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Products of Oxidative Stress Inhibit Aldehyde Oxidation and Reduction Pathways in Dopamine Catabolism Yielding Elevated Levels of a Reactive Intermediate

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Abstract

Dopamine (DA) has been implicated as an endogenous neurotoxin to explain the selective neurodegeneration as observed for Parkinson's disease (PD). In addition, oxidative stress and lipid peroxidation are hypothesized culprits in PD pathogenesis. DA undergoes catabolism by monoamine oxidase (MAO) to 3,4-dihydroxyphenylacetaldehyde (DOPAL), which is further oxidized to 3,4-dihydroxyphenylacetic acid (DOPAC) via aldehyde dehydrogenase (ALDH). As a minor and compensatory metabolic pathway, DOPAL can be reduced to 3,4-dihydroxyphenylethanol (DOPET) via cytosolic aldehyde or aldose reductase (AR). Previous studies have found DOPAL to be significantly more toxic to DA cells than DA and that the major lipid peroxidation products, i.e. 4-hydroxynonenal (4HNE) and malondialdehyde (MDA), potently inhibit DOPAL oxidation via ALDH. The hypothesis of this work is that lipid peroxidation products inhibit DOPAL oxidation, yielding aberrant levels of the toxic aldehyde intermediate. To test this hypothesis, nerve-growth factor differentiated PC6-3 cells were used as a model for DA neurons. Cell viability in the presence of 4HNE and MDA (2-100 μM) was measured by MTT assay and it was found that only 100 μM 4HNE exhibited significant cytotoxicity. Treatment of cells with varying concentrations of 4HNE and MDA resulted in reduced DOPAC production and significant elevation of DOPAL levels, suggesting inhibition of ALDH. In cells treated with 4HNE that exhibited elevated DOPAL, there was a significant increase in DOPET. However, elevated DOPET was not observed for the cells treated with MDA, suggesting MDA to be an inhibitor of AR. Using isolated cytosolic AR, it was found that MDA but not 4HNE inhibited reductase activity toward DOPAL, surprisingly. These data demonstrate that the oxidative stress products 4HNE and MDA inhibit the aldehyde biotransformation step of DA catabolism yielding elevated levels of the endogenous neurotoxin DOPAL, which may link oxidative stress to selective neurodegeneration as seen in PD.

Introduction

Past as well as recent studies implicate oxidative stress and the environment in the etiology of Parkinson's Disease (PD), a degenerative condition involving loss of dopamine (DA) neurons (1-3). Individuals with PD were found to have increased oxidative burden and higher levels of lipid peroxidation as compared to controls (4,5). The process of lipid peroxidation yields several major products, including 4-hydroxy-2-nonenal (4HNE) and malondialdehyde (MDA),

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and these aldehydes are often considered to be biomarkers of oxidative stress (6). However, the mechanistic link between oxidative stress/lipid peroxidation and degeneration of DA neurons is not known.

The lipid peroxidation products 4HNE and MDA are potent inhibitors of mitochondrial aldehyde dehydrogenase (ALDH2) (7-9), an enzyme critical for DA catabolism (10). While 4HNE is a reversible, mixed-type inhibitor of ALDH2, MDA was reported to be an irreversible inhibitor. As shown in Scheme 1, DA undergoes oxidative deamination catalyzed by monoamine oxidase (MAO) initially to 3,4-dihydroxyphenylacetaldehyde (DOPAL), followed by ALDH2-mediated oxidation to 3,4-dihydroxyphenylacetic acid (DOPAC). As noted in a recent review, there are several ALDH enzymes that may compensate for DOPAL oxidation should ALDH2 be inhibited (10).

As a minor pathway, reduction of DOPAL to 3,4-dihydroxyphenylethanol (DOPET) can occur, and such a reaction is catalyzed by cytosolic aldehyde or aldose reductases (AR) (11). The latter was proposed to compensate for DOPAL metabolism in the event of ALDH2 inhibition (12). Therefore, it is predicted that the lipid peroxidation products 4HNE and MDA will impair cellular DA catabolism via inhibition of ALDH2, yielding elevated levels of the aldehyde intermediate. Indeed, two previous studies that utilized isolated model systems (i.e. mitochondria and synaptosomes) reported impairment of DOPAL oxidation via lipid peroxidation products (9,13).

Given the demonstrated cellular toxicity of DOPAL, elevated levels of the aldehyde may be highly significant to degeneration of DA neurons. Previous work found DOPAL to be several-fold more toxic to dopaminergic cells than DA, often considered an endogenous neurotoxin, and other DA metabolites (e.g. DOPAC), both in vitro and in vivo (14,15). The significant toxicity of the aldehyde intermediate may stem from several reported mechanisms including protein reactivity (15-17). Previous work found DOPAL to be reactive toward tissue and protein amines via formation of stable Schiff base adducts.

