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3,4-Dihydroxyphenylacetaldehyde is more efficient than dopamine in oligomerizing and quinonizing alpha-synuclein

Yunden Jinsmaa, Risa Isonaka, Yehonatan Sharabi, and David S. Goldstein

Autonomic Medicine Section, CNP/DIR/NINDS/NIH, Bethesda, MD 20892-1620 (Y.J., R.I., D.G.);

Sackler Faculty of Medicine, Tel Aviv University, Israel (Y.S.)

Running head: DOPAL vs. DA

Corresponding Author: David S. Goldstein, MD PhD, 10 Center Drive, Bldg. 10 Rm. 8N260, Bethesda, MD 20892-1620 USA, Phone: 301-496-2104, Fax: 301-402-0180; iPhone: 301-675-1110; Email: goldsteind@ninds.nih.gov

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Non-standard abbreviations used: ALDH=aldehyde dehydrogenase; AR=aldehyde/aldose reductase; AS=alpha-synuclein; DA=dopamine; DAPI=4,6-diamidino-2-phenylindole; DOPAC=3,4-dihydroxyphenylacetic acid; DOPAL=3,4-dihydroxyphenylacetaldehyde; DOPET=3,4-dihydroxyphenylethanol; FCS=fetal calf serum; GCIs=glial cytoplasmic inclusions; LAAAD=L-aromatic-amino-acid decarboxylase; MAO=monoamine oxidase; MSA=multiple system atrophy; MW=molecular weight; NAC=N-acetylcysteine; nIRF=near infrared fluorescence; PD=Parkinson disease; Tyr=tyrosinase; VMAT=vesicular monoamine transporter

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ABSTRACT

Lewy body diseases such as Parkinson disease (PD) involve intra-neuronal deposition of the protein alpha-synuclein (AS) and depletion of nigrostriatal dopamine. Interactions of AS with dopamine oxidation products may link these neurohistopathologic and neurochemical abnormalities, via two potential pathways—spontaneous oxidation of dopamine to dopamine-quinone and enzymatic oxidation of dopamine catalyzed by monoamine oxidase to form 3,4-dihydroxyphenylacetaldehyde (DOPAL), which is then oxidized to DOPAL-quinone. We compared these two pathways in terms of the ability of dopamine and DOPAL to modify AS. DOPAL was far more potent than dopamine both in oligomerizing and forming quinone-protein adducts with ("quinonizing") AS. The DOPAL-induced protein modifications were enhanced similarly by pro-oxidation with Cu(II) or tyrosinase and inhibited similarly by anti-oxidation with N-acetylcysteine. Dopamine oxidation evoked by Cu(II) or tyrosinase did not quinonize AS. In cultured MO3.13 human oligodendrocytes DOPAL resulted in the formation of numerous intra-cellular quinoproteins that were visualized by near infrared microscopy. We conclude that of the two routes by which oxidation of dopamine modifies AS and other proteins, that via DOPAL is more prominent. The results support developing experimental therapeutic strategies that might mitigate deleterious modifications of proteins such as AS in Lewy body diseases by targeting DOPAL formation and oxidation.

SIGNIFICANCE STATEMENT

Interactions of the protein alpha-synuclein with products of dopamine oxidation in the neuronal cytoplasm may link two hallmark abnormalities of Parkinson disease—Lewy bodies (which contain abundant alpha-synuclein) and nigrostriatal dopamine depletion (which produces the characteristic movement disorder). Of two potential routes by which dopamine oxidation may alter alpha-synuclein and other proteins, that via the autotoxic catecholaldehyde 3,4-dihydroxyphenylacetaldehyde (DOPAL) is more prominent; the results support experimental therapeutic strategies targeting DOPAL formation and DOPAL-induced protein modifications.

INTRODUCTION

Diseases that involve progressive central catecholaminergic neurodegeneration such as Parkinson disease (PD) and multiple system atrophy (MSA) are associated with profound putamen dopamine (DA) depletion (Hornykiewicz, 1966; Tong et al., 2004; Goldstein et al., 2015), deposition of the protein alpha-synuclein (AS) in neurons in PD (Spillantini et al., 1997) and in glial cells in MSA (Tu et al., 1998), and buildup of the catecholaldehyde 3,4-dihydroxyphenylacetaldehyde (DOPAL) (Goldstein et al., 2011b; Goldstein et al., 2013; Goldstein et al., 2015). DOPAL, an obligate intermediate in neuronal DA metabolism, is formed from the enzymatic oxidation of cytoplasmic DA by monoamine oxidase (MAO) in the outer mitochondrial membrane. In cells and animals DOPAL is toxic (Mattammal et al., 1995; Burke et al., 2003; Panneton et al., 2010), so that DOPAL accumulation might contribute to neuronal malfunctions and eventual loss of dopaminergic neurons (Burke et al., 2003; Goldstein et al., 2014).

An almost completely independent line of research has centered on potentially harmful effects of spontaneous oxidation of DA (Hastings, 2009; Surmeier et al., 2011; Burbulla et al., 2017; Herrera et al., 2017). DA can undergo oxidation to form dopamine-quinone (DA-Q) and then a variety of potentially neurotoxic compounds such as aminochrome (Linsenbardt et al., 2009; Paris et al., 2009; Segura-Aguilar, 2019), 5-S-cysteinyldopamine (Montine et al., 1997; Badillo-Ramirez et al., 2019), and isoquinolines (Nagatsu, 1997; Storch et al., 2002).

The catecholamine autotoxicity theory imputes pathologic interactions between catecholamine oxidation products and intra-cellular proteins in the pathogenesis of diseases involving catecholaminergic neurodegeneration (Goldstein et al., 2014). Specifically, DOPAL oligomerizes AS (Burke et al., 2008), and oligomerized AS seems to be pathogenic (Winner et al., 2011; Deas et al., 2016; Gustafsson et al., 2017). Divalent metal cations—especially Cu(II)—augment DOPAL-induced AS oligomerization (Jinsmaa et al., 2014). DOPAL also forms covalent quinone adducts with ("quinonizes") many PD-related proteins, including AS (Jinsmaa et al., 2018). Quinonization may interfere with the functions of these proteins and thereby with numerous intra-cellular processes. In particular, it has been reported that compounds formed after DA oxidation to DA-Q

evoke mitochondrial dysfunction (Jana et al., 2011).

Oxidized DA also can interact with AS (Hasegawa et al., 2006). DA can promote the formation of AS oligomers (Leong et al., 2009; Lee et al., 2011; Saha et al., 2018). Moreover, aminochrome and 5,6-dihydroxyindole, which are products of DA oxidation, can oligomerize AS (Pham et al., 2009; Munoz et al., 2015; Huenchuguala et al., 2019).

The literature on DA oxidation and synucleinopathy has generally overlooked DOPAL (Asanuma et al., 2003; Mazzulli et al., 2006; Leong et al., 2009; Burbulla et al., 2017; Mor et al., 2017; Segura-Aguilar, 2017; Mor et al., 2019), and the literature on DOPAL and synucleinopathy has generally overlooked DA-Q (Burke et al., 2008; Follmer et al., 2015; Jinsmaa et al., 2018; Cagle et al., 2019). In the few studies where DOPAL and DA have been compared directly in terms of oligomerizing AS, DOPAL has been found to be more potent (Burke et al., 2008; Jinsmaa et al., 2014; Jinsmaa et al., 2018). Whether oxidation of DA results in quinonization of AS or of intra-cellular proteins via routes other than DOPAL has not been assessed.

The overall purpose of the present experiments was to compare DOPAL and DA in terms of (1) AS oligomerization and quinonization in 1.5 mL eppendorf tubes experiments; (2) enhancing effects of Cu(II) (Jinsmaa et al., 2014; Dell'Acqua et al., 2017) and mitigating effects of anti-oxidation with N-acetylcysteine (NAC) (Banerjee et al., 2014; Anderson et al., 2016; Goldstein et al., 2017a; Jinsmaa et al., 2018); and (3) quinonization of intra-cellular proteins in cultured cells (Banerjee et al., 2014; Jinsmaa et al., 2018). From these comparisons one would anticipate improved targeting in disease-modification experimental therapeutic trials.

For the cellular experiments we used human MO3.13 oligodendrocytes. Studying MO3.13 oligodendrocytes is relevant to MSA, which is a synucleinopathy that can resemble PD closely clinically but features AS deposition in the cytoplasm of glial cells—especially in oligodendrocytes (Tu et al., 1998; Wakabayashi et al., 1998; Pukass and Richter-Landsberg, 2014). DOPAL is known to aggregate AS in MO3.13 cells (Jinsmaa et al., 2018), but whether DOPAL evokes the formation of intra-cellular quinoproteins has been unknown.

MATERIALS AND METHODS

Reagents and chemicals

Human recombinant AS (WT) was purchased from Calbiochem (La Jolla, CA). DOPAL was from Santa Cruz Biotech (Dallas, TX). Mutant A53T AS, NAC, dithiothreitol, glycine, tyrosinase (Tyr), pargyline-HCl, MAO-B, saponin, and the aldehyde dehydrogenase inhibitor benomyl were from Sigma-Aldrich (St. Louis, MO). The aldehyde reductase inhibitor AL-1576 was a gift from Alcon Laboratories, Fort Worth, TX. Tolcapone was from Orion Pharma (Espoo, Finland). MO3.13 cells were from Cellutions Biosystems Inc. (Burlington, Ontario, Canada). Cell culture medium was from Invitrogen (Camarillo, CA). Phosphate-buffered saline and deionized ultrapure water were from KD Medical (Columbia, MD). Benomyl, AL-1576, and tolcapone were dissolved in dimethylsulfoxide (American Bio, Natick, MA) and stored at -20 ^{o}C. NAC, DOPAL, and DA stock solutions were prepared in ultrapure water and stored at -20 ^{o}C.

