P < 0.001$).

Effects of Acrolein and Hydralazine on GSH Depletion

Intracellular GSH was measured as a function of the specific affinity of MCB to GSH. Thus, it was expressed as the percentage of fluorescence intensity of MCB in the experimental group vs. that of the control group. Applica-

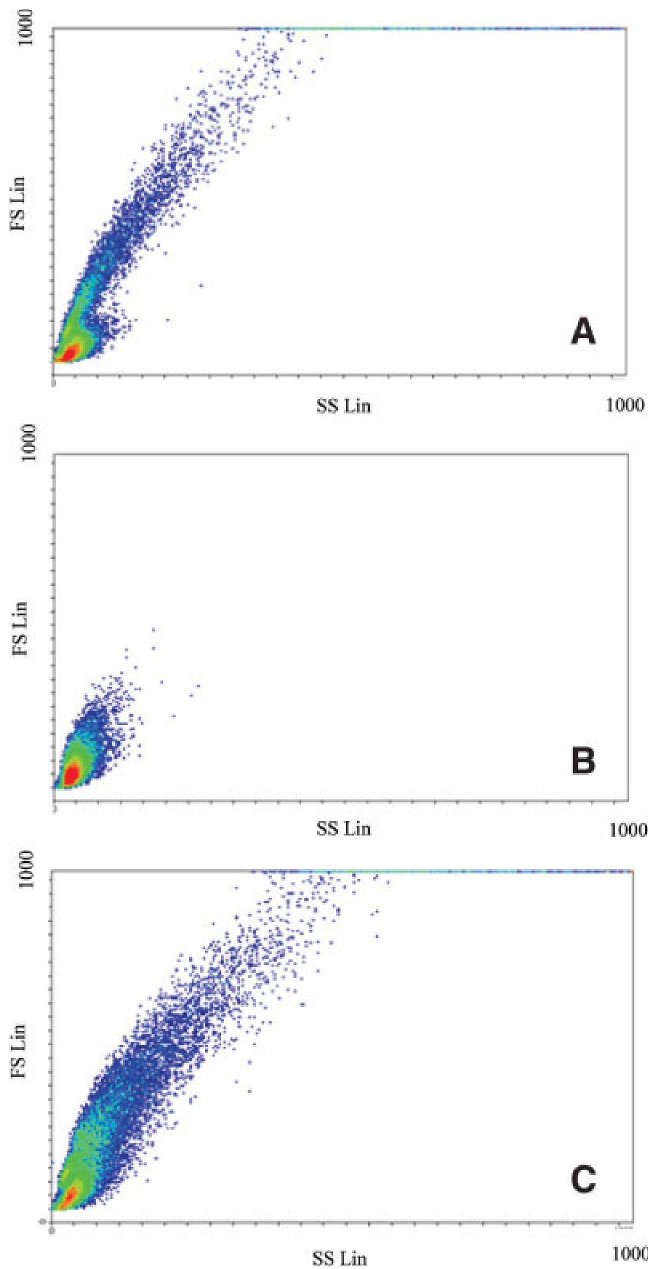

Fig. 2. Light scatter properties of PC12 cells. **A–C** are density plots, with red representing the highest value and blue representing the lowest value. X and Y axes denote the side scatter and forward scatter intensities individually. Each cell is presented as a single dot in these figures. A shows that the untreated PC12 cells had a one-tailed distribution of a wide range along both forward scatter and sideward scatter axes. There were two distinct subpopulations of cells in this group that were different in granularity and size. B: However, when PC12 cells were exposed to 75 μM acrolein for 4 hr, the population shifted to lower values of forward scatter and side scatter intensities. Said another way, the two subpopulations became less distinct. Treatment with 450 μM hydralazine for 4 hr (C) restored the bimodal distribution of the density plot and returned to the extended range characteristic of normal PC12 cells.