The present study was performed to test the hypothesis that the lipid peroxidation products 4HNE and MDA inhibit cellular DA catabolism, yielding elevated levels of the reactive and toxic intermediate DOPAL. To test this hypothesis, nerve growth factor (NGF) differentiated dopaminergic PC6-3 cells, a sub-line of the well-established PC12 cells (18), were utilized as a model for DA catabolism. While this cell line is not of neuronal origin, it is considered to be an appropriate model for DA catabolism, assumes a homogenous population and has been utilized to elucidate mechanisms of toxicity linked to dopaminergic toxicity (19-21). Cells were treated with physiologically-relevant concentrations of 4HNE and MDA and the levels of DOPAL and metabolites determined using HPLC. Both the oxidative as well as the reductive pathways for DA catabolism were studied and the effects of lipid peroxidation products demonstrated. Such work is of importance as it may elucidate a mechanistic link between oxidative stress/lipid peroxidation and the generation of a neurotoxin endogenous to DA neurons at aberrant and harmful levels.

Experimental Procedures

Materials

DOPAL was biosynthesized via an established procedure involving enzyme-catalyzed conversion of DA to DOPAL by rat liver MAO (17). 4HNE was synthesized as previously described (22). MDA was synthesized by acidification of 1,1,3,3-tetramethoxypropane with hydrochloric acid (1:1) and diluting into 50 mM sodium phosphate buffer (pH 7.4). Concentrations of 4HNE and MDA were determined spectrophotometrically. The DOPAL concentration was determined using an ALDH assay with NAD (23) and HPLC analysis, as

described below. 3,4-Dihydroxyphenylethanol (DOPET) was obtained via reduction of DOPAL with a 10-fold excess of sodium borohydride. DA, DOPAC and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Cell Culture

PC6-3 cells were cultured in RPMI 1640 medium supplemented with heat-inactivated 10% horse serum, 5% fetal bovine serum, and penicillin (10 IU/ml) and streptomycin (10 mg/ml). Cells were grown in 100 mm^2 tissue culture dish at 37°C in a humid atmosphere containing 5% CO_2. PC6-3 cells (6×10^4) were seeded into six-well plates at final volume of 2 ml/well and were incubated at 37°C for 3 days in 5% of CO_2. NGF was added into each well at a final concentration of 50 ng/ml for cell differentiation, and plates were kept at the same condition for 4 days before being used for studies. For experiments involving treatment of cells with 4HNE or MDA, the media was removed and replaced with HEPES-buffered media containing: 115 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose, 1 mM NaH_2PO_4 and 15 mM HEPES (pH 7.4).

Cytotoxicity Assay

Cell viability was determined using the MTT reduction assay. The assay is based on conversion of yellow tetrazolium salt MTT into purple formazan by active mitochondrial reductase. Briefly, NGF-differentiated cells were treated with different concentration of 4HNE (2, 10, 25, 50 and 100 μM) and MDA (2, 10, 25, 50 and 100 μM) for 1 hr. Yellow MTT (0.5 mg/ml) was added into each well and incubated for 2 h at 37 °C. After this, the medium was removed and purple formazan dissolved in DMSO. The absorbance for the formazan reduction product was measured at 570 nm using a Molecular Devices Spectra-Max plate-reader. Absorbance values were compared to a control (without 4HNE or MDA). It should be noted that 4HNE and MDA were incubated with MTT alone and absorbance read over time to rule out the possibility that 4HNE and MDA directly interacted with MTT.

Treatment of Cells with 4HNE and MDA

NGF-differentiated PC6-3 cells (6×10^4 per well) in HEPES-buffered media (pH 7.4) were pretreated with DA (100 μM) for 15 min at 37 °C to initiate DA catabolism. After the preincubation period, 4HNE (2, 10, 25, 50 and 100 μM) or MDA (2, 10, 25, 50 and 100 μM) was added to the cells, and the control group did not contain any 4HNE or MDA. All groups (i.e. control and treated) were incubated at 37 °C for 1 hr. Aliquots of the extracellular media were removed at 0, 15, 30, 45, and 60 min and placed in microcentrifuge tubes containing perchloric acid (5% v/v) to terminate the reaction and precipitate the protein. Precipitated protein was removed via centrifugation at 10000*g* for 3 min. The supernatant was analyzed for extracellular DA metabolites using the HPLC method as described below.

For analysis of intracellular metabolites, the buffered-media was removed and cells quickly rinsed once with buffered media, followed by removal of the media. The cells were triturated with a pipette and lysed using hypotonic buffer (10 mM sodium phosphate, pH 7.4) containing 1% triton X-100 (v/v).

HPLC Analysis of Intra- and Extra-cellular DA Metabolites

Extracellular DA metabolites were measured via sampling the media, which permitted time-course analysis of DA catabolism in cells. The amount of DA and metabolites in the extracellular space was found to be much greater than that in the intracellular space. Separation and quantification of DA and its metabolites was performed using an Agilent 1100 Series Capillary HPLC system with a photodiode array detector set to absorbance at 202 and 280 nm. Twenty μL of sample solution was injected and separation was achieved using a Phenomenex

C18 Luna microbore column (1 × 150 mm, 100 Å) and a mobile phase consisting of 0.1% trifluoroacetic acid (v/v) in HPLC-grade water and 6% acetonitrile (v/v) at a flow rate of 50 μL/min. Retention times for DA, DOPAL, DOPET and DOPAC were determined to be 3.8, 7.3, 8.8, and 12.3 min, respectively, using standards samples (DA, DOPAL, DOPET and DOPAC). Quantification of metabolites was determined using a calibration curve of standards where peak area was converted to concentration units.