Eppendorf tube experiments

Experiments in 1.5 mL eppendorf plastic sample tubes were done to compare DA with DOPAL in terms of oligomerization and quinonization of AS and effects of various conditions augmenting or mitigating these actions.

Effects of treatment with DOPAL or DA on AS protein modifications were assessed by incubation of AS (3 μM) with DOPAL (30 μM) or DA (30 μM) in 50 mM phosphate buffer (pH 7.4) at 37 ^{o}C in a water bath for 2 or 5 hours depending on the experiment. Samples (15 μL) were taken at different time points and heated in NuPAGE LDS sample buffer with 50 mM dithiothreitol for 5 min at 70 ^{o}C . The reaction mixtures (5 μL or 15 μL) were then electrophoresed on NuPAGE 4-12% Bis-Tris gels.

Protein modifications were detected by near-infrared fluorescence (nIRF) spectrometry (15 μL applications to gels) and western blotting (5 μL applications to gels). The nIRF signals were read with excitation wavelength 685 nm using an Odyssey infrared imager (Li-Cor Biosciences, Lincoln, NE) (Jinsmaa et al., 2018). For western blotting the proteins were transferred to nitrocellulose membranes using an iBlot dry blotting system (Novex Life Technologies, ThermoFisher Scientific,

Waltham, MA). AS detection was done with mouse anti-human AS antibody (Invitrogen, Camarillo, CA, 1:200), with the secondary antibody being goat anti-mouse IRDye 800CW (Abcam, Cambridge, MA, 1:10,000), and read using the Odyssey imaging system. Protein staining was done with AcquaStain (Bulldog Bio, Portsmouth, NH). There was 1 experiment, with 3-4 replicates at each observation point (Jinsmaa et al., 2018).

Effects of enzymatic oxidation of DOPAL or DA on AS protein modifications were examined in two experiments. In one experiment DA (30 μM) and DOPAL (30 μM) were kept at room temperature for 20 min without and with Tyr (69 U/mL), followed by incubation of the mixture with AS (3 μM) for 1 hour. In another experiment different concentrations of DOPAL (10, 30, and 100 μM) or DA (10, 30, and 100 μM) were incubated with Tyr and then incubated with AS. Protein modifications were detected as described above. There were 2 experiments, with 3-4 replicates at each observation point.

Effects of Cu(II) on AS protein modifications elicited by DOPAL and DA were studied in two experiments. In one experiment, AS (3 μM) and DOPAL (30 μM) were incubated with various concentrations of Cu(II) (1-100 μM) at 37 ^{o}C in a water bath for 1 hour. Different concentrations of NAC (100-300 μM) were added at the start of the incubation. There were 3-4 replicates. In another experiment, to compare DOPAL and DA, AS (3 μM) and DOPAL (30 μM) or DA (30 μM) were incubated with Cu(II) (1 μM or 30 μM) at 37 ^{o}C in a water bath for 1 hour. To assess the time courses of the reactions, samples (15 μL) were taken at different time points (0, 5, 15, 30, 45, and 60 min). There were 3-4 replicates. Protein modifications were detected as described above.

Human wild-type (WT) and A53T mutant forms of AS were compared in terms of effects of DOPAL+Cu(II) on AS oligomerization and quinonization. Each protein (3 μM) was incubated with DOPAL (30 μM) and Cu(II) (30 μM) at 37 ^{o}C in a water bath for 1 hour. Protein modifications were detected as described above. There was 1 experiment, with 3-4 replicates.

Cellular experiments about DOPAL- and DA-induced protein modifications

Using nIRF spectroscopy and microscopy we compared DOPAL and DA in terms of quinonization of intracellular proteins in MO3.13 human oligodendrocytes. The cells were cultured

in high glucose Dulbecco modified Eagle's medium containing 10% fetal calf serum (FCS). MO3.13 cells were plated in 12-well plates (1.5 x 10^5 cells/well) or 2-well/slides (8 x 10^4 cells/well). The cells were incubated for 24 hours with 10 μM tolcapone added to the medium to block catechol-O-methyltransferase. On the day of the acute experiments the medium used was Dulbecco's modified Eagle's medium containing 10 μM each of tolcapone and benomyl and 1 μM AL-1576. The cells were then further exposed to other compounds as described below.

To assess Cu(II) effects on DOPAL-quinonized proteins in MO3.13 cells, after 24 hours of incubation with medium containing tolcapone the cells were treated with DOPAL (100 μM) and Cu(II) (10 and 30 μM) for 5 hours at 37 ^{o}C in a CO_2 incubator. The cells were then lysed with RIPA buffer (Millipore, Temecula, CA) containing 1 tablet per 10 mL of Complete Mini protease inhibitors (Roche Diagnostics, Indianapolis, IN). The proteins (15 μL) were heated in NuPAGE LDS sample buffer with 50 mM dithiothreitol for 5 min at 70 ^{o}C. The reaction mixtures were then electrophoresed on NuPAGE 4-12% Bis-Tris gels, and the nIRF signals were quantified using the Odyssey infrared imaging system as described previously (Jinsmaa et al., 2018).

To assess effects of NAC on DOPAL-quinonized intracellular proteins, MO3.13 cells were exposed to DOPAL+Cu(II) (30 μM each) with different concentrations of NAC (10, 30, 100, and 300 μM) for 5 hours at 37 ^{o}C in a CO_2 incubator. The cells were then lysed and processed as described above.

To visualize intracellular DOPAL-induced quinoproteins in MO3.13 oligodendrocytes, the cells were exposed to DOPAL (10, 30, and 100 μM) for 5 hours in the presence of 30 μM Cu(II). The cells were then fixed with ice-cold methanol for 20 min and washed with cold PBS 3 times. Permeabilization and blocking were done with 15 drops/slide of TrueBlack IF Background Suppressor System (Biotium Inc, Fremont, CA) for 15 min. Slides were then incubated with human anti-tubulin antibody (Abcam, 1:1500) overnight at 4 ^{o}C, followed by incubation with the secondary antibody Alexa FluorR 488 goat anti-mouse (Molecular Probes, Thermo Scientific, Waltham, MA, 1:500) for 1 hour at room temperature. Cells were also stained for DAPI (Abcam, 1:2000) to visualize cell nuclei. Images were obtained using a Zeiss 880 confocal microscope. nIRF was

detected at 587-686 nm. There was 3 experiments with 1 set of observations.

Data analysis and statistics

Mean values were expressed ± standard error of the mean. Statistical analyses were done by one-way factorial analyses of variance with Dunnett's post-hoc test to compare experimental with control (or DOPAL alone) mean values (GraphPad Software, La Jolla, CA). T-testing was done to compare two independent groups. Statistical significance was defined by $p<0.05$.

RESULTS

DOPAL oligomerizes and quinonizes AS.

DOPAL time-dependently quinonized and oligomerized AS (Fig. 1). AS dimer and trimer and quinonized AS monomer were detectable by about 30 minutes of incubation (Fig. 1A) and increased further with increasing times of incubation (Fig. 1B and 1C). Oligomerization and quinonization occurred approximately concurrently. As the incubation time increased, DOPAL resulted in an intense high molecular weight (MW) "smear" by both western blotting and nIRF (Fig. 1B and 1C). Incubation of AS with DA resulted in a small amount of AS dimer, a faint "smear" of high MW AS at long incubation times, and complete absence of quinonized AS (Fig. 1B and 1C). Protein staining showed that DOPAL time-dependently decreased AS monomer (Fig. 1D), indicating substantial reactivity with AS to form other compounds, whereas DA did not.

DOPAL and DA were treated with Tyr to catalyze oxidation of the catechols. For both DOPAL and DA the oxidation started immediately, and within 5-10 minutes of the reaction it was obvious by the change of the color of the solutions that both catechols had been oxidized (data not shown). After Tyr-induced oxidation of DOPAL and DA for 20 min, western blotting demonstrated AS oligomerization by both catechols, with DOPAL having greater potency (Fig. 2B and 2D). Incubation of AS with Tyr alone was without effect (Fig. 2A and 2B). The "smear" of high MW AS noted above was also seen when AS was incubated with DA and Tyr (Fig. 2B and 2D); however, no detectable quinonized AS was seen even at the highest DA concentration (100 μM, Fig. 2C).

Cu(II) enhances DOPAL-induced AS quinonization and oligomerization.

Cu(II) at concentrations as low as 1 μM augmented DOPAL-induced AS quinonization compared to DOPAL alone (Fig. 3A and 3B, Fig. 4A and 4C). The effects of Cu(II) were concentration-dependent (Fig. 3A). At the 30 μM concentration, Cu(II) enhanced and accelerated both DOPAL-induced quinonization and oligomerization of AS, with AS dimer and quinonized AS monomer detectable in as little as 5 minutes of incubation (Fig. 3A and Fig. 4B and 4C). DA failed to produce any quinonized AS signals under the same experimental conditions (Fig. 4B and 4C). NAC concentration-dependelty mitigated the effects of DOPAL+Cu(II) (Fig. 3C and 3D). Cu(II)

alone did not oligomerize or quinonize AS (data not shown).

Mutant A53T AS is more vulnerable than WT AS to DOPAL effects.

Mutant A53T AS was more vulnerable to DOPAL+Cu(II) treatment than was WT AS (Fig. 5). Thus, for A53T AS the amount of nIRF signal with DOPAL+Cu(II) vs. DOPAL alone was about twice as large as that for WT AS ($t=2.81$, $p=0.0049$).

DOPAL induces the formation of cellular quinoproteins.