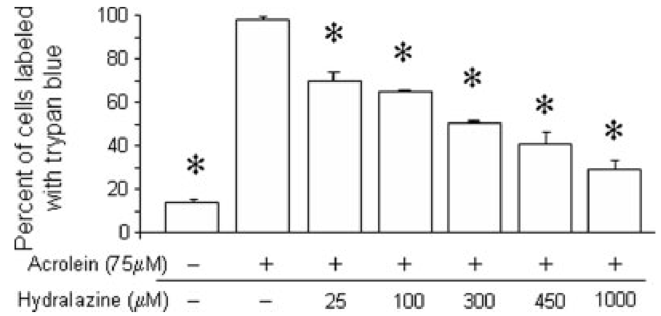

Fig. 3. Exclusion of trypan blue after hydralazine treatment. PC12 cells were incubated for 4 hr in the absence or presence of 75 μM acrolein and five different concentrations of hydralazine in individual experiments (25 μM, 100 μM, 300 μM, 450 μM, and 1 mM). The data are expressed as a percentage of trypan blue-stained cells over the total number of cells. Data are shown as mean ± SD (bars) (n = 4). The control group and the groups treated with both acrolein and hydralazine were significantly different from the group treated with acrolein only (*$P < 0.001$).

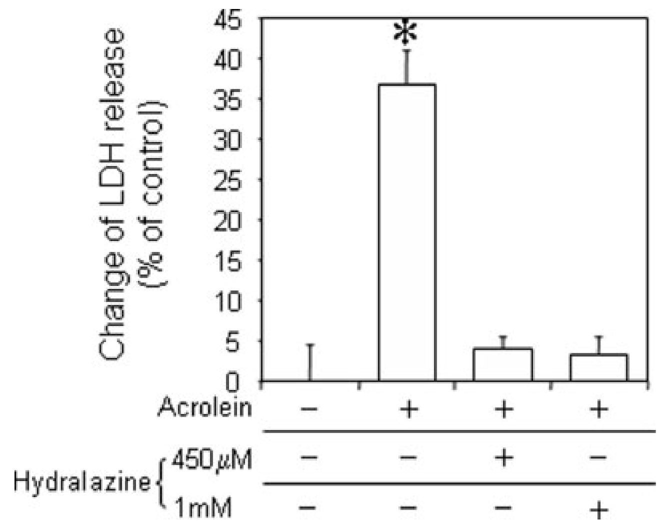

Fig. 4. Effects of acrolein and hydralazine post-treatment on membrane integrity. The percentage of lactate dehydrogenase (LDH) release from cytosol to the extracellular medium is a direct indicator of membrane permeability. Membrane integrity as a function of LDH release was measured 2 hr after exposure to 75 μM acrolein. Among three treatment groups, two groups were treated with 450 μM or 1 mM hydralazine after a 15-min delay. The percent LDH release has been normalized to zero for the control group. Data are presented as the discrepancy between the treatment groups and the control group. Data are shown as mean ± SD (bars; n = 3). The acrolein treated group was significantly different from the control group (*$P < 0.001$).

tion of acrolein at all concentrations above 1 μM significantly reduced the GSH levels of PC12 cells by 1, 2, and 4 hr, respectively, compared with the level of the control group ($P < 0.001$, n = 4; Fig. 6). Intracellular levels of GSH were thus very sensitive to acrolein. When PC12 cells were exposed to 10 μM acrolein, a concentration too

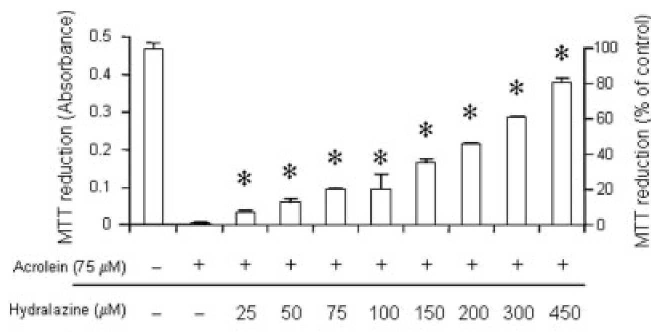

Fig. 5. Effect of hydralazine on cell viability by MTT assay. Cells were incubated with 75 μM acrolein for 12 hr, and some treated were with hydralazine after a 15-min delay ranging from 25 to 450 μM. Cell viability was determined by absorbance of the MTT reduction product at 550 nm. Values are expressed as the percentage of control cells. Results are denoted as mean ± SD (bars; n ≥ 3). Cell viability was significantly improved by hydralazine treatment in a concentration-dependent manner compared with the group treated with acrolein only (*$P < 0.05$).