For separation and electrochemical detection of intracellular DA and metabolites, a Waters Alliance HPLC system with an ESA Coulochem III coulometric electrochemical detector was used. Sixteen μL of sample was injected and separation was achieved using a Nova-Pak C18 column (3.9 × 150 mm, 100 Å) and a mobile phase consisting of 0.3% trifluoroacetic acid in HPLC-grade water containing 2% ACN (v/v) at a flow rate of 0.6 mL/min. For electrochemical detection, the guard cell, E1 and E2 were set to +400, -180 and +200 mV, respectively, for detection of catechol-containing compounds.

Measurement of ALDH Activity and Change in [DOPAL]

The initial linear slopes for the time-dependent formation of DOPAC were calculated via GraphPad Prism 4.0 (GraphPad Prism Software, San Diego, CA). Comparison of initial linear slopes for cells treated with 4HNE or MDA to those of the contemporaneous control yielded the % ALDH activity (i.e. % Control Activity (DOPAC)). The % change in concentration of DOPAL from 0 to 60 min was determined via $([DOPAL]_{60\ min} — [DOPAL]_{0\ min})_{4HNE/MDA\ Treated}$ divided by $([DOPAL]_{60\ min} — [DOPAL]_{0\ min})_{Control}$ times 100.

Linear Regression and Statistical Analysis

All linear regression and statistical analyses were performed using the software GraphPad Prism 4.0. For ALDH (i.e. DOPAL oxidation) and AR activity and levels of DOPAL, data for cells treated with 4HNE or MDA were compared to the controls and significant differences determined using an unpaired two-tailed t-test ($p < 0.05$). For intracellular DA and metabolites, an ANOVA was performed using the Tukey's post-test.

Measurement of AR Activity

AR activity was measured using PC6-3 cellular AR. Briefly, 100 mm^2 tissue cell dish with confluent, undifferentiated PC6-3 cells was washed twice with 100 mM phosphate buffer (pH=7.0) and collected in 500 μl of phosphate buffer using a cell scraper. The collected cells were sonicated for 15 sec in ice-cold bath, centrifuged at 45,000g for 1 hr at 4°C, and the supernatant was used for AR activity.

AR activity was measured at 37°C in the presence of 1 mM NADPH using cell lysate at a final protein concentration of 1 mg/ml. 10 μM DOPAL was used as substrate for AR. Various concentrations of MDA (2, 10, 25 μM) or 25μM of 4HNE was incubated with cell lysate, NADPH and DOPAL in 100 mM phosphate buffer (pH 7.0). DOPAL was added just before the incubation to initiate the reaction. Aliquots were collected at 0 and 60 min of incubation time and placed in microcentrifuge tubes containing 5% of perchloric acid to precipitate protein and stop the reaction. Samples were spun at 10000g for 3 min to pellet protein, and HPLC (described above) was used to determine and quantify the reaction products (e.g. DOPET).

Results

Cytotoxicity of 4HNE and MDA in PC6-3 cells

To rule out cytotoxicity of the lipid peroxidation products 4HNE and MDA as a confounder for experiments involving NGF-differentiated PC6-3 cells, the cytotoxicity was determined

for various concentrations (i.e., 0, 2, 10, 25, 50 and 100 μM) of these lipid peroxidation products using a 60 min period of exposure. Cytotoxicity was measured via the MTT assay as described in the Experimental Procedures section. As shown in Figure 1, MDA at all concentrations tested did not exhibit any significant toxicity on cells in comparison with the control group; however, 4HNE at higher levels did exert toxicity. Specifically, minimal cell loss was observed for lower concentrations of 4HNE (0 to 50 μM), and only 100 μM 4HNE showed significant reduction in cell viability with only 22% of surviving cells ($p < 0.05$).

Inhibition of ALDH by 4HNE and MDA in PC6-3 cells

As shown in Figures 2A,B and 3A,B, treatment of PC6-3 cells with MDA or 4HNE (0, 2, 10, 25, 50 and 100 μM) for 60 min resulted in a concentration-dependent decrease in the level of DOPAC formed over time (i.e. lower DOPAL oxidation), which indicates inhibition of cellular ALDH enzymes by the lipid aldehydes. All concentrations tested, with the exception of 2 μM 4HNE, yielded a decrease in time-dependent formation of DOPAC with a significant effect starting from 10 μM of the lipid aldehydes, i.e. approximately 60% decrease for both the MDA and 4HNE groups ($p < 0.05$). At 10μM, 4HNE and MDA decreased DOPAC production to a similar degree; however, at higher concentrations (e.g. 50μM), 4HNE was a better inhibitor (<10% remaining) than MDA (~20% remaining).

Inhibition of DOPAC production (i.e. cellular ALDH) yielded a significant increase in the level of extracellular DOPAL that was found to be concentration (i.e. 4HNE or MDA) and time-dependent (Figures 2C,D and 3C,D). Overall, MDA-treated cells had a higher concentration of DOPAL after the 60 min treatment than those incubated with 4HNE.