Cu(II) (30 μM) augmented DOPAL-induced formation of cellular quinoproteins in MO3.13 oligodendrocytes (Fig. 6A and 6B). No quinoproteins were detected in DA-treated cells (data not shown). NAC attenuated the formation of quinoproteins evoked by DOPAL+Cu(II) (Fig. 6C and 6D).

nIRF microscopy was used to visualize intracellular catechol-induced quinoproteins (Fig. 7). DOPAL exposure resulted in the appearance of intra-cellular quinoproteins, in the form of myriad pinpoint spots in nuclei and as aggregates that seemed extracellular (or were remnants of dead cells). DA did not result in detectable intra-cellular quinoproteins (data not shown).

DISCUSSION

It has long been suspected that DA oxidation products contribute to the pathogenesis of PD (Blaschko, 1952; Carlsson and Fornstedt, 1991; Mattammal et al., 1995; Burbulla et al., 2017) and in particular interact with AS to challenge dopaminergic neuronal homeostasis (Mazzulli et al., 2007; Goldstein et al., 2014; Mor et al., 2017; Mor et al., 2019)

There are two general routes by which this could happen—(1) non-enzymatic oxidation of DA to DA-quinone (DA-Q), with subsequent formation of DA-Q-derived compounds such as aminochrome (Linsenbardt et al., 2012; Segura-Aguilar, 2019), isoquinolines (Storch et al., 2002), and 5-S-cysteinyldopamine (Montine et al., 1997; Badillo-Ramirez et al., 2019); and (2) MAO-catalyzed enzymatic oxidation of DA to form DOPAL (Mattammal et al., 1995), with spontaneous oxidation of DOPAL to DOPAL-quinone (DOPAL-Q) (Anderson et al., 2011; Follmer et al., 2015; Jinsmaa et al., 2018) (see Visual Abstract). These two routes have been studied almost completely separately by different research groups. Here we comprehensively compared DOPAL with DA in terms of oligomerizing and quinonizing AS. Both types of protein modifications may be deleterious (Jana et al., 2011; Winner et al., 2011).

DOPAL is the main mediator of DA-dependent AS oligomerization and quinonization.

The present results confirm previous reports that DOPAL is more potent than DA in oligomerizing AS (Burke et al., 2008; Jinsmaa et al., 2014). A major new finding is that unlike DOPAL, DA does not quinonize AS, even in the setting of oxidation of DA by Cu(II) or tyrosinase.

The differences in potencies of DOPAL and DA in oligomerizing and quinonizing AS may be explained by their different chemical structures (Rees et al., 2009). Whereas DA has a terminal amine group, DOPAL has a reactive aldehyde group that can bind covalently to lysine residues (Anderson et al., 2016), which are abundant in the AS molecule (Rees et al., 2009; Follmer et al., 2015; Plotegher et al., 2017). Occupation of lysine residues completely prevents DOPAL-induced oligomerization and quinonization of AS (Jinsmaa et al., 2018).

DA and especially DOPAL incubation resulted in the formation of AS oligomers in discrete bands at low molecular weights and poorly defined AS “smears” at high molecular weights (e.g.,

Figs. 1C, 2B, and 2D). Since anti-oxidation with NAC mitigated these AS modifications, the results raise the possibility that oxidation products of DOPAL and DA can polymerize (Lee et al., 2007) and bind to AS, resulting in a large variety of high molecular weight forms of the bound AS. AS oligomers linked by DOPAL condensation products (Werner-Allen et al., 2016; Werner-Allen et al., 2017; Werner-Allen et al., 2018) may also polymerize. These possibilities are not mutually exclusive, and the present data cannot distinguish between them. Since AS oligomerization and quinonization occurred approximately simultaneously, we also cannot draw inferences about the relationship between quinonization and oligomerization of AS.

Because of the novelty of the discovery that DOPAL induces quinonization of numerous intracellular proteins, there is relatively scant literature about how this happens. Citraconic acid, which occupies lysine residues, prevents DOPAL-induced quinonization of alpha-synuclein (Jinsmaa et al., 2018). This indicates that the site of the quinonization is lysines, which are relatively abundant in the alpha-synuclein molecule. N-acetylcysteine inhibits DOPAL-induced quinonization of alpha-synuclein (Jinsmaa et al., 2018), consistent with dependence of the quinonization on oxidation of DOPAL to DOPAL-quinone. Since dopamine and dopamine-related catechols do not quinonize proteins nearly as potently as does DOPAL, it seems likely DOPAL-induced protein quinonization results from reaction of the aldehyde with the amines of the lysines in alpha-synuclein. Whether DOPAL-quinone is actually incorporated in the molecule has not yet been determined. This could be addressed by experiments using tracer-labeled DOPAL. We also do not know whether quinonization is a step in the oligomerization of alpha-synuclein, since the present experiments indicated approximately simultaneous quinonization and oligomerization of alpha-synuclein by DOPAL. Cu(II) accentuates DOPAL- and DA-induced protein modifications.

The possible roles of copper and copper-protein interactions with AS in PD pathogenesis are gaining increasing research attention (Montes et al., 2014; Saha et al., 2018). We reported previously that anti-oxidation with NAC prevents the augmenting effect of Cu(II) on DOPAL-induced AS oligomerization (Jinsmaa et al., 2018). Now we report analogous enhancement by Cu(II) of DOPAL-induced quinonization of AS and mitigation of this enhancement by NAC. As part of the current

experiments we tested other metal ions, including Fe(II), Mn(II), and Ca(II). Cu(II) was the most potent in terms of enhancing DOPAL-induced quinonization of AS (unpublished observations).

Collectively the present findings suggest that potential deleterious effects of Cu(II) at physiologically attainable concentrations are related to DOPAL more than to DA and might be ameliorated by anti-oxidant treatment.

A53T AS is more susceptible than wild-type AS to quinonization by DOPAL.

The extent of quinonization of AS by Cu(II)+DOPAL was about twice as great with the A53T mutant form of AS than with human WT AS. This finding suggests that, at least in the setting of Cu(II), neurons expressing mutant forms of AS may be more vulnerable to DOPAL-induced quinonization than neurons expressing WT AS.

DOPAL quinonizes numerous intra-cellular proteins; DA does not.

In MO3.13 oligodendrocytes DOPAL—but not DA—resulted in the formation of numerous intra-cellular quinoproteins. Incubation of cells with hydrogen peroxide or in serum-free medium to evoke oxidative stress does not result in the formation of cellular quinoproteins (unpublished observations). Therefore, DOPAL-induced intra-cellular protein quinonization does not seem to be the non-specific result of oxidative stress. nIRF microscopy revealed myriad punctate sites of DOPAL-derived signal corresponding in position to nuclei, as well as large, high-intensity deposits of nIRF signal in extracellular fluid or remnants of necrotic cells. The identities of the quinoproteins are unknown but might be revealed by a proteomics technical approach.

We doubt that the lack of effect of dopamine on visualized intracellular quinoproteins was from the dopamine not being taken up into the cells. Glial cells express the cell membrane norepinephrine transporter (NET) and the extreneuronal transporter mediating the uptake-2 process (Takeda et al., 2002). Since dopamine is a better substrate for the NET than is norepinephrine (Eisenhofer, 2001), it is reasonable to presume that dopamine was taken up. In separate experiments we measured intracellular dopamine in cultured oligodendrocytes incubated with dopamine. We detected a large amount of dopamine and little if any DOPAL in the cells (unpublished observations), which we attribute to relatively little MAO activity in the cells.

Although DOPAL evokes substantial neurotoxicity (Panneton et al., 2010), interferes with key catecholaminergic functions such as tyrosine hydroxylation (Mexas et al., 2011) and decarboxylation of DOPA to form dopamine (Jinsmaa et al., 2018), and evokes quinonization of numerous intracellular proteins, so far there has been no demonstration that DOPAL-induced cytotoxicity is mediated by protein quinonization.

Implications and Conclusions

Protein modifications such as AS oligomerization and quinonization induced by DOPAL could result in functional abnormalities—e.g., decreased neurotransmitter synthesis, vesicular storage, and recycling (Goldstein et al., 2011a; Goldstein et al., 2013; Goldstein et al., 2017b; Goldstein et al., 2019) that increasingly challenge neuronal homeostasis during a long prodromal phase.

The present results may have implications for disease-modification therapeutic strategies in conditions involving catecholaminergic neurodegeneration. Inhibition by NAC of protein modifications induced by DOPAL-Q may help explain the finding that in PD patients NAC improves scores on the Uniform Parkinson Disease Rating Scale and results of striatal dopaminergic neuroimaging (Monti et al., 2016; Monti et al., 2019). A rational target for future experimental therapeutics may be the sequence of enzymatic oxidation of DA to form DOPAL via MAO and the spontaneous oxidation of DOPAL to form DOPAL-Q.

In conclusion, of the two routes by which DA oxidation modifies AS and other proteins, that via DOPAL is more prominent.

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Authorship Contributions

Participated in research design: Yunden Jinsmaa, David S. Goldstein, Yehonatan Sharabi

Conducted experiments: Yunden Jinsmaa, Risa Isonaka

Performed data analysis: Yunden Jinsmaa

Wrote or contributed to the writing of the manuscript: Yunden Jinsmaa, David S. Goldstein, Yehonatan Sharabi

REFERENCES

Anderson DG, Florang VR, Schamp JH, Buettner GR and Doorn JA (2016) Antioxidant-mediated modulation of protein reactivity for 3,4-dihydroxyphenylacetaldehyde, a toxic dopamine metabolite. *Chem Res Toxicol* **29**:1098-1107.

Anderson DG, Mariappan SV, Buettner GR and Doorn JA (2011) Oxidation of 3,4-dihydroxyphenylacetaldehyde, a toxic dopaminergic metabolite, to a semiquinone radical and an ortho-quinone. *J Biol Chem* **286**:26978-26986.

Asanuma M, Miyazaki I and Ogawa N (2003) Dopamine- or L-DOPA-induced neurotoxicity: the role of dopamine quinone formation and tyrosinase in a model of Parkinson's disease. *Neurotox Res* **5**:165-176.