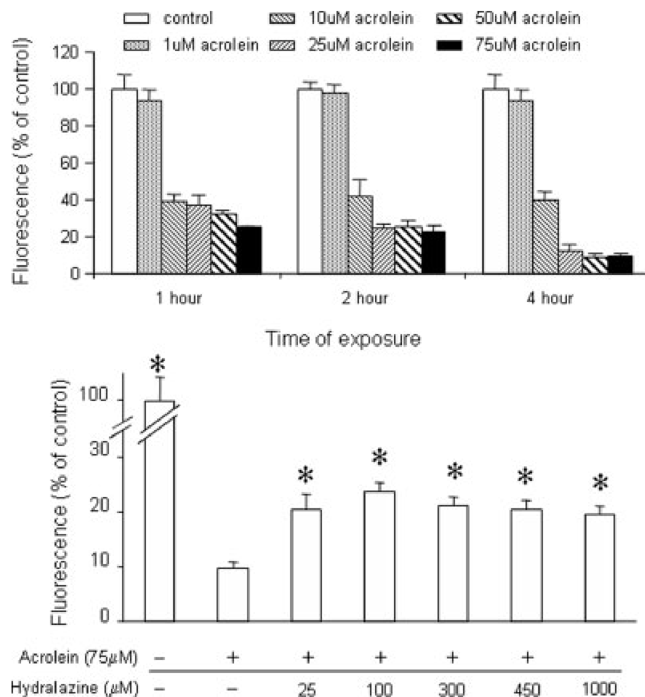

Fig. 6. Effect of acrolein on glutathione depletion (**A**) and neuroprotection by hydralazine (**B**). The intracellular level of glutathione was determined by fluorescence from cell extracts. In A, PC12 cells were incubated with six different concentrations of acrolein (0, 1, 10, 25, 50, 75 μM) for 1, 2, and 4 hr. In B, cells were incubated for 4 hr with hydralazine (0, 25, 100, 300, 450, 1,000 μM) in the absence or presence of 75 μM acrolein. Values for the treated groups (acrolein and/or hydralazine) are expressed as percentage of control group values [mean ± SD (bars); n = 4]. Significance of glutathione level between control group or hydralazine-treated groups and acrolein-treated group was *$P < 0.001$.

low to induce the cell death, the intracellular GSH level as a function of fluorescence intensity decreased to only 39.1% of normal value as early as 1 hr. Compared with the group treated with 75 μM acrolein (9.6%), the fluorescence intensities of MCB in the groups treated with increasing concentrations of hydralazine (25 μM to 1 mM) increased significantly (19.1%, $P < 0.001$).

Effects of Acrolein and Hydralazine on Intracellular ATP Level

Intracellular levels of ATP were linearly related to the emitted light/luminescence (relative light units). The level of ATP was expressed as the percentage of luminescence in the experimental groups vs. the control group. Similar to the changes observed in GSH levels, intracellular ATP levels decreased dramatically after exposure to 25 μM, 50 μM, and 75 μM of acrolein by 1, 2, and 4 hr, respectively ($P < 0.001$, n = 4; Fig. 7). One exception was noted: the tested concentration of 25 μM acrolein at 1 hr postexposure. When PC12 cells were treated with different concentrations of hydralazine, the ATP levels increased significantly ($P < 0.001$, n = 4; Fig. 8). The ATP levels of groups treated with 300 μM and 1 mM hydralazine were not statistically different from the level of the control group.

Relative Cytotoxicity of Acrolein, HNE, and MDA and Neuroprotection by Hydralazine

Given the results reported above, the MTT test was chosen to compare the effects of acrolein with those of the other toxic products of LPO, MDA and HNE. The concentrations of all three aldehydes were 75 μM. The concentration of hydralazine used in the study to attempt to attenuate their toxicities was 450 μM. As shown previously, the cell viability is proportional to the absorbance of formazan products in the MTT assay. Similarly to the results we attained previously, acrolein, HNE, and MDA at 75 μM all decreased cell viability by only 4 hr after the exposure. Among these three toxins, acrolein-induced cytotoxicity (absorbance = 0.135) was the most damaging to PC12 cells, followed by HNE (absorbance = 0.203) and MDA (absorbance = 0.447). Each of these differs significantly from the others ($P < 0.001$, n = 4). Once PC12 cells were treated with 450 μM hydralazine (as usual, 15 min after the application of 75 μM acrolein/HNE/MDA), cell viabilities increased significantly compared with the paired control groups ($P < 0.001$, n = 4). By 4 hr, the MTT indices of cell viability of the three groups treated with 450 μM hydralazine were not statistically different from each other.