DOPET in MDA and 4HNE-treated PC6-3 cells

A compensatory pathway for DOPAL metabolism is via reduction of the aldehyde to an alcohol (i.e. DOPET) by AR, found in the cytoplasm. As shown in Fig. 4A, DOPET increased over time, compared to a control, for cells treated with 10 μM 4HNE and higher concentrations. While the differences in the level of DOPET are not significantly different for cells treated with ≥10 μM 4HNE, it appears that there is a trend such that higher 4HNE yields a higher concentration of DOPET.

However, treatment of the cells with MDA resulted in a much different outcome, as shown in Figure 4B. Specifically, cells incubated with MDA (2 to 100 μM) yielded only a slight increase in the DOPET levels compared to the control. While not statistically significant, the trend suggests that higher [MDA] resulted in lower [DOPET]. Such a finding suggests that MDA inhibits AR-mediated reduction of DOPAL.

It should be noted that extracellular DA did not change significantly over the course of the reactions.

Inhibition of cellular AR by MDA but 4HNE

The effect of 4HNE and MDA on AR activity was determined using the cytosolic fraction from PC6-3 cells. Incubation of the cell cytosol (1 mg/mL) with 10 μM DOPAL and 1 mM NADPH for 60 min yielded significant production of DOPET, as measured via the HPLC method described above. Compared to this control, there was a significant decrease in the time-dependent formation of DOPET (i.e. DOPAL reduction) for cytoplasmic samples containing various concentrations of MDA (2 to 25 μM), as shown in Figure 5. The inhibition of AR was concentration-dependent for MDA, with only ~10% of activity remaining for 25 μM of the aldehyde. In contrast, 4HNE at 25 μM yielded no inhibition of AR activity toward DOPAL, compared to the control. Such findings demonstrate that MDA but not 4HNE inhibits AR-mediated reduction of DOPAL both in intact cells and cytosolic extracts. It should be noted

that this work was performed using cytosol from undifferentiated cells, and therefore as a control, these experiments were repeated using cytosol from differentiated PC6-3 cells. The sensitivity of AR for MDA and resistance of the enzyme(s) toward 4HNE was not significantly different for cytosol from differentiated versus undifferentiated cells (data not shown).

It was found that MDA was a substrate for AR as judged via NADPH oxidation in the presence of various concentrations of MDA, and therefore, the lipid peroxidation product appears to be a competitive, substrate inhibitor of the enzyme(s) (data not shown).

Level of intracellular DA and DA-metabolites

As shown in Figure 6, the levels of intracellular DA and DOPAL were the same for the control and cells treated with varying concentrations of MDA. Such a finding (i.e. no change in intracellular [DOPAL]) is surprising given that MDA treatment causes extracellular levels of DOPAL to increase dramatically compared to a control (Figure 3C and D). As found for extracellular DOPAC, intracellular [DOPAC] appeared to decrease with increasing [MDA], and the levels of DOPAC for treated cells was demonstrated to be significantly different compared to the control ($p < 0.05$).

Discussion

It has been hypothesized that the DA catabolite DOPAL is an endogenous neurotoxin relevant to PD pathogenesis and serving as a chemical trigger for the disease (15,24). Previous work demonstrated the toxicity of DOPAL toward dopaminergic cells via various mechanisms, including production of oxidative stress, induction of the mitochondrial transition pore and protein modification (14,25-27). Subsequent studies showed that 4HNE and/or MDA at low μM inhibited human recombinant ALDH2 and impaired DOPAL oxidation in rat brain mitochondria and crude rat striatal synaptosomes, with the latter yielding an increase in the concentration of the aldehyde and elevated levels of catechol adducts on proteins (9,13). In addition, it was demonstrated that 4HNE inhibited ALDH2 but not MAO. Evidence was presented indicating that the catechol adducts were due to elevated DOPAL, including the dependence of protein modification (i.e. catechol adducts) on MAO activity and significantly greater reactivity of DOPAL toward proteins than DA or DOPAC (13).

The current study extends these previous important findings to a dopamingeric cell model (i.e. NGF-treated PC6-3 cells), which includes both the oxidative and reductive pathways for DA catabolism. Data are presented supporting a mechanistic link between oxidative stress, hypothesized to be involved in PD pathogenesis, and generation of DOPAL at elevated levels. Specifically, both of the lipid peroxidation products 4HNE and MDA at low μM were found to significantly inhibit DA catabolism at the aldehyde oxidation and/or reduction step. It should be noted that these findings were performed using cells in the presence of 5% CO_2; however, these experiments were repeated under atmospheric conditions at 37 °C (i.e. without 5% CO_2) to rule out the possibility that the CO_2 influenced the reported outcomes (data not shown).

4HNE and MDA inhibited cellular ALDH-mediated oxidation of DOPAL to DOPAC, and both of these lipid peroxidation products have been demonstrated to be significant inhibitors of ALDH's. However, 4HNE and MDA inhibited ALDH with comparable potency as shown in Figure 1, which was not expected. Previous studies using simpler model systems (i.e. mitochondria, synaptosomes) found 4HNE to be a better inhibitor of mitochondrial ALDH than MDA (9,13). Given the higher reactivity of 4HNE toward protein nucleophiles than MDA and that these lipid aldehydes were provided exogenously to the cells, the effective (i.e. intracellular or mitochondrial) concentration of 4HNE may be significantly lower than that for MDA, yielding the results displayed in Figure 2. Also, dopaminergic cells have effective metabolic enzymes for biotransformation of 4HNE, including ALDH and AR (28). A recent

report demonstrated the ability of DA treatment to increase levels of γ-glutamylcysteine ligase and cellular and mitochondrial GSH (29). DOPAL, especially at elevated concentrations as observed in this study, might also serve as an inducer for cellular antioxidant systems.