Badillo-Ramirez I, Saniger JM and Rivas-Arancibia S (2019) 5-S-cysteinyl-dopamine, a neurotoxic endogenous metabolite of dopamine: Implications for Parkinson's disease. *Neurochem Int* **129**:104514.

Banerjee K, Munshi S, Sen O, Pramanik V, Roy Mukherjee T and Chakrabarti S (2014) Dopamine cytotoxicity involves both oxidative and nonoxidative pathways in SH-SY5Y cells: Potential role of alpha-synuclein overexpression and proteasomal inhibition in the etiopathogenesis of Parkinson's disease. *Parkinsons Dis* **2014**:878935.

Blaschko H (1952) Amine oxidase and amine metabolism. *Pharmacological reviews* **4**:415-458.

Burbulla LF, Song P, Mazzulli JR, Zampese E, Wong YC, Jeon S, Santos DP, Blanz J, Obermaier CD, Strojny C, Savas JN, Kiskinis E, Zhuang X, Kruger R, Surmeier DJ and Krainc D (2017) Dopamine oxidation mediates mitochondrial and lysosomal dysfunction in Parkinson's disease. *Science* **357**:1255-1261.

Burke WJ, Kumar VB, Pandey N, Panneton WM, Gan Q, Franko MW, O'Dell M, Li SW, Pan Y, Chung HD and Galvin JE (2008) Aggregation of alpha-synuclein by DOPAL, the monoamine oxidase metabolite of dopamine. *Acta Neuropathol* **115**:193-203.

Burke WJ, Li SW, Williams EA, Nonneman R and Zahm DS (2003) 3,4-Dihydroxyphenylacetaldehyde is the toxic dopamine metabolite in vivo: implications for

Parkinson's disease pathogenesis. *Brain Res* **989**:205-213.

Cagle BS, Crawford RA and Doorn JA (2019) Biogenic Aldehyde-Mediated Mechanisms of Toxicity in Neurodegenerative Disease. *Curr Opin Toxicol* **13**:16-21.

Carlsson A and Fornstedt B (1991) Possible mechanisms underlying the special vulnerability of dopaminergic neurons. *Acta Neurol Scand Suppl* **136**:16-18.

Deas E, Cremades N, Angelova PR, Ludtmann MH, Yao Z, Chen S, Horrocks MH, Banushi B, Little D, Devine MJ, Gissen P, Klenerman D, Dobson CM, Wood NW, Gandhi S and Abramov AY (2016) Alpha-Synuclein Oligomers Interact with Metal Ions to Induce Oxidative Stress and Neuronal Death in Parkinson's Disease. *Antioxid Redox Signal* **24**:376-391.

Dell'Acqua S, Bacchella C, Monzani E, Nicolis S, Di Natale G, Rizzarelli E and Casella L (2017) Prion Peptides Are Extremely Sensitive to Copper Induced Oxidative Stress. *Inorg Chem* **56**:11317-11325.

Eisenhofer G (2001) The role of neuronal and extraneuronal plasma membrane transporters in the inactivation of peripheral catecholamines. *Pharmacol Ther* **91**:35-62.

Follmer C, Coelho-Cerqueira E, Yatabe-Franco DY, Araujo GD, Pinheiro AS, Domont GB and Eliezer D (2015) Oligomerization and membrane-binding properties of covalent adducts formed by the interaction of alpha-synuclein with the toxic dopamine metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL). *J Biol Chem* **290**:27660-27679.

Goldstein DS, Holmes C, Kopin IJ and Sharabi Y (2011a) Intra-neuronal vesicular uptake of catecholamines is decreased in patients with Lewy body diseases. *J Clin Invest* **121**:3320-3330.

Goldstein DS, Jinsmaa Y, Sullivan P and Sharabi Y (2017a) N-Acetylcysteine prevents the increase in spontaneous oxidation of dopamine during monoamine oxidase inhibition in PC12 cells. *Neurochem Res* **42**:3289-3295.

Goldstein DS, Kopin IJ and Sharabi Y (2014) Catecholamine autotoxicity. Implications for pharmacology and therapeutics of Parkinson disease and related disorders. *Pharmacol Ther* **144**:268-282.

Goldstein DS, Pekker MJ, Eisenhofer G and Sharabi Y (2019) Computational modeling reveals

multiple abnormalities of myocardial noradrenergic function in Lewy body diseases. *JCI Insight* **5**.

Goldstein DS, Sullivan P, Holmes C, Kopin IJ, Basile MJ and Mash DC (2011b) Catechols in post-mortem brain of patients with Parkinson disease. *Eur J Neurol* **18**:703-710.

Goldstein DS, Sullivan P, Holmes C, Kopin IJ, Sharabi Y and Mash DC (2015) Decreased vesicular storage and aldehyde dehydrogenase activity in multiple system atrophy. *Parkinsonism Relat Disord* **21**:567-572.

Goldstein DS, Sullivan P, Holmes C, Mash DC, Kopin IJ and Sharabi Y (2017b) Determinants of denervation-independent depletion of putamen dopamine in Parkinson's disease and multiple system atrophy. *Parkinsonism Relat Disord* **35**:88-91.

Goldstein DS, Sullivan P, Holmes C, Miller GW, Alter S, Strong R, Mash DC, Kopin IJ and Sharabi Y (2013) Determinants of buildup of the toxic dopamine metabolite DOPAL in Parkinson's disease. *J Neurochem* **126**:591-603.

Gustafsson G, Lindstrom V, Rostami J, Nordstrom E, Lannfelt L, Bergstrom J, Ingelsson M and Erlandsson A (2017) Alpha-synuclein oligomer-selective antibodies reduce intracellular accumulation and mitochondrial impairment in alpha-synuclein exposed astrocytes. *J Neuroinflammation* **14**:241.

Hasegawa T, Matsuzaki-Kobayashi M, Takeda A, Sugeno N, Kikuchi A, Furukawa K, Perry G, Smith MA and Itoyama Y (2006) Alpha-synuclein facilitates the toxicity of oxidized catechol metabolites: implications for selective neurodegeneration in Parkinson's disease. *FEBS Lett* **580**:2147-2152.

Hastings TG (2009) The role of dopamine oxidation in mitochondrial dysfunction: implications for Parkinson's disease. *J Bioenerg Biomembr* **41**:469-472.

Herrera A, Munoz P, Steinbusch HWM and Segura-Aguilar J (2017) Are Dopamine Oxidation Metabolites Involved in the Loss of Dopaminergic Neurons in the Nigrostriatal System in Parkinson's Disease? *ACS Chem Neurosci* **8**:702-711.

Hornykiewicz O (1966) Dopamine (3-hydroxytyramine) and brain function. *Pharmacological*

reviews **18**:925-964.

Huenchuguala S, Sjodin B, Mannervik B and Segura-Aguilar J (2019) Novel Alpha-Synuclein Oligomers Formed with the Aminochrome-Glutathione Conjugate Are Not Neurotoxic. *Neurotox Res* **35**:432-440.

Jana S, Sinha M, Chanda D, Roy T, Banerjee K, Munshi S, Patro BS and Chakrabarti S (2011) Mitochondrial dysfunction mediated by quinone oxidation products of dopamine: Implications in dopamine cytotoxicity and pathogenesis of Parkinson's disease. *Biochim Biophys Acta* **1812**:663-673.

Jinsmaa Y, Sharabi Y, Sullivan P, Isonaka R and Goldstein DS (2018) 3,4-Dihydroxyphenylacetaldehyde-induced protein modifications and their mitigation by N-acetylcysteine. *J Pharmacol Exp Ther* **366**:113-124.

Jinsmaa Y, Sullivan P, Gross D, Cooney A, Sharabi Y and Goldstein DS (2014) Divalent metal ions enhance DOPAL-induced oligomerization of alpha-synuclein. *Neurosci Lett* **569**:27-32.

Lee H, Dellatore SM, Miller WM and Messersmith PB (2007) Mussel-inspired surface chemistry for multifunctional coatings. *Science* **318**:426-430.

Lee HJ, Baek SM, Ho DH, Suk JE, Cho ED and Lee SJ (2011) Dopamine promotes formation and secretion of non-fibrillar alpha-synuclein oligomers. *Exp Mol Med* **43**:216-222.

Leong SL, Cappai R, Barnham KJ and Pham CL (2009) Modulation of alpha-synuclein aggregation by dopamine: a review. *Neurochem Res* **34**:1838-1846.

Linsenbardt AJ, Breckenridge JM, Wilken GH and Macarthur H (2012) Dopaminochrome induces caspase-independent apoptosis in the mesencephalic cell line, MN9D. *J Neurochem* **122**:175-184.

Linsenbardt AJ, Wilken GH, Westfall TC and Macarthur H (2009) Cytotoxicity of dopaminochrome in the mesencephalic cell line, MN9D, is dependent upon oxidative stress. *Neurotoxicology* **30**:1030-1035.

Mattammal MB, Haring JH, Chung HD, Raghu G and Strong R (1995) An endogenous dopaminergic neurotoxin: implication for Parkinson's disease. *Neurodegeneration* **4**:271-281.

Mazzulli JR, Armakola M, Dumoulin M, Parastatidis I and Ischiropoulos H (2007) Cellular oligomerization of alpha-synuclein is determined by the interaction of oxidized catechols with a C-terminal sequence. *J Biol Chem* **282**:31621-31630.

Mazzulli JR, Mishizen AJ, Giasson BI, Lynch DR, Thomas SA, Nakashima A, Nagatsu T, Ota A and Ischiropoulos H (2006) Cytosolic catechols inhibit alpha-synuclein aggregation and facilitate the formation of intracellular soluble oligomeric intermediates. *J Neurosci* **26**:10068-10078.