DISCUSSION

Cytotoxicity of Acrolein

In this study, we demonstrate that acrolein, a product of membrane lipid peroxidation, disrupts membrane integrity, depletes intracellular glutathione level, disturbs mitochondrial functions and energy generation, and con-

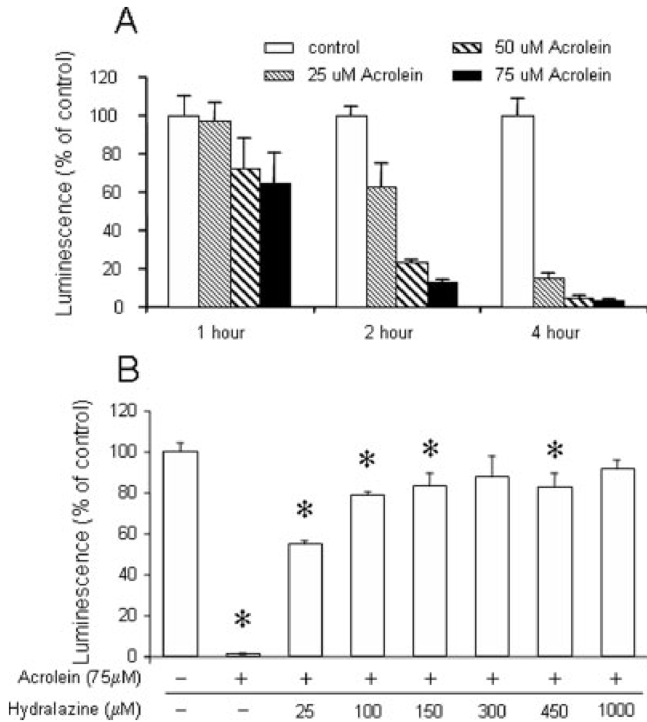

Fig. 7. Acrolein dose-dependent effect on ATP loss and neuroprotection by hydralazine. ATP levels in **A** were examined in the groups treated at four different concentrations of acrolein (0, 25, 50, 75 μM) for 1, 2, and 4 hr, respectively. In **B**, PC12 cells were incubated for 4 hr in the absence or presence of 75 μM acrolein and at six different concentrations of hydralazine (0, 25, 100, 150, 300, 450, and 1,000 μM). The results are expressed as percentage of control group values. They are shown as mean ± SD (bars; n = 4). ATP levels in the treated groups (acrolein and/or hydralazine) were significantly different from the control groups (*$P < 0.001$).

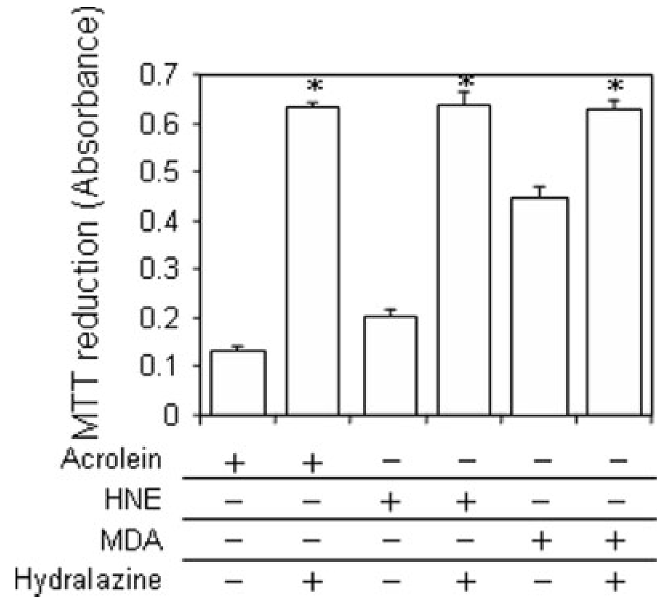

Fig. 8. Hydralazine (HYD) reduces cell death induced by 2-hydro-4-nonenal (HNE), malondealdehyde (MDA), and acrolein. Cells were tested with one of three lipid peroxidation products (HNE, MDA or acrolein) in each group. The final concentration of the three toxins was 75 μM. Hydralazine was 450 μM. Cell viability was determined by the absorbance of MTT reduction product (formazan) at 550 nm. Values are expressed as a percentage of control group value. Results are mean ± SD (bars; n = 3). Hydralazine significantly reduced cell death as a function of MTT assay induced by lipid peroxidation products (*$P < 0.001$).