Although both 4HNE and MDA apparently inhibited ALDH-mediated DOPAL oxidation to a similar extent, the concentration of DOPAL after 60 min was significantly higher for MDA than 4HNE. Subsequent analysis revealed the compensating effect of AR for elevated levels of DOPAL, i.e. enzyme-catalyzed reduction of DOPAL to DOPET, but surprisingly, cells treated with 4HNE but not MDA exhibited a dose-dependent increase in the alcohol product. Based on the observed results, it appeared that MDA inhibited both ALDH and AR, yielding a higher concentration of DOPAL than 4HNE, which targets only the ALDH.

To investigate this issue further, the cytosolic cell fraction containing AR, was obtained and incubated with DOPAL, NADPH and MDA or 4HNE. As shown in Figure 5, MDA at all concentrations (2 to 25 μM) inhibited AR activity using DOPAL as substrate, while 25 μM 4HNE had no effect. Given that 4HNE is an excellent substrate for the AR enzymes (30-32), which are unusual in that aldose reductase (AKR1B1) requires GSH binding to 4HNE and other substrates for activity (30), one might predict competitive inhibition to some extent; however, this was not observed for 4HNE. Perhaps at higher concentrations of 4HNE (>25 μM), competitive inhibition might be possible.

A previous study showed that MDA or 4HNE treatment of rat aortic smooth muscle cells lead to an increase in aldehyde reductase gene expression (33). However, it does not appear there are any reports documenting sensitivity or resistance of AR toward MDA. It is possible and probable that AR is irreversibly inhibited by MDA given the presence of critical Lys residues in these enzymes that could be targeted by the aldehyde (34,35); however, as noted in the results section, the inhibition appears to be reversible. In addition, the identity of the AR(s), e.g. AKR1A1 or AKR1B1, sensitive to MDA is unknown, and studies are currently in progress to address these issues.

Initially, only extracellular levels of DA metabolites were measured, as shown in Figures 2-4, however, subsequent experiments were performed to determine the intracellular concentrations of such compounds. As shown in Figure 6, the intracellular level of DA did not change with MDA treatment, suggesting that MDA did not affect the trafficking or metabolism of DA. As one might expect, the intracellular concentration of DOPAC decreased with increasing MDA, as was observed for the extracellular environment.

However, it was surprising that while extracellular levels of DOPAL were elevated several-fold after 4HNE or MDA exposure at the 60 min time-point (Figures 2 and 3), the intracellular concentration of DOPAL was the same for control and treated cells (Figure 6). Such a result might suggest transport of the aldehyde (intracellular) against a gradient (extracellular), and indeed, a previous study found that DOPAL efflux/export was sensitive to mazindol, indicating a role for the dopamine transporter (27). These findings raise the possibility that cells neighboring dopamine neurons, e.g. astrocytes, microglia, may experience elevated concentrations of DOPAL during or subsequent to oxidative stress. How these other cells and cell types react to the toxic aldehyde is currently unknown and warrants further investigation, especially given that PD pathogenesis may involve neurons and glia (36).

A possible consequence of elevated DOPAL is protein modification given the potential reactivity of the aldehyde. Such a result was observed in a previous study using rat striatal synaptosomes as a model for DA metabolites and indicates increased levels of DOPAL-protein adducts (13). Because it is structurally analogous to DA, DOPAL could interact with proteins that have affinity for DA, including the vesicular monoamine transporter or tyrosine hydroxylase. It is conceivable that other proteins important for DA neuron homeostasis may

be targeted, and also of question, is the functional consequence of protein adduction. A previous study reported that α-synuclein was modified by DOPAL resulting in significant aggregation of the protein (37).

Because it contains both a catechol, that could undergo auto-oxidation to a reactive ortho-quinone as observed for DA (38-41), and an amine-reactive aldehyde, DOPAL has the potential to modify Cys and/or Lys residues on target cellular proteins. Previous work demonstrated the reactivity of the DOPAL aldehyde toward Lys using a model peptide and the stability of the resulting Schiff-base adduct (13). In addition, this same study found that DOPAL but not DA significantly modified bovine serum albumin (BSA) (13), and it should be noted that BSA contains only one free Cys but sixty Lys residues . Given these data and the possibility that the ortho-quinone of DOPAL may be unstable and prone to rapid rearrangement/cyclization, as determined for the DA-quinone (42,43), protein modification may be largely due to Lys adduction. However, neuronal tissue contains prostaglandin H synthase, which catalyzes oxidation of DA to its quinone form especially in the presence of hydrogen peroxide (44), and it is possible that the enzyme can oxidize DOPAL yielding significant levels of the quinone form of this DA metabolite. Therefore, it is not clear at this point which adduct (Lys or Cys) predominates in DA cells and is responsible for toxicity.

