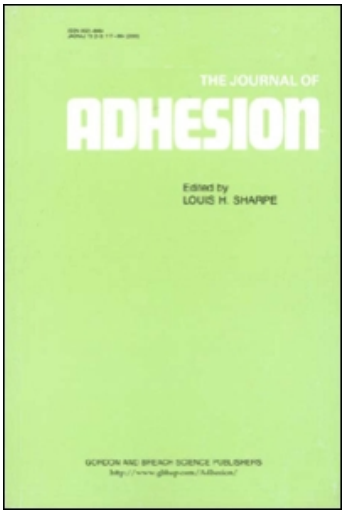

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Evidence that DOPA-Derivatives are Generated After L-DOPA Incorporation into Proteins by Mammalian Cells

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The adhesive and cohesive properties of the amino acid L-3,4-dihydroxyphenylalanine (DOPA) have been widely explored as a potential material for adhesion, based, among other things, on the biological system of blue mussel extracellular byssal threads and foot proteins. Proteins containing DOPA are generated within mammalian cells by oxidation of tyrosine residues during periods of oxidative stress. By generating proteins containing DOPA, in vitro*, through the (mis)incorporation of DOPA during protein synthesis, we are able examine the role and fate of DOPA-containing proteins in mammalian cells. We demonstrate a decrease in catabolism of long half-life cell proteins and an increase in cellular autofluorescence when DOPA is present in cell proteins. We provide evidence for the formation of DOPA derivatives which can be detected in proteins after ^{14}C-DOPA incorporation by HPLC analysis. Additionally, we demonstrate that the cells upregulate the expression of genes required to handle damaged proteins and protein aggregates under these conditions. Substantial evidence for DOPA derivatives and cross-linking has previously been shown in extracellular blue mussel byssal threads; here we provide evidence for cell-associated DOPA derivatives in mammalian cells.*

Keywords: DOPA; DOPA derivatives; Protease-resistance; Protein cross-links

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1. INTRODUCTION

Extensive study has been directed towards the development of versatile adhesives that are water resistant and yet biocompatible. Towards this goal much attention has been given to biological systems such as Californian blue mussels (*Mytilus edulis*) that are able to adhere with their byssal thread foot proteins to both organic and inorganic surfaces with remarkable strength and water resistance [1–3]. The versatility and strength of this bond is due almost exclusively to a major amino acid constituent of mussel foot proteins (Mfp) L-3,4-dihydroxyphenylalanine (DOPA), a hydroxylated derivative of tyrosine. Mfps are known to contain up to 30 mol% DOPA, created by posttranslational modifications to existing tyrosine residues [4]. The adhesive properties of DOPA to inorganic surfaces are believed to be due to the unique ability of DOPA to displace water and either directly interact through hydrogen bonds and van der Waal's forces or indirectly adhere through the chelation of metals, particularly iron [3,5]. Additionally, DOPA has the ability to adhere to organic surfaces through covalent bonds made *via* a quinone intermediate [3]. In addition to the adhesive properties of the foot proteins, the strength of the byssal thread is greatly increased due to its high content of DOPA. Firstly, a corresponding gradient of DOPA and chelated iron increase the cohesive strength, while other DOPA residues form cross-links with neighboring amino acids such as cysteine, histidine, and lysine to further increase the internal cohesive strength of the fibrous byssal thread. Beyond the Mfps, a diverse range of tissues utilize the unique chemical, mechanical, and optical properties of DOPA. In the protease-resistant squid beak the gradient of beak stiffness corresponds to the chemical gradient of DOPA in conjunction with the number of DOPA-histidine cross-links [6,7].

In contrast to marine invertebrates, which synthesize proteins containing DOPA and utilize their unique properties particularly in the extracellular space [8], mammals do not synthesize proteins containing DOPA. Free DOPA is synthesized *in vivo* only by specialized mammalian cells, such as dopaminergic neurones, where it is a precursor of the neurotransmitter dopamine, and in melanocytes, where it is incorporated into the (non-protein) pigment melanin [9]. Proteins containing DOPA, however, are formed in an unregulated manner in mammalian tissues during periods of oxidative stress [10] and, consequently, have been detected in cells and tissues from a range of pathologies associated with aging or inflammation such as atherosclerosis [11], cataractogenesis [12], and during the aging

process itself [13,14]. Tyrosine residues in proteins [15] and peptides [16] are one of the major targets of hydroxyl radicals and, thus, appreciable amounts of proteins containing DOPA can be generated *in vivo*. While mammalian cells contain significant levels of antioxidants and reductants to protect the cell against hydroxyl radicals, proteins may still be oxidized in the extracellular matrix and then internalized for catabolism, intercellularly in specific compartments of the cell such as the lysosome or mitochodria, or during times of oxidative stress.