sequently causes cell death in PC12 cell culture. As shown in our companion paper, acrolein (100 μM) can cause necrotic cell death by 4 hr after the exposure in vitro. Acrolein at 200 μM damaged axonal membrane integrity and interrupted action potential conduction along the isolated guinea pig spinal cord (Shi et al., 2002). Here, we reduced the application of acrolein to 75 μM when exposing PC12 cells in vitro culture to the toxins; however, it still induced membrane damage and cell death. The collapse of the cell anatomy and physiology was also shown by the LDH release assay and the MTT assay. The results from MTT and the ethidium homodimer-1 (EthD-1) staining assay (data not shown) also indicated that the incidence of cell death is proportional to the concentration of applied acrolein. It is not an ''all-or-none'' phenomenon.

Acrolein Induced Necrosis in PC12 Cell

Morphological changes in PC12 cells included swollen cell bodies and loss of filapodia and neurites and were consistent with our previous studies (see companion paper). Additionally, acrolein caused significant reduction in ATP production at all concentrations tested (25, 50, and 75 μM), and the reduction is time and concentration dependent. By 4 hr after acrolein exposure, ATP production in the 75 μM acrolein-treated group maintained only 3.23% of the normal value. Intracellular ATP level, functioning as a switch, is critical for signaling cell death pathways. Apoptosis can be initiated and can progress only when the ATP level remains relatively normal. Apoptosis will be avoided if the ATP level goes too low. The evidence presented in our companion paper and here shows that the PC12 cell death induced by acrolein is necrosis, but acrolein at extremely low concentrations has also been reported to induce apoptotic death in other cell types (Rudra and Krokan, 1999; Tanel and Averill-Bates, 2005).

The mechanism by which acrolein inhibited mitochondrial bioenergetics is still not clear. However, studies on isolated mitochondria have indicated that acrolein might inhibit mitochondrial respiration directly. Picklo and Montine (2001) showed that acrolein specifically inhibited mitochondrial complex I and, consequently, the state 3 respiration. It is also suggested that this inhibition was due to the formation of a Michael adduct of acrolein with mitochondria proteins (Zollner, 1973). The inhibition could also be an indirect effect, insofar as acrolein has been shown to induce oxidative stress that eventually causes uncoupling of oxidative phosphorylation and ATP production (Harper et al., 2004; Luo and Shi, 2005).

Acrolein Depleted Intracellular GSH

It has been reported by Horton et al. (1997) that acrolein at a very low concentration depletes glutathione without affecting cell viability. In our study, similar results were demonstrated when intracellular reduced glutathione (GSH) was measured by MCB. The lowest concentration of acrolein that induced significant reduction of GSH was 10 μM, far below the concentration required to reduce the ATP level (25 μM). At 10 μM concentration, MCB fluorescence dropped to approximately 40% of control values and then remained relatively stable over 4 hr. However, GSH reduction was even more profound over time when the acrolein concentration was increased to 25, 50, and 75 μM.

As an active eletrophile, acrolein reacts rapidly with GSH, forming a glutathione-acrolein adduct, which simultaneously inactivates GSH functioning. Reduction in GSH level aggravates oxidative stress, because GSH itself is a critical component of the endogenous antioxidant defense system. Simply by this action, acrolein can initiate a vicious cycle of free radical generation and lipid peroxidation, which further reduces intracellular GSH.

Hydralazine significantly increased GSH level at all concentrations used (from 25 μM to 1 mM). It is likely that the action of hydralazine protects GSH from adducting with acrolein. In these studies, there have been a few surprises. For one, the overall change in GSH was modest. MCB fluorescence changed only from 9.6% to about 20% of control values. Increase in the concentration of hydralazine, however, failed to improve GSH levels further. It may be that the reaction of acrolein and GSH happens rapidly and irreversibly. A 15-min delay in hydralazine application was not able to spare the portion of GSH that had reacted with acrolein.