In summary, the present study demonstrated that low μM levels of oxidative stress products, i.e. 4HNE and MDA, significantly inhibited DA catabolism, yielding elevated levels of the reactive intermediate DOPAL. Cellular ALDH's were sensitive to both 4HNE and MDA, and interestingly, cellular AR was found to be inhibited by MDA. Because MDA affected both ALDH's and AR, the level of DOPAL was higher for cells treated with MDA versus 4HNE. These data demonstrate that low levels of lipid peroxidation products will yield aberrant levels of the reactive and toxic intermediate, DOPAL, and suggest a link between oxidative stress and generation of a neurotoxin endogenous to DA neurons. Such findings may provide a mechanism for selective neurotoxicity or neurodegeneration, as seen for PD. In addition to lipid peroxidation products impairing DA catabolism, it was also found that treatment of PC6-3 cells with the pro-oxidant iron (50 and 200 μM) yielded an increase in DOPAL, ~8-fold over the control (data not shown).

Pesticides associated with PD incidence, such as the organochlorine dieldrin, were shown to generate oxidative stress in cellular models of DA neurons as well as DA neurons in mice. It should be noted that dieldrin can disrupt vesicular DA stores causing release of the neurotransmitter (20), which could yield elevated DOPAL. Therefore, it is of question whether or not exposure to a pesticide (e.g. dieldrin) associated with PD yields elevated DOPAL, and work is in progress to address this issue. As noted in a previous paper, DOPAL may serve as a "chemical trigger" for selective neurodegeneration, such as PD (24).

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Abbreviations

4HNE, 4-hydroxy-2-nonenal
ALDH, aldehyde dehydrogenase
ALDH2, mitochondrial aldehyde dehydrogenase
AR, aldehyde or aldose reductase
DA, dopamine
BSA, bovine serum albumin
DOPAC, 3,4-dihydroxyphenylacetic acid

DOPAL, 3,4-dihydroxyphenylacetaldehyde
DOPET, 3,4-dihydroxyphenylethanol
MAO, monamine oxidase
MDA, malondialdehyde
MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NGF, nerve-growth factor
PD, Parkinson's disease
ROS, reactive oxygen species

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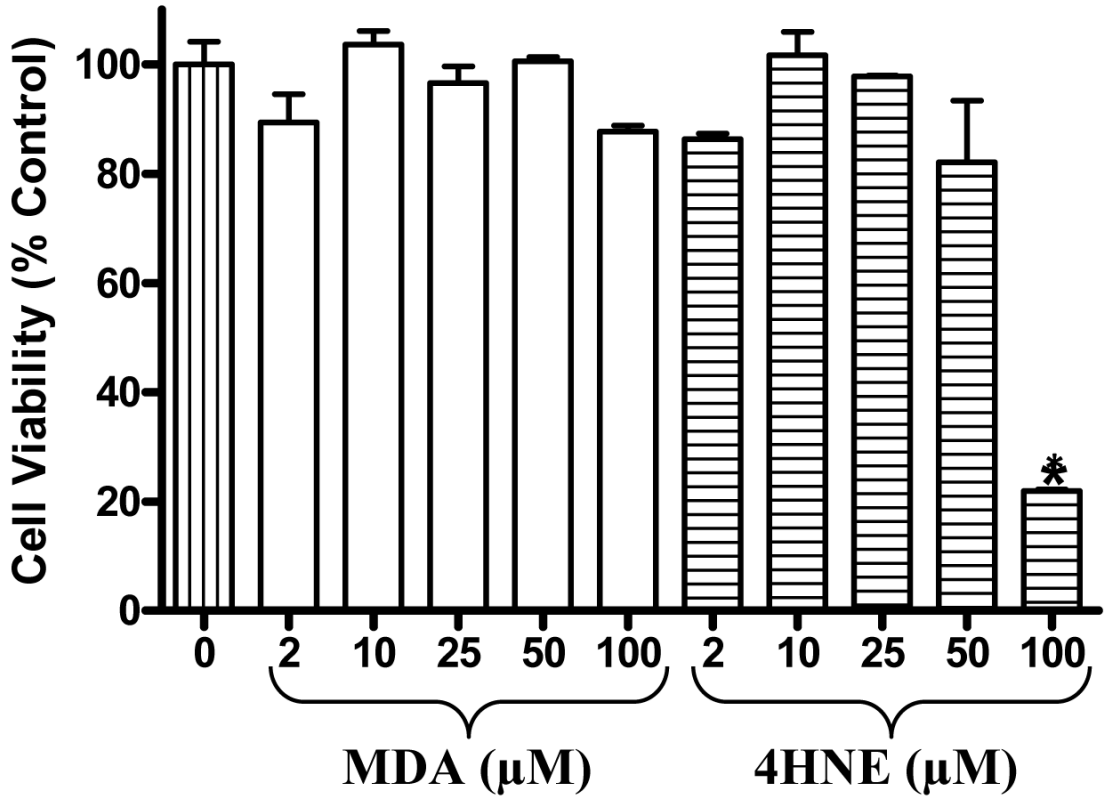


Figure 1.

MDA- and 4HNE-induced cytotoxicity in PC6-3 cells. The cells were treated with varying concentrations of MDA or 4HNE (2 to 100 μM) for 60 min. Cell viability was measured via the MTT assay and compared to a control (i.e. 0 μM MDA/4HNE). Values represent mean ± SEM (n = 2, except for the control where n = 4). *, significantly different from the control (p < 0.05).