Waite and colleagues demonstrated that enzymatic oxidation of tyrosine residues in isolated neuropeptides and model decapeptides resulted in peptide crosslinking with dimer and trimer formation. While the exact nature of these protein crosslinks was not fully characterized based on preliminary mass spectrometry and UV-Vis absorption data, the most likely structure is a diDOPA crosslink, consistent with previously published results [17]. To what extent any of these potential crosslinking species are generated in mammalian cells is not known. Furthermore, DOPA's ability to crosslink with neighboring amino acids seems to be highly dependent on the abundance of available nucleophiles such as thiolates and amines. As extensively studied by Waite and colleagues, DOPA has been shown similarly to crosslink with histidine in Squid Beaks [6], cysteine in Mfp's [18], and other DOPA residues in byssus [19].

In contrast to the studies of Waite and colleagues in which extracellular DOPA-containing proteins are secreted into an oxidizing environment where crosslinking and aggregation is desirable for the adhesive, chemical, and biochemical stability of the peptides, protein-bound DOPA present in mammalian tissues is primarily intracellular and, thus, present in a reducing environment, where crosslinking is not "desired" or expected, and can create problems for the cell. We have shown that proteins containing DOPA can be generated *in vitro* from the (mis)incorporation of L-DOPA. L-DOPA differs from the protein amino acid tyrosine only in that it contains an additional hydroxyl group, and it can be activated by tyrosyl-tRNA synthetase [20] and so incorporated into cellular proteins by mammalian cells *in vitro* [21,22]. This provides us with a novel approach to generate DOPA-containing proteins selectively (in the virtual absence of other oxidative modifications) in mammalian cells and to study their metabolism. Unlike many other oxidative modifications to protein side chains which generate relatively inert chemical species, DOPA is capable of initiating secondary reactions and transferring damage to other biomolecules [23], as well as replenishing levels of reduced metals (for review see [10]). The primary mechanism for the removal of oxidized proteins in biological systems is complete enzymatic

hydrolysis to the constituent amino acids [10,24], but our previous studies have shown that DOPA-containing proteins can accumulate in cells [25].

Since the ability of DOPA to crosslink proteins has been previously shown by Waite, Messersmith, and others, it is possible that DOPA generated during periods of oxidative stress in mammalian cells could also be involved in similar crosslinking reactions which could stabilize protein aggregates in the cytosol of the cell. In this paper, we aim to understand the fate of DOPA-containing proteins in mammalian cells and to investigate the possibility that DOPA derivatives are formed which could prevent their efficient catabolism. In these studies, we provide evidence that DOPA-derivatives are formed in mammalian cells under conditions in which cells exhibit autofluorescence and protease-resistant, DOPA-containing, proteins are generated. We also examine changes in gene expression in the cells under these conditions and demonstrate that a range of proteins involved in handling modified proteins are expressed at higher levels in cells in which DOPA-containing proteins are present.

2. MATERIALS AND METHODS

2.1. Materials

All radiochemicals were obtained from Amersham Life Science (Buckinghamshire, England). Culture medium was from SAFC biosciences (Lenexa, KS, USA). Water was from a Milli Q 4-stage system (Millipore-Waters, Lane Cove, NSW, Australia). Other chemicals, solvents, and chromatographic materials were AR- or HPLC-grade.

2.2. Cell Culture

J774 cells (a mouse macrophage cell line from American Tissue and Cell Culture (ATCC), Manassas, VA, USA) were maintained in 750 cm^2 flasks in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% FCS.

2.3. L-DOPA Incorporation Studies

For studies involving the incorporation of L-DOPA, culture medium was replaced with modified tyrosine and leucine-free Eagle's Minimal Essential Media (EMEM) (SAFC Biosciences, Lenexa, Kansas, USA) containing 10% FCS containing either L-DOPA or ^{14}C-L-DOPA. After 2 h (short half-life) or 24 h (long half-life) the medium was removed,

cells washed three times with PBS, and recovered by centrifugation. DOPA incorporation into protein was measured by liquid scintillation counting or HPLC analysis of the hydrolyzed proteins.

2.4. Degradation Studies Using DOPA and ^{14}C-leucine

Cell proteins were labeled with ^{14}C-leucine and DOPA by incubating J774 cells for 24 hours in tyrosine and leucine-free EMEM containing ^{14}C-leucine (0.3 μM) and DOPA (0, 30, 100, 300, 600, and 900 μM). The labeling medium was then replaced with degradation medium (DMEM containing 10% FCS and 5 mM tyrosine and 5 mM leucine). To monitor predominantly short half-life proteins, a 2-hour incubation was used and to monitor long-half proteins a 8-hour labeling period was used. Cells were then washed in PBS, pelleted by centrifugation, lysed with 0.1% Triton® X-100, and the radioactivity in the culture medium and cell lysates measured by liquid scintillation counting. Trichloroacetic acid (TCA, Sigma, Sydney, Australia) (50%) was added to give a final concentration of 10% and free amino acid (TCA soluble) and protein incorporated (TCA precipitable) radiolabel in cell lysates and culture medium quantified by liquid scintillation counting [21]. Cell pellets were washed three times with 5% TCA and the pellet dissolved in formic acid.