Montes S, Rivera-Mancia S, Diaz-Ruiz A, Tristan-Lopez L and Rios C (2014) Copper and copper proteins in Parkinson's disease. *Oxid Med Cell Longev* **2014**:147251.

Monti DA, Zabrecky G, Kremens D, Liang TW, Wintering NA, Bazzan AJ, Zhong L, Bowens BK, Chervoneva I, Intenzo C and Newberg AB (2019) N-Acetyl Cysteine Is Associated With Dopaminergic Improvement in Parkinson's Disease. *Clin Pharmacol Ther*.

Monti DA, Zabrecky G, Kremens D, Liang TW, Wintering NA, Cai J, Wei X, Bazzan AJ, Zhong L, Bowen B, Intenzo CM, Iacovitti L and Newberg AB (2016) N-Acetyl cysteine may support dopamine neurons in Parkinson's disease: Preliminary clinical and cell line data. *PLoS One* **11**:e0157602.

Montine TJ, Picklo MJ, Amarnath V, Whetsell WO, Jr. and Graham DG (1997) Neurotoxicity of endogenous cysteinylcatechols. *Exp Neurol* **148**:26-33.

Mor DE, Daniels MJ and Ischiropoulos H (2019) The usual suspects, dopamine and alpha-synuclein, conspire to cause neurodegeneration. *Mov Disord* **34**:167-179.

Mor DE, Tsika E, Mazzulli JR, Gould NS, Kim H, Daniels MJ, Doshi S, Gupta P, Grossman JL, Tan VX, Kalb RG, Caldwell KA, Caldwell GA, Wolfe JH and Ischiropoulos H (2017) Dopamine induces soluble alpha-synuclein oligomers and nigrostriatal degeneration. *Nat Neurosci* **20**:1560-1568.

Munoz P, Cardenas S, Huenchuguala S, Briceno A, Couve E, Paris I and Segura-Aguilar J (2015) DT-Diaphorase Prevents Aminochrome-Induced Alpha-Synuclein Oligomer Formation and Neurotoxicity. *Toxicol Sci* **145**:37-47.

Nagatsu T (1997) Isoquinoline neurotoxins in the brain and Parkinson's disease. *Neurosci Res* **29**:99-111.

Panneton WM, Kumar VB, Gan Q, Burke WJ and Galvin JE (2010) The neurotoxicity of DOPAL: behavioral and stereological evidence for its role in Parkinson disease pathogenesis. *PLoS One* **5**:e15251.

Paris I, Lozano J, Perez-Pastene C, Munoz P and Segura-Aguilar J (2009) Molecular and neurochemical mechanisms in PD pathogenesis. *Neurotox Res* **16**:271-279.

Pham CL, Leong SL, Ali FE, Kenche VB, Hill AF, Gras SL, Barnham KJ and Cappai R (2009) Dopamine and the dopamine oxidation product 5,6-dihydroxylindole promote distinct on-pathway and off-pathway aggregation of alpha-synuclein in a pH-dependent manner. *J Mol Biol* **387**:771-785.

Plotegher N, Berti G, Ferrari E, Tessari I, Zanetti M, Lunelli L, Greggio E, Bisaglia M, Veronesi M, Girotto S, Dalla Serra M, Perego C, Casella L and Bubacco L (2017) DOPAL derived alpha-synuclein oligomers impair synaptic vesicles physiological function. *Sci Rep* **7**:40699.

Pukass K and Richter-Landsberg C (2014) Oxidative stress promotes uptake, accumulation, and oligomerization of extracellular alpha-synuclein in oligodendrocytes. *J Mol Neurosci* **52**:339-352.

Rees JN, Florang VR, Eckert LL and Doorn JA (2009) Protein reactivity of 3,4-dihydroxyphenylacetaldehyde, a toxic dopamine metabolite, is dependent on both the aldehyde and the catechol. *Chem Res Toxicol* **22**:1256-1263.

Saha S, Khan MAI, Mudhara D and Deep S (2018) Tuning the Balance between Fibrillation and Oligomerization of alpha-Synuclein in the Presence of Dopamine. *ACS Omega* **3**:14213-14224.

Segura-Aguilar J (2017) On the role of endogenous neurotoxins and neuroprotection in Parkinson's disease. *Neural Regen Res* **12**:897-901.

Segura-Aguilar J (2019) On the role of aminochrome in mitochondrial dysfunction and endoplasmic reticulum stress in Parkinson's disease. *Front Neurosci* **13**:271.

Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R and Goedert M (1997) Alpha-

synuclein in Lewy bodies. *Nature* **388**:839-840.

Storch A, Ott S, Hwang YI, Ortmann R, Hein A, Frenzel S, Matsubara K, Ohta S, Wolf HU and Schwarz J (2002) Selective dopaminergic neurotoxicity of isoquinoline derivatives related to Parkinson's disease: studies using heterologous expression systems of the dopamine transporter. *Biochem Pharmacol* **63**:909-920.

Surmeier DJ, Guzman JN, Sanchez-Padilla J and Goldberg JA (2011) The origins of oxidant stress in Parkinson's disease and therapeutic strategies. *Antioxid Redox Signal* **14**:1289-1301.

Takeda H, Inazu M and Matsumiya T (2002) Astroglial dopamine transport is mediated by norepinephrine transporter. *Naunyn Schmiedebergs Arch Pharmacol* **366**:620-623.

Tong J, Fitzmaurice PS, Ang LC, Furukawa Y, Guttman M and Kish SJ (2004) Brain dopamine-stimulated adenylyl cyclase activity in Parkinson's disease, multiple system atrophy, and progressive supranuclear palsy. *Ann Neurol* **55**:125-129.

Tu PH, Galvin JE, Baba M, Giasson B, Tomita T, Leight S, Nakajo S, Iwatsubo T, Trojanowski JQ and Lee VM (1998) Glial cytoplasmic inclusions in white matter oligodendrocytes of multiple system atrophy brains contain insoluble alpha-synuclein. *Ann Neurol* **44**:415-422.

Wakabayashi K, Yoshimoto M, Tsuji S and Takahashi H (1998) Alpha-synuclein immunoreactivity in glial cytoplasmic inclusions in multiple system atrophy. *Neurosci Lett* **249**:180-182.

Werner-Allen JW, DuMond JF, Levine RL and Bax A (2016) Toxic dopamine metabolite DOPAL forms an unexpected dicatechol pyrrole adduct with lysines of alpha-synuclein. *Angew Chem Int Ed Engl* **55**:7374-7378.

Werner-Allen JW, Levine RL and Bax A (2017) Superoxide is the critical driver of DOPAL autoxidation, lysyl adduct formation, and crosslinking of alpha-synuclein. *Biochem Biophys Res Commun* **487**:281-286.

Werner-Allen JW, Monti S, DuMond JF, Levine RL and Bax A (2018) Isoindole linkages provide a pathway for DOPAL-mediated cross-linking of alpha-synuclein. *Biochemistry* **57**:1462-1474.

Winner B, Jappelli R, Maji SK, Desplats PA, Boyer L, Aigner S, Hetzer C, Loher T, Vilar M, Campioni S, Tzitzilonis C, Soragni A, Jessberger S, Mira H, Consiglio A, Pham E, Masliah E,

Gage FH and Riek R (2011) In vivo demonstration that alpha-synuclein oligomers are toxic. *Proc Natl Acad Sci USA* **108**:4194-4199.

FOOTNOTE

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FIGURE LEGENDS

Fig. 1: Time courses of DOPAL and dopamine (DA) effects on alpha-synuclein (AS) quinonization and oligomerization. (A) AS (3 μM) was incubated with DOPAL or DA (30 μM each) at 37 °C and samples were taken at for up to 120 minutes (A) and 300 minutes (B) of incubation. (A and B) Quinonized AS was detected by near infrared fluorescence (nIRF) spectroscopy (red). (A and C) Oligomerized AS was detected by Western blotting (green). (D) Protein staining was used to demonstrate decreased AS monomer during incubation of AS with DOPAL. Lanes=order of the gel lanes, Groups: 1=AS alone as a control (CON), 2=DOPAL, 3=DA. DOPAL time-dependently increased AS quinonization and oligomerization, whereas DA did not elicit AS quinonization and produced a slight “smear” of high MW AS.

Fig. 2: Effects of enzymatic oxidation of DA and DOPAL with tyrosinase on AS quinonization and oligomerization. DA or DOPAL (30 μM each) was incubated with or without tyrosinase ("+Tyr" or "no Tyr") for 20 minutes at room temperature and then incubated with AS (3 μM) for 1 hour at 37 °C. (A and B) Concentration course of DA or DOPAL (10, 30 and 100 μM each) oxidation with Tyr. (A and C) Quinonized AS was detected by near infrared fluorescence (nIRF) spectroscopy (red). (B and D) Oligomerized AS was detected by Western blotting (green). N=number of replicates, 1=DA, 2=DOPAL. Enzymatic oxidization augmented DOPAL-induced oligomerization and quinonization of AS. Incubation of AS with DA and Tyr resulted in a “smear” of high MW AS. DA did not quinonize AS even in the setting of enzymatic oxidation by Tyr.

Fig. 3: Effects of Cu(II) on DOPAL-induced AS quinonization and N-acetylcysteine (NAC) effect. (A and B) AS (3 μM) was incubated with 30 μM DOPAL and 1-100 μM Cu(II) for 1 hour at 37 °C. (C and D) AS was incubated with DOPAL and 30 μM Cu(II) and 0-1000 μM NAC for 1 hour at 37 °C. Quinonized AS was detected by near infrared fluorescence (nIRF) spectroscopy (red). N=number of replicates. Fx=fractions of integrated intensities of AS monomers compared to DOPAL alone. Statistical analyses were done by one-way ANOVA

with Dunnett's post-hoc test. Mean values are expressed ± SEM. ****P<0.0001, ***P<0.001 compared to DOPAL alone; †††p<0.001 vs. DOPAL+Cu(II) compared to no NAC. Cu(II) concentration-dependently augmented DOPAL-induced AS quinonization and oligomerization. NAC attenuated this effect.