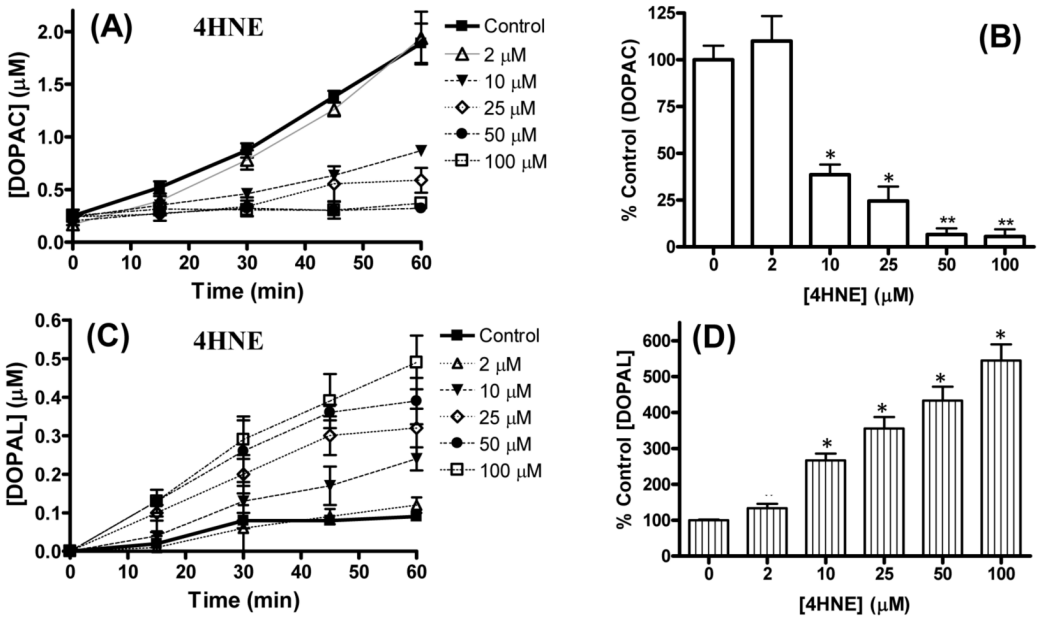


Figure 2.

Treatment of PC6-3 cells with 4HNE resulted in decreased DOPAC formation and increased DOPAL. (A) The averaged time-course demonstrating 4HNE-mediated inhibition of DOPAC production (i.e. DOPAL oxidation). Shown are the results of $n = 3$ experiments (done in triplicate). (B) Based on comparison of slopes for initial linear formation DOPAC, 4HNE inhibits DOPAL oxidation in a dose-dependent manner. Values shown represent the mean ± SEM ($n = 3$, triplicate). *, significantly different from the control ($p < 0.05$). **, not significantly different from zero ($p > 0.05$). (C) The averaged time course demonstrating 4HNE-mediated increase in DOPAL. Shown are the results of $n = 3$ experiments. (D) Treatment of cells with 4HNE yields a dose-dependent increase in DOPAL based on the comparison of total DOPAL produced over 60 min. Values shown represent mean ± SEM ($n = 3$, triplicate). *, significantly different from the control ($p < 0.05$).

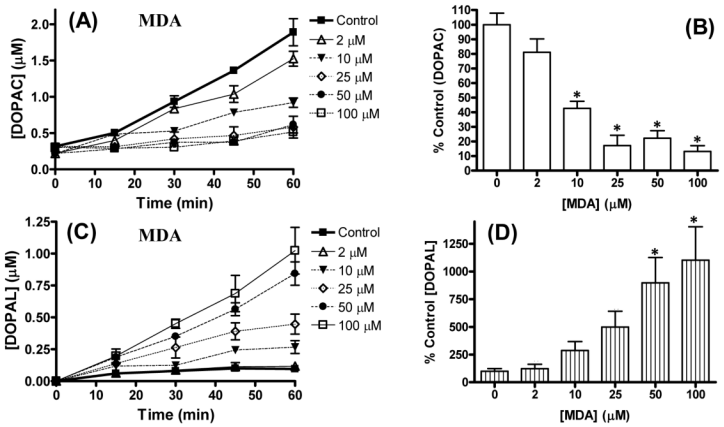


Figure 3.

Treatment of PC6-3 cells with MDA resulted in decreased DOPAC formation and increased DOPAL. (A) The averaged time-course demonstrating MDA-mediated inhibition of DOPAC production (i.e. DOPAL oxidation). Shown are the results of $n = 3$ experiments. (B) Based on comparison of slopes for initial linear formation DOPAC, MDA inhibits DOPAL oxidation in a dose-dependent manner. Values shown represent the mean ± SEM ($n = 3$). *, significantly different from the control ($p < 0.05$). (C) The averaged time course demonstrating MDA-mediated increase in DOPAL. Shown are the results of $n = 3$ experiments. (D) Treatment of cells with MDA yields a dose-dependent increase in DOPAL based on the comparison of total DOPAL produced over 60 min. Values shown represent mean ± SEM ($n = 3$, that is 3 wells of cells). *, significantly different from the control ($p < 0.05$).