2.5. Protein Hydrolysis and HPLC Analysis

Cells proteins in lysates were precipitated with TCA (10%), washed, and delipidated by re-suspending the pellet twice in TCA (10%) containing sodium deoxycholate (0.02%) and sodium borohydride (25 μg/mL) and then washed twice in ice cold acetone and once in diethyl ether. The delipidated protein samples were then freeze-dried and hydrolyzed under anaerobic conditions using a standard gas-phase acid catalyzed method (HCl containing mercaptoacetic acid) [15]. HPLC analysis of DOPA was performed on a LC-10A HPLC system (Shimadzu, Rydalmere, New South Wales, Australia) equipped with a column oven (Waters, 30°C) with methods developed in our laboratory as described in detail previously [12]. System operation was automated by Class LC-10 software. Chromatography was on a Zorbax ODS column (Agilent, Forest Hill, Australia) (250 × 4.6 mm) with a Pelliguard guard column (LC-18) (Sydney, Australia). The mobile phase was a gradient of solvent A [10 mM sodium phosphate buffer (pH 2.5) with 200 mM sodium perchlorate] and solvent B (80% v/v methanol) at a flow rate of 1 mL/min. The following gradient was used: isocratic elution with 100% A for 12 min, then to 80% A over

8 min; elution at 80% A for 3 min before changing to 50% A in 3 min; isocratic elution at 50% A for a further 3 min then re-equilibration with 100% A for 10 min. The eluate was monitored by a UV detector (Shimadzu) and a fluorescence detector (Hitachi F-1080) in series. The fluorescence detector was set at an excitation wavelength of 280 nm and an emission wavelength of 320 nm. The unmodified tyrosine was quantified by UV measurement when there was an off-scale response by fluorescence detection. The elution positions of the amino acids and oxidized amino acids were defined on the basis of standards using both UV and fluorescent spectra.

2.6. Detection of ^{14}C-DOPA and Derivatives by HPLC

The HPLC gradient was modified slightly for radiometric detection. Briefly, a gradient from 0 to 1% B was held over 25 min; an isocratic flow of 50% B for 2 min; isocratic elution of 100% B for 3 min, then re-equilibration with 100% A for 10 min. A In/US Systems Radio HPLC Detector model 4 (v4.0x) (Tampa, FL, USA) was used with a 500 μL cell volume. The HPLC was run with a 1 mL/min flow rate while the β-ram detector was run with a 3 mL/min UltimaGold (Perkin Elmer, Waltham, MA, USA) scint flow resulting in a 7.5 sec residence time.

2.7. RNA Array Studies

To identify genes regulated by DOPA-containing proteins, cDNA was hybridized to a BD Atlas™ Mouse Cancer nylon array containing 1,176 genes (BD Clontech, Palo Alto, CA, USA, catalogue #634547). Total RNA was isolated from both control and DOPA-treated cultures using the Atlas Pure Total RNA Labeling System according to the manufacturer's instructions (BD Clontech, San Jose, CA, USA). RNA purity was determined by measuring the ratio of protein (280 nm) to RNA (260 nm) in a UV/VIS spectrometer (Lambda 40, Perkin Elmer, Wellesley, MA, USA) and samples with a ratio of <1.7 were excluded. RNA concentration was determined using the A260 obtained in purity measurements and calculated according to the manufacturer's instructions. RNA quality was determined by running 1 μg RNA on a 1% agarose gel and visualizing with ethidium bromide. cDNA probe synthesis was undertaken according to the manufacturer's instructions (Atlas cDNA Expression Arrays User Manual, Protocol #PT3140-1, version #PROX591) using PowerScript™ Reverse Transcriptase (BD Biosciences, North Ryde, Australia) as the DNA polymerase and α-[^{32}-P]–dATP (3000 Ci/mmol 10 μCi/μL, Amersham Biosciences,

Rydalmere, NSW, Australia) as the label. Probes were purified by column chromatography according to the manufacturer's instructions, and the specific activity determined via liquid scintillation counting (Canberra Packard, Schwadorf, Austria). Probes with an activity less than 1×10^6 cpm were not used for hybridization. Membranes were pre-hybridized with ExpressHyb Hybridization Solution (BD Biosciences Clontech, catalogue #636831) for 3 h at 68°C. Probes were prepared for hybridization by boiling in a water bath with Cot-1 DNA, then loaded onto membranes, and hybridization was performed in roller bottles overnight at 68°C. The following day, membranes were washed thoroughly according to the manufacturer's instructions, then wrapped tightly in cling film, securely taped to the inside of a BAS Cassette 2040 (Fuji Photo Film Co., Pty, Ltd., Tokyo, Japan), and exposed to image plates (Fujifilm BAS-MS 20×40 cm) for up to 72 h at RT, depending on the specific activity of the probe. Plates were scanned on the Fujix Bas 1500 Phosphorimager (Fuji, Tokyo) using Image Reader v1.8 (Berthold, Bundoora, Vic, Australia). Differential expression ($\geq$1.5 fold increase) was calculated for genes where signals appeared on both duplicate membranes when the signal was at least 50% above background and normalized to the house keeping gene β-actin. Fold changes were calculated in Atlas Image v2.7 (BD Biosciences Clontech).