Fig. 4: Comparisons of DA- vs. DOPAL-induced AS modifications in the presence of Cu(II). (A) Incubation of AS (3 μM) with 30 μM each of DA and DOPAL and 1 μM Cu(II). (B) Incubation of AS with DA or DOPAL and 30 μM Cu(II). (A,B and C) Quinonized AS was detected by near infrared fluorescence (nIRF) spectroscopy (red). (A and B) Oligomerized AS was detected by Western blotting (green). △Integrated intensity= the difference in integrated intensity of signal at each time point minus the integrated intensity at 0 minutes. Lanes=order of the gel lanes. Cu(II) at 30 μM accelerated and enhanced DOPAL-induced oligomerization and quinonization of AS. Incubation of Cu(II) (30 μM) with 30 μM DA and AS resulted in a "smear" of high MW AS.

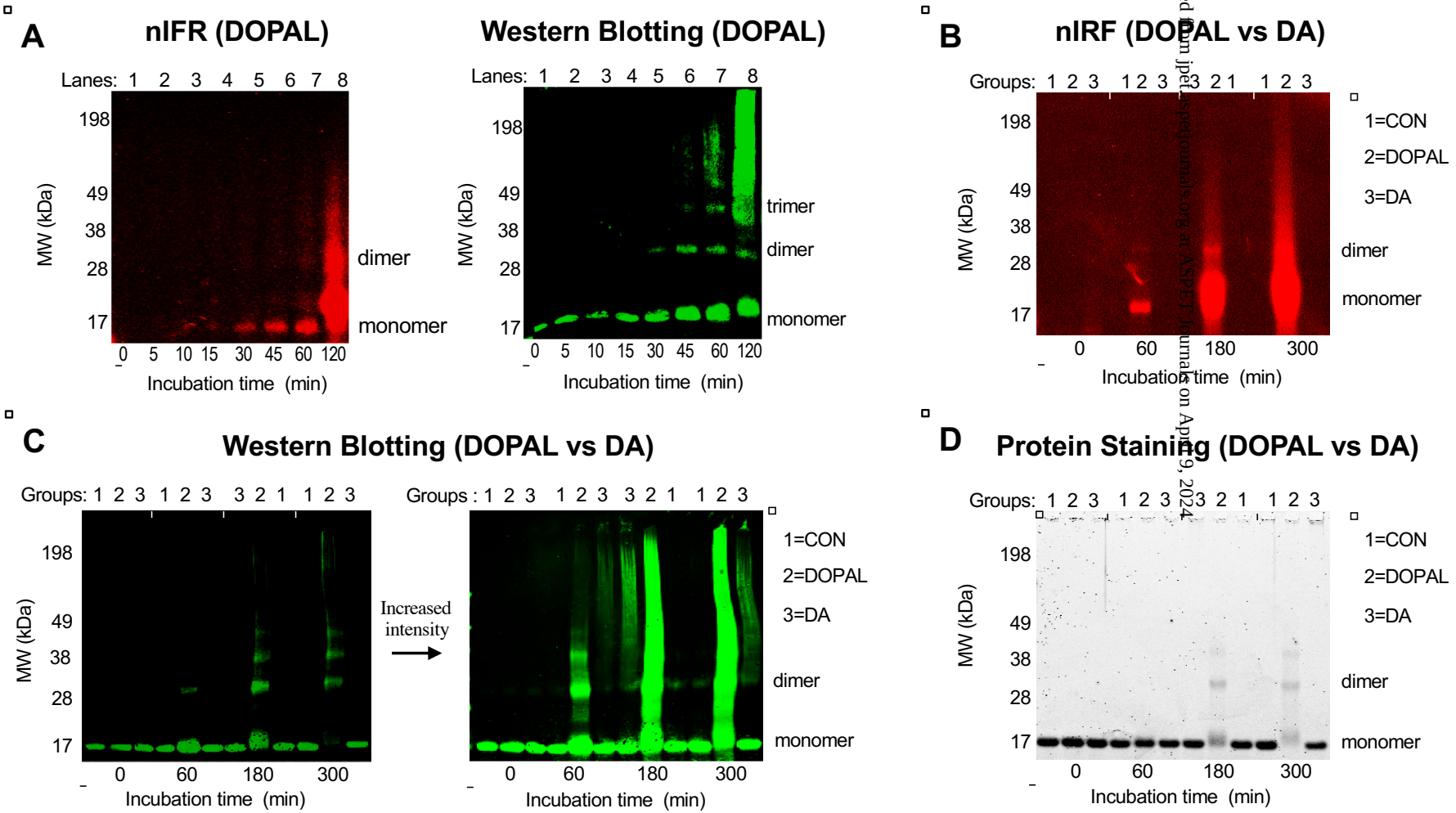
Fig. 5: DOPAL-induced quinonization of mutant A53T vs. wild type AS. Wild type (WT) or A53T mutant AS (3 μM) was incubated with 30 μM each of DOPAL and Cu(II) (without or with) or 300 μM NAC (without or with) for 1 hour at 37 °C. Quinonized AS was detected by near infrared fluorescence (nIRF) spectroscopy. Fx=fractions of integrated intensities of AS monomers compared to DOPAL alone. N=numbers of replicates. Statistical analyses were done by one-way ANOVA with Dunnett's post-hoc test. Mean values are expressed ± SEM. ***p<0.001, **p<0.01 compared to DOPAL alone; †††p<0.001, ††p<0.05 compared to no NAC; ++ p<0.01 for A53T compared to WT. DOPAL quinonized both A53T and WT AS, with about twice as large an effect on A53T AS. The enhancing effects were attenuated by NAC.

Fig. 6: DOPAL-induced quinonization of intracellular proteins in MO3.13 cells and NAC effect. MO3.13 cells ($1.5x10^5$ cells/well) were exposed to (A) DOPAL (100 μM) with or without Cu(II) (10 and 30 μM) for 24 hours and then lysed in RIPA buffer with protease inhibitors. (B) MO3.13 cells were exposed to DOPAL and 30 μM Cu(II), with NAC (0-300

μM) added at the start of incubation. DOPAL-quinonized proteins were detected and quantified by infrared fluorescence (nIRF) spectroscopy (red). Fx=fractions of integrated intensities of each column compared to CON (B) or DOPAL alone (D) groups normalized to the protein of each lanes. N=number of replicates. Statistical analyses were by one-way ANOVA with Dunnett's post-hoc test. Mean values are expressed ± SEM. **$p<0.001$ compared to DOPAL alone; $^{\dagger\dagger\dagger}p<0.001$, $^{\dagger\dagger}p<0.01$ compared to no NAC. Cu(II) augmented DOPAL-induced quinonization of intracellular proteins, and NAC attenuated these effects.

Fig. 7: Visualization of intracellular DOPAL-induced quinoproteins. MO3.13 cells were cultured in slide chambers ($8x10^4$ cells/slides) for 24 hours and treated with Cu (II) (30 μM) and 0-100 μM DOPAL for 5 hours. Cells were then stained with DAPI (1:2000) (blue) and human tubulin antibody (1:1500) (green). Immunofluorescence and near infrared fluorescence (nIRF) were visualized microscopically. Scale bar in images is 20 μm. Treatment with DOPAL produced nIRF signals, suggesting the presence of quinoproteins.