Overall, acrolein could provoke oxidative stress by GSH depletion in addition to generation of ROS (Nardini et al., 2002; Luo and Shi, 2004, 2005). The redox imbalance can act together with acrolein to affect multiple cell functions. This could explain why application of an antioxidant can attenuate cytotoxicity of acrolein under the condition of GSH depletion (Nardini et al., 2002).

Acrolein, HNE, and MDA

Acrolein, HNE, and MDA are three major end products of lipid peroxidation (Esterbauer et al., 1991). It has been reported that acrolein has a long half-life (days) and high reactivity (Ghilarducci and Tjeerdema, 1995). Here, we tested these three toxins via MTT assay to compare cytotoxicities of the three aldehydes. Acrolein, as expected, was the worst among the three as indicated by the most reduced mitochondrial function.

Hydralazine Rescues PC12 Cells From Death Caused by Acrolein

The observation made by Lalich and Paik (1974) directed people's attention to hydralazine, an antihypertensive drug, for this possible function. In their study, they found that hydralazine protected rats from side effects after long exposure to allyamine. This drug was later determined to be transformed to acrolein by alcohol dehydragenase in the liver (Jaeschke et al., 1987).

Our study provides direct evidence of the protective effects of hydralazine on acrolein-induced PC12 cell death. Hydralazine attenuated membrane damage, disruption of mitochondrial functions, intracellular GSH depletion, and ATP reduction. The protective effects most likely are mediated by the ability of hydralazine, a strong nucleophile, to react with acrolein, forming hydrazone.

Our study also shows that the neuroprotective effects of hydralazine are not specific to acrolein. In fact, hydralazine improved mitochondrial functions in HNE- and MDA-treated cell populations as well. This finding is very important, in that it is well known that lipid peroxidation yields multiple end products, including acrolein.

Applications of Hydralazine in CNS Trauma and Neurodegenerative Diseases

With HPLC or antibodies that specifically recognized protein-aldehyde adducts, researchers have found that the level of aldehydes increases after spinal cord injury (Springer et al., 1997; Lucas et al., 2002; Luo et al, 2005), traumatic brain injury (Zhang et al., 1999), various neurodegenerative diseases such as Alzheimer's disease (Zarkovic, 2003), cardiovascular disease (Uchida, 2000), and cancer (Skrzydlewska et al., 2005). Thus an increase in carbonyl stress is rather a universal secondary injury pathway leading to cell malfunction and death. The potential benefit of application of hydralazine is no longer limited to spinal cord injury then. Oxidative stress coexists in all of these diseases, with carbonyl stress raising the implication of close interaction between these two pathologies. However, these two processes are also independent of each other in many aspects as well.

It is widely accepted that all CNS trauma and neurodegenerative diseases are mediated by multiple mechanisms. Thus, it is not realistic to rely on a single therapy meant to slow or stop such varied pathological processes. The ultimate treatment will likely be a cocktail therapy targeting individual pathologies and pathways specifically. For example, all of the clinical trials that have focused on antioxidant treatment of CNS trauma have failed by far to show efficacy (Tolias and Bullock, 2004). This strongly implies that inhibition of oxidative stress is probably not sufficient to stop all of the pathological mechanisms leading to cell death, including carbonyl stress. Application of hydralazine generally improved most cellular functions tested but did not improve GSH levels. Perhaps hydralazine combined with antioxidant will protect cells better from acrolein-mediated injury. Other than hydralazine, cross-linking breakers might provide further protection by breaking down the cross-linking that has already formed. Even though such a drug has been tested only for diabetic complications, it would be worth testing this idea for other disorders (Vasan et al., 2003).

Carbonyl stress occurs not only by lipid peroxidation but also by glycation/glycosalation that produces several carbonyls as intermediate products. One study has shown

that hydralazine inhibited production of AGEs by inhibiting semicarbazide-sensitive amine oxidase (SSAO; Nordquist, 2002). Thus hydralazine probably provides a possibly wider protection rather than just lipid peroxidation products. Further study will be valuable to test this concept. Because hydralazine is an antihypertensive drug, this might impose problems on patients with normal or even low blood pressure, as occurs after trauma. Thus, developing a drug that maintains carbonyl scavenging properties without interfering with blood pressure is desirable.

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