2.8. Real-Time PCR

RNA for real-time PCR was conducted as described previously [25]. Sequences for the forward and reverse primers were as follows:

Hsp90 forward, 5′-TTTGGTTGCTGAGAAAGTGACTG-3′,
Hsp90 reverse, 5′-CCTTTGTTCCACGACCCATTG-3′,
18S forward, 5′-CGGCTACCACATCCAAGGAA-3′,
18S reverse, 5′-GCTGGAATTACCGCGGCT-3′.

3. RESULTS AND DISCUSSION

3.1. The Presence of DOPA in Long Half-Life Proteins Can Make Them Resistant to Proteolysis in J774 Cells

We have previously shown that L-3,4-dihydroxyphenylalanine (DOPA) can be (mis)incorporated into cell proteins in place of tyrosine by mammalian cells and that this process is prevented by inhibitors of protein synthesis [21,22]. Using a standard metabolic labeling approach which allows us to selectively incorporate ^{14}C-leucine into predominantly short or long half-life proteins, we demonstrated that the presence of protein-incorporated DOPA increased the turnover of

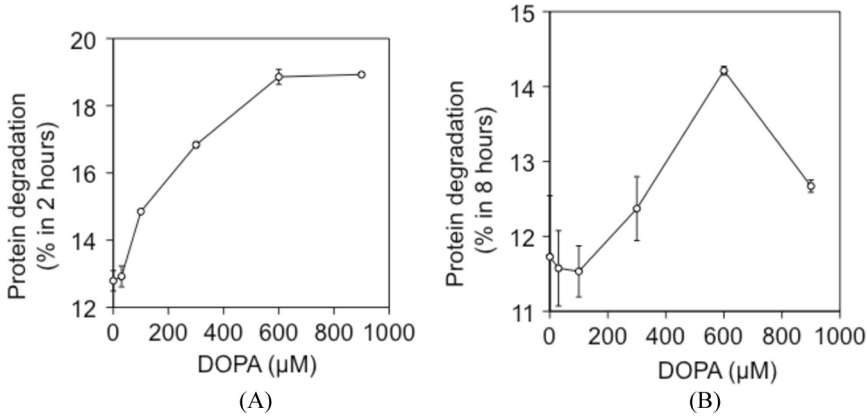


FIGURE 1 Turnover of (A) short half-life and (B) long half-life ^{14}C-leucine labeled proteins by mouse macrophages (J774) after incorporation of DOPA into proteins. Cells were incubated in media containing ^{14}C-leucine (0.3 μM) and DOPA (0, 30, 100, 300, 600 or 900 μM) for (A) 2 hrs or (B) 8 hrs. Cells were then washed and placed in degradation media and the radioactivity was measured by scintillation counting. Rates of degradation of (A) short half-life proteins increased with the amount of DOPA supplied in the culture medium. At higher concentrations of DOPA, the degradation of (B) long half-life proteins decreased again. Figure is representative of three separate experiments.

short half-life proteins at all DOPA concentrations (Fig. 1A). Under the conditions used, 12.8% of ^{14}C-leucine labeled proteins were degraded in 2 h and this increased to 19% when DOPA was also present in proteins. Incorporation of DOPA into long half-life proteins produced a bi-phasic degradation profile (Fig. 1B). We know from studies using a cell-free protein expression system that incorporation of DOPA can cause protein unfolding [26] and this generally renders proteins more susceptible to degradation [27]. These data raise the possibility that the ability of DOPA to form crosslinks with other amino acid residues in proteins, as has been reported to occur in a range of tissues from marine organisms, could be responsible for the protease-resistance observed for long half-life DOPA-containing proteins in mammalian cells.

3.2. The Relative Recovery of DOPA Decreases at Higher Levels of DOPA Incorporation into Proteins by Mammalian Cells

Using similar conditions to those previously used to generate long half-life proteins, DOPA (5 to 500 μM, of which 0.2% was ^{14}C labeled)

was incorporated into cell proteins for 24 h, proteins were precipitated, washed thoroughly, and the amount of radiolabel in the proteins measured by liquid scintillation counting. The amount of recovered radiolabel in proteins increased as the amount of DOPA supplied to the cells increased (Fig. 2A) and there was a linear correlation over most of the range of DOPA concentrations. The experiment was repeated and the amount of DOPA present in the total protein lysate was assessed from the amount of DOPA recovered by HPLC (Fig. 2B). It was clear that at higher levels of DOPA incorporation, a smaller proportion of incorporated DOPA was recoverable as DOPA itself, suggesting that DOPA derivatives were being generated. As we have shown previously [24], DOPA incorporation into proteins can be associated with the generation of fluorescent material in cells. In the present studies autofluorescence was also present in cells after incubation with 500 μM DOPA for 24 h (Fig. 3) but not in cells incubated in medium alone. This is likely to be due to the formation of aggregates in cells and supports the view that this process is initiated or enhanced by DOPA. The incomplete recovery of DOPA by HPLC is consistent with DOPA derivates being generated in proteins, and this corresponds to an increase in autofluorescence in cells indicative of the formation of large aggregates.