Fig. 1



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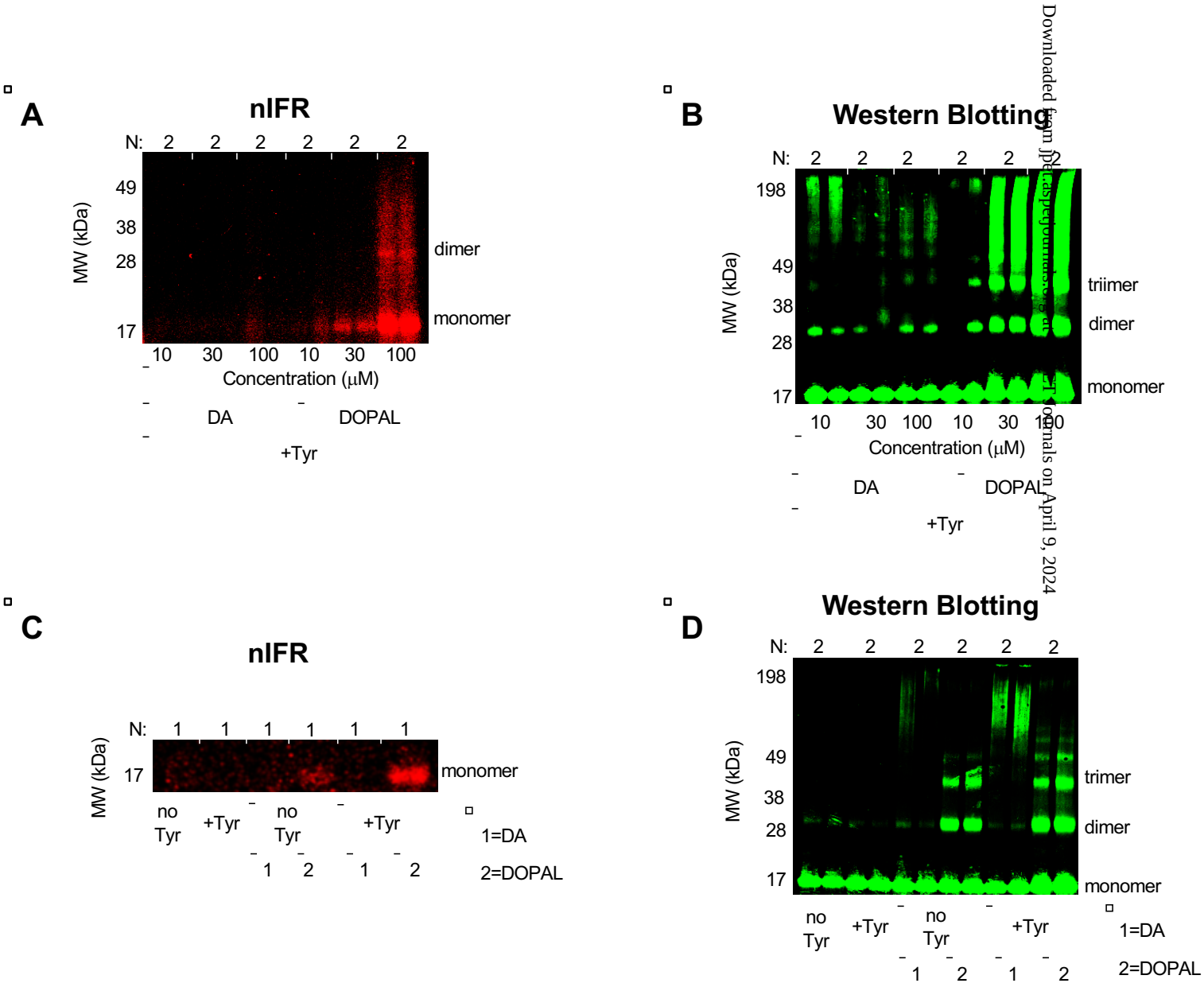
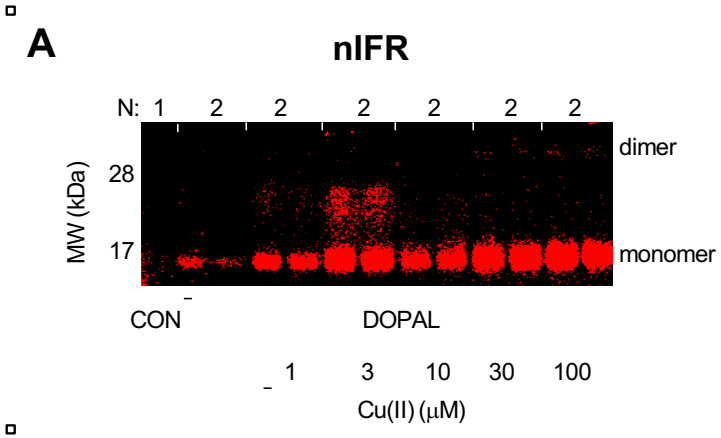
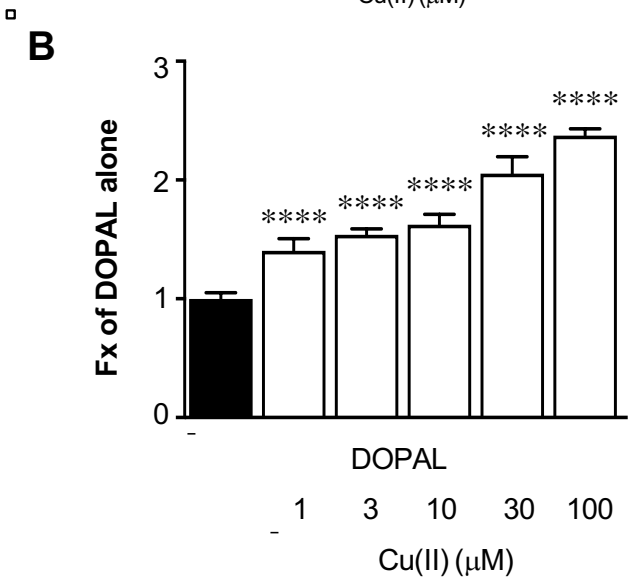
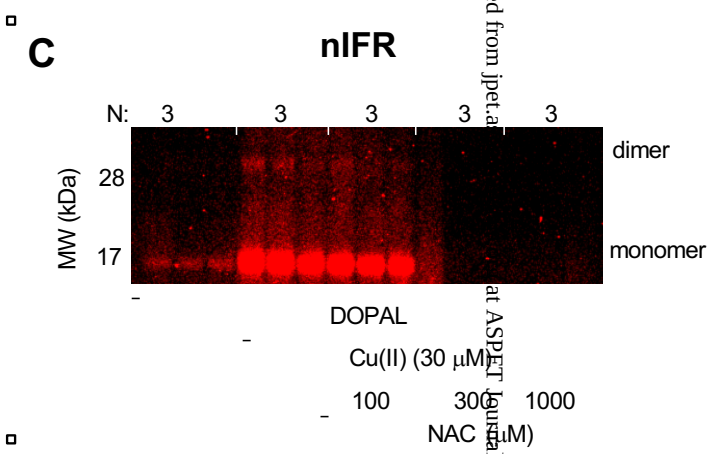
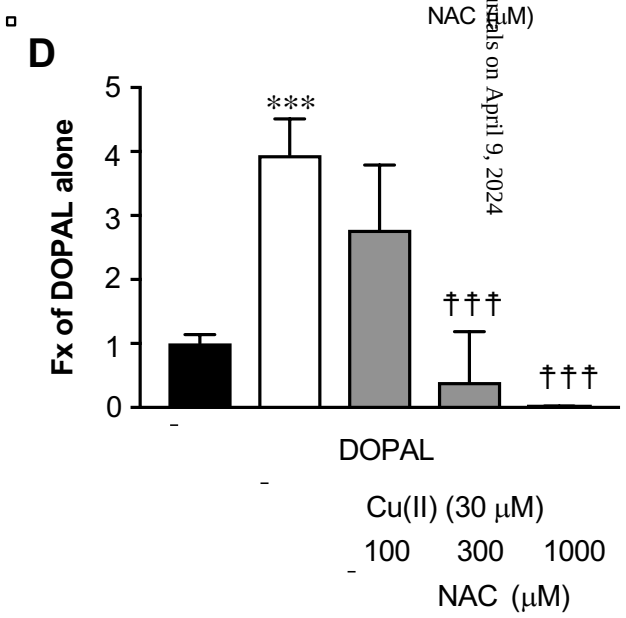


Fig. 3









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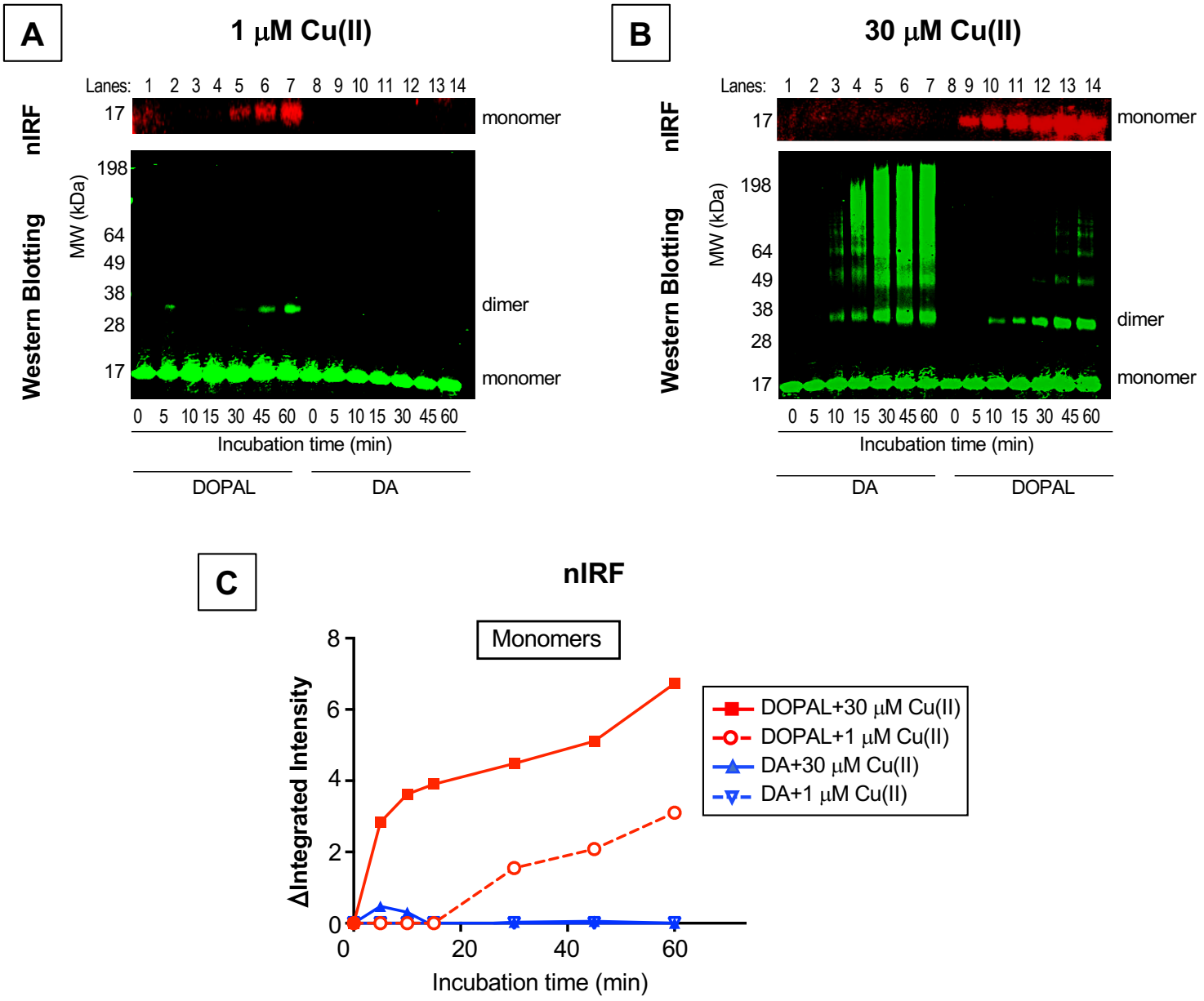
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DA+30 μM Cu(II)
DA+1 μM Cu(II)