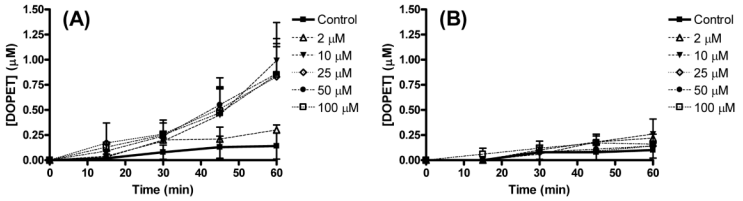


Figure 4.

Production of DOPET in PC6-3 cells treated with (A) 4HNE or (B) MDA. Only incubation with 4HNE yielded a significant increase in DOPET. Shown are the results of $n = 3$, experiment performed in triplicate.

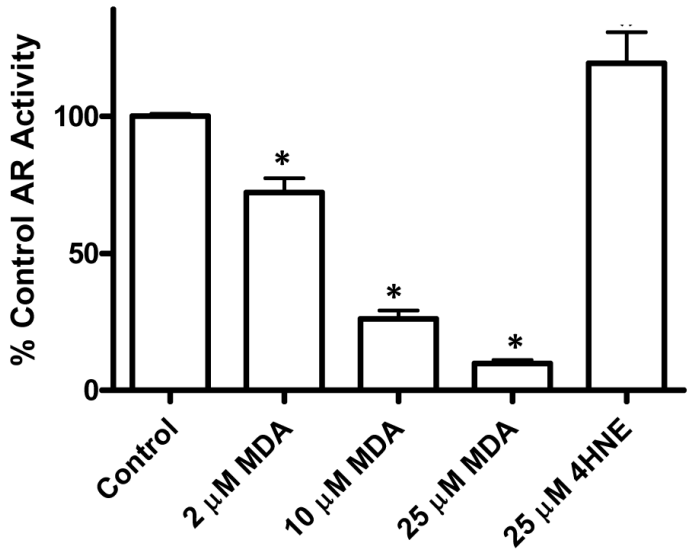


Figure 5.

MDA but not 4HNE inhibits reduction of DOPAL to DOPET via cytosolic AR. Isolated PC6-3 cytosol was treated with 2, 10 or 25 μM MDA or 25 μM 4HNE. The activity toward 10 μM DOPAL (i.e. DOPAL reduction to DOPET) was measured and compared to a control containing no 4HNE or MDA (% Control AR Activity). Values shown represent the mean ± SEM (n = 6). *, significantly different from the control ($p < 0.05$).

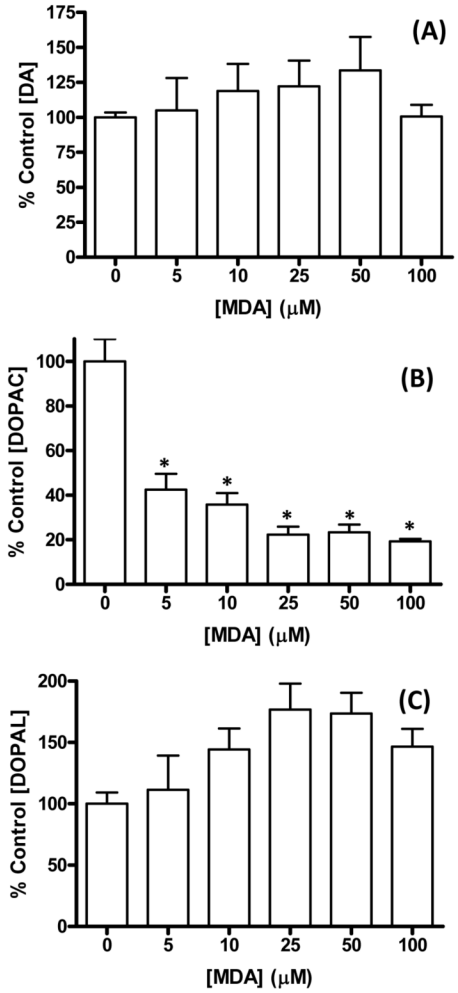
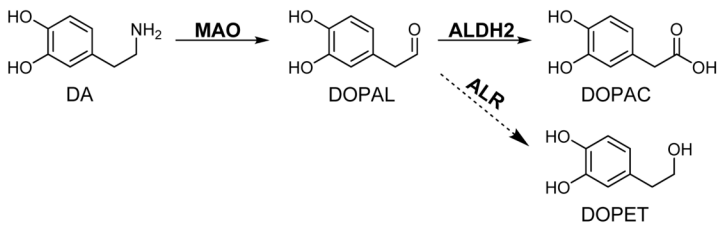


Figure 6.

Levels of intracellular (A) DA, (B) DOPAC and (C) DOPAL in PC6-3 cells treated with varying concentrations of MDA (0 to 100 μM). Values shown represent the mean ± SEM (n = 6, experiment performed twice in triplicate). The levels of DOPAC but not DA or DOPAL changed significantly following MDA treatment for 60 min, compared to a control ($p < 0.05$), based on two-tailed t-test and ANOVA with Tukey post-test. *, significantly different from the control ($p < 0.05$).



Scheme 1.
DA catabolism involving formation of proposed toxin and amine and aldehyde biotransformation.