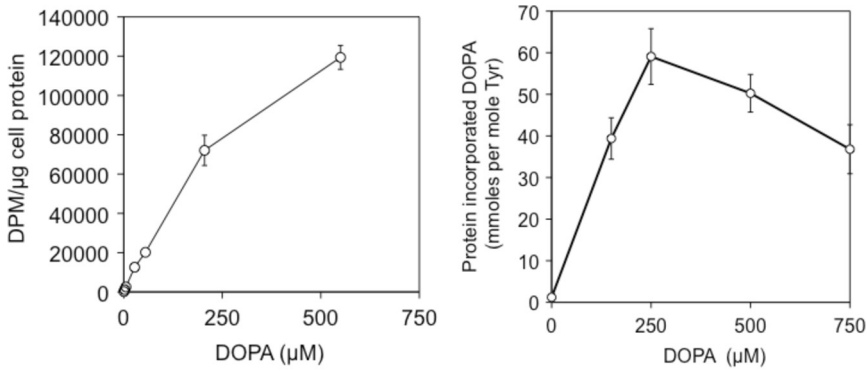


FIGURE 2 (A) Incorporation of ^{14}C-DOPA into proteins by mouse macrophages (J774). DOPA (5 to 500 μM of which 0.2% was ^{14}C-DOPA) was incubated with J774 cells in tyrosine-depleted medium for 24 hrs after which the cells were washed, precipitated and the radiolabel measured by scintillation counting (DPM per μg cell protein). (B) Recovery of DOPA from proteins after hydrolysis. Cell proteins were isolated, hydrolyzed, and the chemical level of DOPA measured by HPLC and expressed as mmoles to moles of tyrosine.

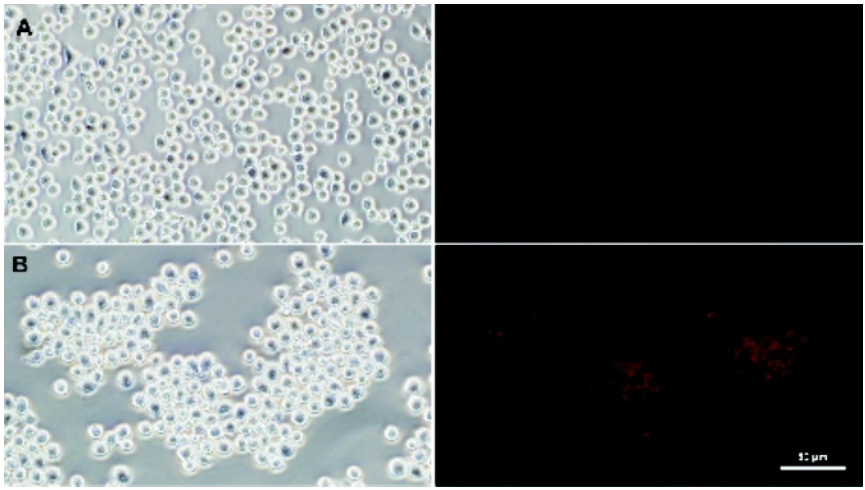
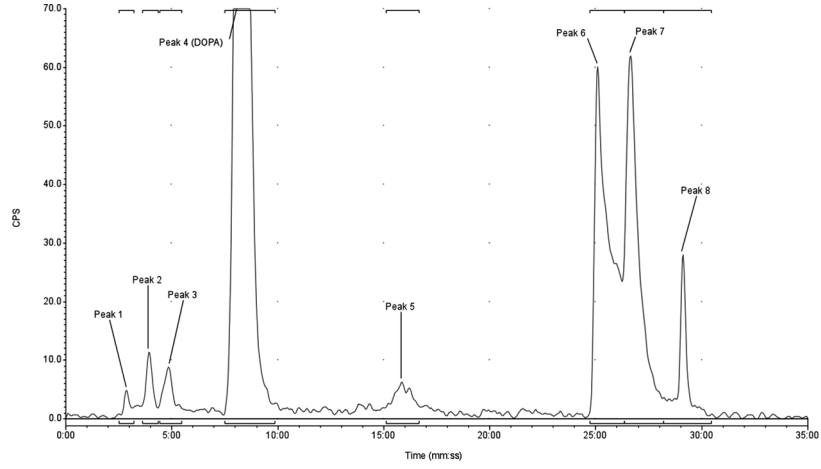