Fig. 5

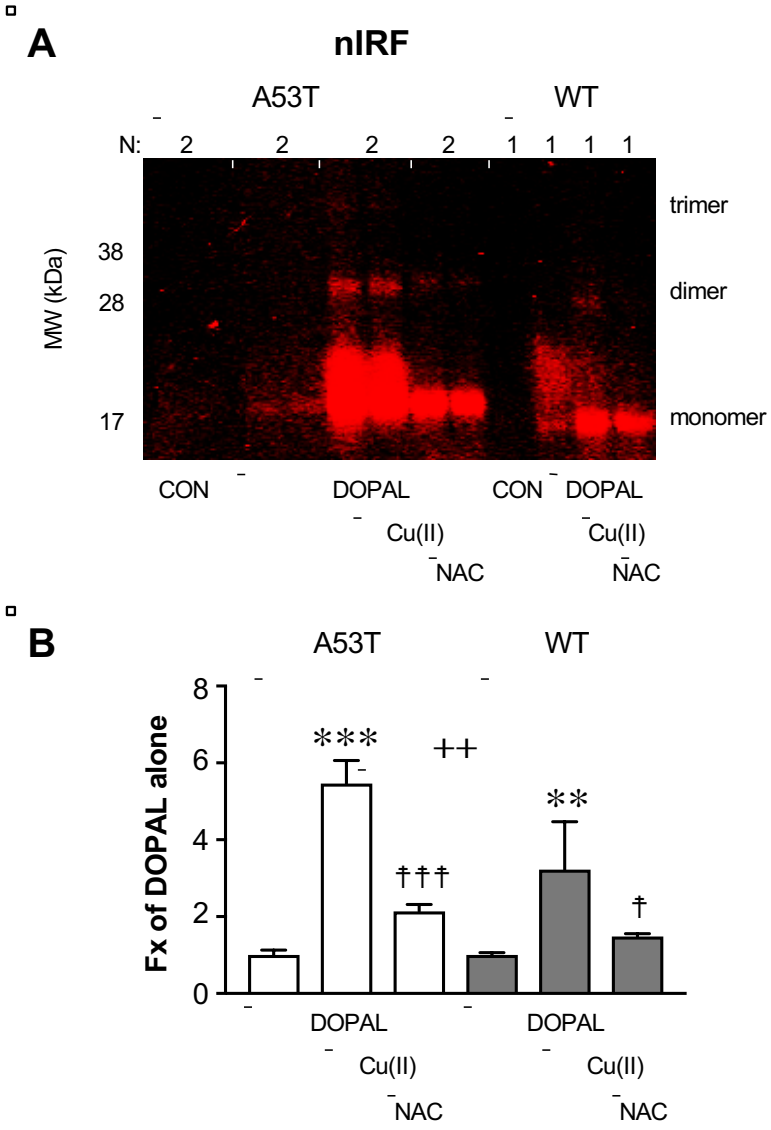
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Fig. 6

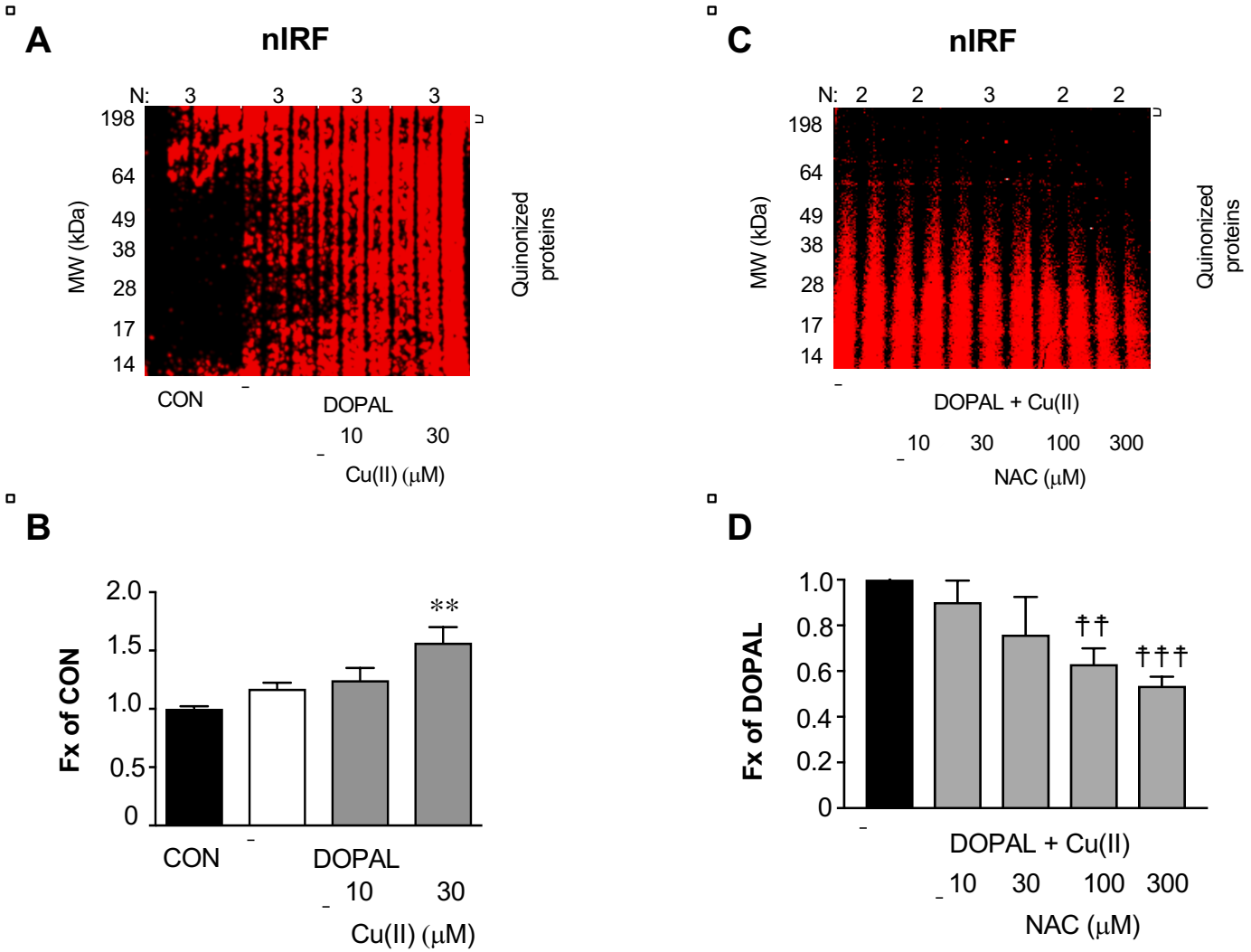
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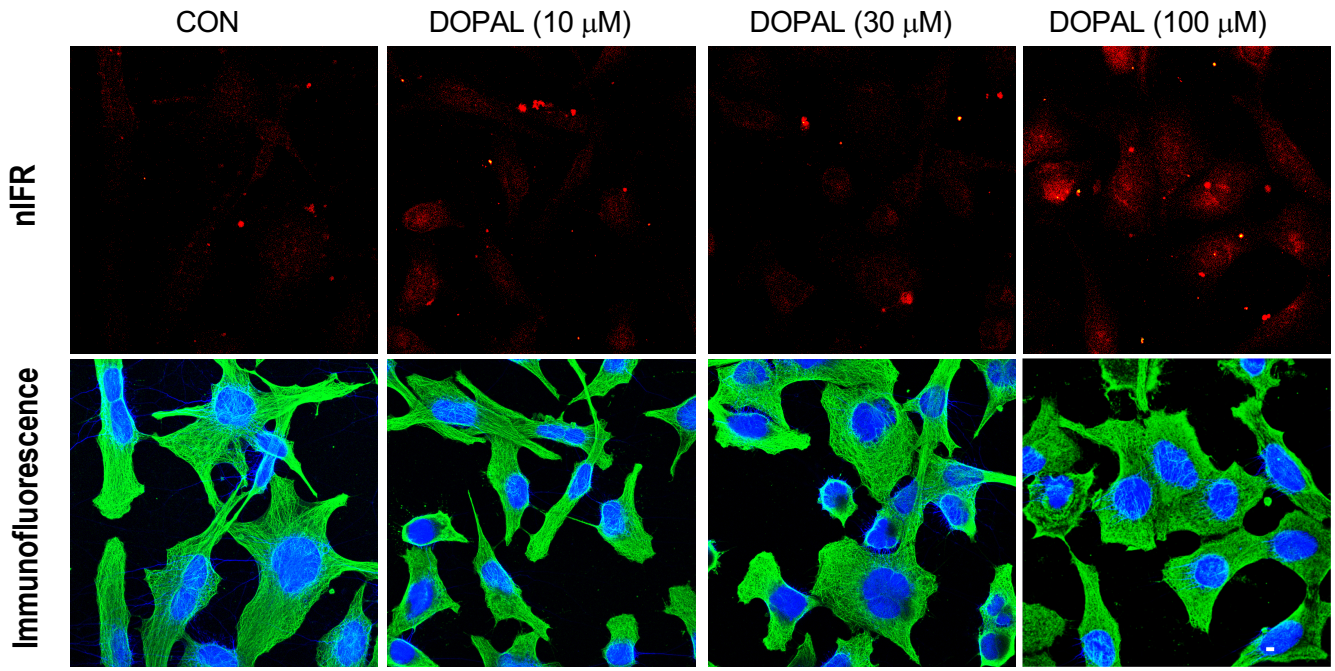
Fig. 7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