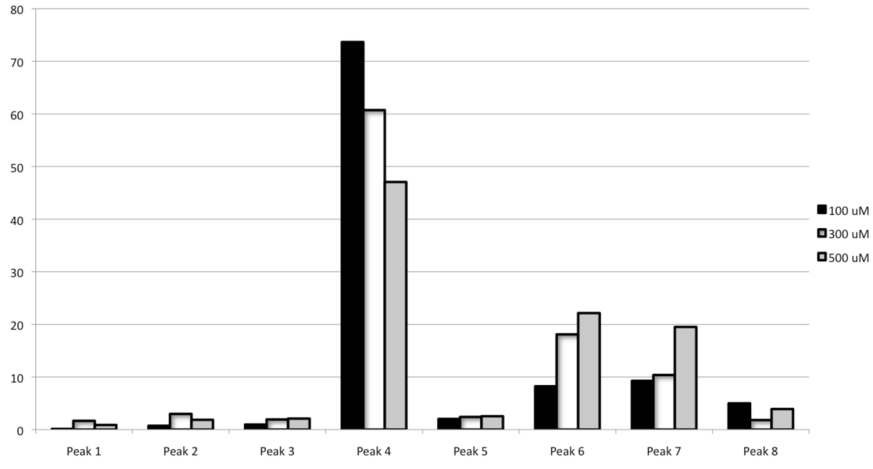
FIGURE 3 Autofluorescence in J774 cells (A) untreated or (B) incubated for 24 hours with 500 μM ^{14}C-DOPA. Digital images are shown of J774 cells after incubation with DOPA. Left hand panels shows the bright field image of J774 cells, right hand panels show natural fluorescence using an excitation wavelength of 545–580 nm with a 610 nm emission filter. Scale marker is 50 μM.

3.3. DOPA Derivatives Can be Recovered from Long Half-Life Cell Proteins After Incorporation of ^{14}C-L-DOPA into Proteins

While it has been proposed that DOPA derivatives might crosslink proteins and stabilize protein aggregates in mammalian cells making them protease resistant [25], there is at present no direct evidence of the existence of these species. To address this issue we allowed mammalian macrophages (J774) to incorporate ^{14}C DOPA (100, 300, and 500 μM ^{14}C-DOPA) into proteins in tyrosine-depleted medium; we then removed the DOPA-containing medium allowing cells to degrade some of the labeled proteins for 4 h, thus, enriching the cells in material that is not readily catabolized and likely to contain DOPA derivatives. Cell proteins were hydrolyzed and examined by HPLC using a flow-through radiometric detector. At the lower level of DOPA supply (100 μM), 74% of radiolabel was recovered as DOPA; around seven additional radiolabeled peaks were detectable which accounted for the remaining 26% of the radiolabel (Fig. 4B). Increasing the amount of DOPA supplied to cells to 300 and 500 μM increased the amount of DOPA derivatives formed and decreased the amount of radiolabel



(A)



(B)

FIGURE 4 (A) Representative chromatogram using radiometric detection of hydrolyzed cell proteins isolated from J774 cells treated with 500 μM ^{14}C-DOPA. A range of radiolabeled peaks were detected after hydrolysis of proteins (assigned numbers 1 to 8). Protein bound DOPA is shown as truncated peak 4. All of the peaks were present in cells treated with 100 μM and 300 μM. (B) Relative abundance of peaks detected after 24-hour treatment of increasing concentrations of ^{14}C-DOPA. J774 cells were treated with 100, 300, or 500 μM of ^{14}C-DOPA in EMEM deficient media for 24 hours, the cells were lysed, and the proteins precipitated and hydrolyzed overnight. The hydrolysate was then separated by HPLC and the relative abundance of radioactive peaks was measured. Peak 4 = DOPA.

recovered as DOPA to 61% and 47%, respectively. The same additional radiolabeled species were seen at all of the DOPA concentrations used (shown for 500 μM, Fig. 4A) but the relative amounts varied (Fig. 4B). These DOPA-derived products were present in insufficient quantities in the present study to identify by mass spectrometry but they provide the first direct evidence that DOPA derivatives are produced in mammalian cells following DOPA incorporation into proteins. It is possible that peaks 6 and 7 contain oxidative products of DOPA (and might not be homogeneous), since species with a similar retention times can be formed in the presence of Cu^{2+} and DOPA alone (not shown). It is likely these intermediates of DOPA are necessary for further crosslinking since it has been shown that the quinone is the reactive species of DOPA and very susceptible to nucleophilic attack by amino acids such as cysteine, histidine, and DOPA. While additional work is necessary to identify these DOPA derivatives with mass spectrometry, peak 5, which shows a small increase in percentage with increasing DOPA concentration, has retention time consistent with a 5-S-cysteinyl-DOPA standard (a gift from Prof. Kazumasa Wakamatsu, Fujita Health University, Toyoake Aichi, Japan). As well as being a metabolite of melanin and a marker of melanoma progression [28] 5-S-cysteinyl-DOPA might also be generated by nucleophilic attack from cysteine on the electron-poor DOPA-quinone and is potentially a protein crosslinking species where both cysteine and DOPA are present in proteins.

3.4. Microarray Analysis of Changes in Gene Expression in Cells in Which DOPA-Containing Proteins Were Present

To examine the cellular response to DOPA-containing proteins we used microarray analysis and compared gene expression in cells which were synthesizing DOPA-containing proteins with those that were synthesizing only native proteins. We observed increased expression of genes from several functional classes including proteases, DNA repair, and stress response (data not shown). For the purpose of this study, we focused on the increased expression of genes from the "heat shock and chaperones" functional classes (Table 1). Cells treated with DOPA exhibited increased expression of a number of heat shock proteins (HSPs) such as HSP70 and HSP90. The importance of HSPs in protein catabolism is often overlooked but they play critical roles in recognizing non-native proteins and delivering them to the major proteolytic machinery of the cell (lysosomes and proteasomes) for degradation [29]. Quantitative analysis of mRNA levels supported the microarray data and confirmed that HSP90 expression was increased in cells in which DOPA-containing proteins were accumulating (Fig. 5).

TABLE 1 Changes in the Expression of Heat Shock Proteins and Chaperones in Response to L-DOPA. Total RNA was Extracted and Converted to cDNA, Labeled with α-[^{32}-P]-dATP, and Hybridized Overnight. The Membrane was Exposed to a Phosphorimaging Screen for 72 hours at Room Temperature. Changes in Spot Intensity were Analyzed with Atlas Image v2.7 Where an Increase of $\geq$1.5 was Considered Significant. Increases in Gene Expression were Normalized to β-actin

Heat Shock Proteins & Chaperones				
Gene name	Protein name	Differential expression ratio n = 3	GenBank accession no.	Swiss-Prot. no.
Heat shock cognate 71kD	HSPA8	2.43	Y00371	PI1142
90-kDa heat-shock protein gene	HSP90	1.86	M16660	P08238
Mus musculus breast heat shock 73 protein (hsc73)	Hspa8	1.78	U27129	P63017
Mouse 84 kD heat shock protein	HSP90-beta	1.90	M18186	P11499
CTT7/CCTH CCT eta subunit (chaperonin containing TCP-1)	TCP-1-eta	2.04	Z31399	P80313
CCT5/CCTE (chaperonin containing TCP-1) epsilon subunit	TCP-1-epsilon	2.10	Z31555	P80316

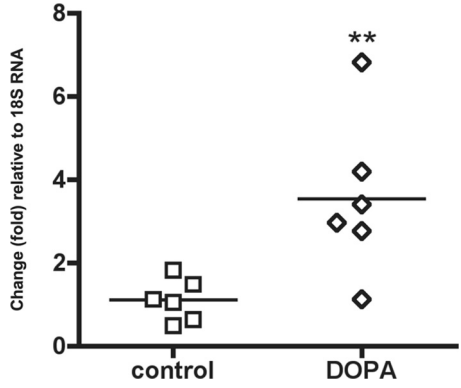


FIGURE 5 Real-time PCR analysis of expression of Hsp90 after 24-hour incubation with 500 μM DOPA. RNA was extracted, reverse transcribed to cDNA, and the expression of Hsp90 determined. Differential expression was normalized to 18S RNA and the fold change calculated according to the method of Livak [33] (Statistical analyses was conducted using unpaired two-tailed Student's T-tests with Mann-Whitney post-hoc analysis where $p < 0.05$ was considered significant.) $^{**}p < 0.01$.

4. SUMMARY

The many elegant studies that have lead to the characterization of DOPA derivatives and DOPA interactions in a range of marine organisms have highlighted the potential importance of this oxidative modification to proteins in mammalian cells [11–14]. The inability of cells to remove damaged or misfolded proteins can adversely affect cell function and this has been highlighted in many neurodegenerative disorders [30,31], whereas clearly such resistance to proteolytic removal is important and advantageous in extracellular adhesive molecules. We have previously provided evidence that proteins containing incorporated DOPA are present in Parkinson's disease (PD) patients who take L-DOPA (levodopa) as a therapeutic agent [32] and it is possible, therefore, that a gradual accumulation of proteins containing DOPA in PD patients might occur over a long period of time and could eventually have a detrimental effect on neuronal cell function.

Unlike many oxidative modifications to amino acid side chains that generate inert species, DOPA is reactive, and hence it can subsequently, by crosslinking or other reactions, render proteins resistant to proteolysis thus blocking the major pathway available for their removal. In the present study we demonstrate that by incorporating DOPA into long half-life proteins in macrophages by biosynthesis, we generate proteins which resist degradation and accumulate in cells. Only a proportion of the DOPA incorporated into proteins is recoverable from proteins as DOPA, and we demonstrate that a number of DOPA derivatives are generated and increase in relative abundance as the level of DOPA in proteins increases. Protein-bound DOPA is often used as a marker of protein oxidation *in vivo*; however, since DOPA can form additional species, some of which might have the ability to crosslink proteins, identification of these species could provide valuable bio-markers of protein aggregation *in vivo*.

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ABBREVIATIONS

DOPA—L-3,4-dihydroxyphenylalanine
Mfp—Mussel foot proteins
DMEM—Dulbecco's minimal essential medium
FCS—Fetal calf serum
TCA—Trichloroacetic acid
PD—Parkinson's Disease

REFERENCES

[1] Waite, J. H., *Ann. NY Acad. Sci.* **875**, 301–309 (1999).
[2] Zhao, H. and Waite, J. H., *J. Biol. Chem.* **281**, 26150–26158 (2006).
[3] Lee, H., Scherer, N. F., and Messersmith, P. B., *Proc. Natl. Acad. Sci. USA* **103**, 12999–13003 (2006).
[4] Waite, J. H., *Methods Enzymol.* **258**, 1–20 (1995).
[5] Monahan, J. and Wilker, J. J., *Chem. Commun. (Camb.)* 1672–1673 (2003).
[6] Miserez, A., Schneberk, T., Sun, C., Zok, F. W., and Waite, J. H., *Science* **319**, 1816–1819 (2008).
[7] Messersmith, P. B., *Science* **319**, 1767–1768 (2008).
[8] Waite, J. H., *Comp. Biochem. Physiol. B* **97**, 19–29 (1990).
[9] Land, E. J., Ramsden, C. A., and Riley, P. A., *Methods Enzymol.* **378**, 88–109 (2004).
[10] Rodgers, K. J. and Dean, R. T., *Int. J. Biochem. Cell Biol.* **32**, 945–955 (2000).
[11] Fu, S., Davies, M. J., Stocker, R., and Dean, R. T., *Biochem. J.* **333**, 519–525 (1998).
[12] Fu, S., Dean, R., Southan, M., and Truscott, R., *J. Biol. Chem.* **273**, 28603–28609 (1998).
[13] Ames, B. N., Shigenaga, M. K., and Hagen, T. M., *Proc. Nat. Acad. Sci. USA* **90**, 7915–7922 (1993).
[14] Linton, S., Davies, M. J., and Dean, R. T., *Exp. Gerontol.* **36**, 1503–1518 (2001).
[15] Gieseg, S. P., Simpson, J. A., Charlton, T. S., Duncan, M. W., and Dean, R. T., *Biochem.* **32**, 4780–4786 (1993).
[16] Burzio, L. A. and Waite, J. H., *Protein Sci.* **10**, 735–740 (2001).
[17] McDowell, L. M., Burzio, L. A., Waite, J. H., and Schaefer, J., *J. Biol. Chem.* **274**, 20293–20295 (1999).
[18] Zhao, H. and Waite, J. H., *Biochem.* **44**, 15915–15923 (2005).
[19] Burzio, L. A. and Waite, J. H., *Biochem.* **39**, 11147–11153 (2000).
[20] Calendar, R. and Berg, P., *Biochem.* **5**, 1690–1695 (1966).
[21] Rodgers, K. J., Wang, H., Fu, S., and Dean, R. T., *Free Radic. Biol. Med.* **32**, 766–775 (2002).
[22] Rodgers, K. J., Hume, P. M., Dunlop, R. A., and Dean, R. T., *Free Radic. Biol. Med.* **37**, 1756–1764 (2004).
[23] Simpson, J. A., Narita, S., Gieseg, S., Gebicki, S., Gebicki, J. M., and Dean, R. T., *Biochem. J.* **282**, 621–624 (1992).
[24] Dunlop, R. A., Rodgers, K. J., and Dean, R. T., *Free Radic. Biol. Med.* **33**, 894–906 (2002).
[25] Dunlop, R. A., Dean, R. T., and Rodgers, K. J., *Biochem. J.* **410**, 131–140 (2008).
[26] Ozawa, K., Headlam, M. J., Mouradov, D., Watt, S. J., Beck, J. L., Rodgers, K. J., Dean, R. T., Huber, T., Otting, G., and Dixon, N. E., *Febs. J.* **272**, 3162–3171 (2005).
[27] Stadtman, E. R. and Levine, R. L., *Ann. NY Acad. Sci.* **899**, 191–208 (2000).
[28] Wakamatsu, K., Kageshita, T., Furue, M., Hatta, N., Kiyohara, Y., Nakayama, J., Ono, T., Saida, T., Takata, M., Tsuchida, T., Uhara, H., Yamamoto, A., Yamazaki, N., Naito, A., and Ito, S., *Melanoma Res.* **12**, 245–253 (2002).
[29] Liberek, K., Lewandowska, A., and Zietkiewicz, S., *EMBO J.* **27**, 328–335 (2008).
[30] Uversky, V. N., *Curr. Protein Pept. Sci.* **9**, 507–540 (2008).
[31] Soto, C. and Estrada, L. D., *Arch. Neurol.* **65**, 184–189 (2008).
[32] Rodgers, K. J., Hume, P. M., Morris, J. G., and Dean, R. T., *J. Neurochem.* **98**, 1061–1067 (2006).
[33] Livak, K. J. and Schmittgen, T. D., *Methods* **25**, 402–408 (2001).