Oxidized protein deposition and accumulation have been implicated in the aetiology of a wide variety of age-related pathologies. Protein oxidation in vivo commonly results in the in situ modification of amino acid side chains, generating new oxidized amino acid residues in proteins. We have demonstrated previously that certain oxidized amino acids can be (mis)incorporated into cell proteins in vitro via protein synthesis. In the present study, we show that incorporation of o- and m-tyrosine resulted in increased protein catabolism, whereas dopa incorporation generated proteins that were inefficiently degraded by cells. Incorporation of higher levels of L-dopa into proteins resulted in an increase in the activity of lysosomal cathepsins, increased autofluorescence and the generation of high-molecular-mass SDS-stable complexes, indicative of protein aggregation.

These effects were due to proteins containing incorporated L-dopa, since they were not seen with the stereoisomer D-dopa, which enters the cell and generates the same reactive species as L-dopa, but cannot be incorporated into proteins. The present study highlights how the nature of the oxidative modification to the protein can determine the efficiency of its removal from the cell by proteolysis. Protection against the generation of dopa and other species that promote resistance to proteolysis might prove to be critical in preventing toxicity from oxidative stress in pathologies associated with protein deposition, such as atherosclerosis, Alzheimer’s disease and Parkinson’s disease.

Key words: aging, cathepsin B, dopa, lysosome, oxidized protein, proteasome.

Although all amino acids are targets for modification by reactive oxygen species, the aromatic residues are particularly susceptible [17]. Hydroxyl radical attack of phenylalanine residues results in the addition of a hydroxy group at C-2 to generate 2-hydroxyphenylalanine (o-tyrosine) or at C-3 to generate 3-hydroxyphenylalanine (m-tyrosine) [18]. These species are stable and are commonly used as markers of oxidative damage in diseased tissue [3–6]. Hydroxyl radical addition to the aromatic ring of tyrosine generates dopa, which, unlike m-tyrosine or o-tyrosine, is not an end product, but a reactive species capable of further modification [16,19,20]. In the presence of cellular antioxidants, dopa can redox-cycle to produce additional reactive species [21], some of which are stable enough to diffuse from the site of formation to damage distant macromolecules, hence amplifying the initial insult. Elevated levels of o-tyrosine, m-tyrosine and dopa have been reported in pathologies associated with protein deposition, including cataract [5,6], atherosclerotic plaque [3,4], myocardial ischaemia and reperfusion [22,23], and in peripheral lymphocytes from Parkinson’s disease patients taking L-dopa as pharmacotherapy [24].

The aims of the present study were to generate proteins containing incorporated o-tyrosine, m-tyrosine and dopa and to compare the turnover rates of these proteins in intact cells. We demonstrate that all three oxidized amino acids are incorporated into the polypeptide chain of proteins and have dramatically different effects on protein turnover. We provide evidence for a role of protein-incorporated dopa in the impaired removal of proteins and propose a role for this species in protein accumulation and aggregation, a hallmark of aging and age-related pathologies.

INTRODUCTION

Oxidized protein deposition and accumulation have been implicated in the aetiology of a wide variety of age-related pathologies, including neurodegenerative diseases (for a review, see [1]), atherosclerosis [2–4], cataractogenesis [5,6], macular degeneration [7], amyotrophic lateral sclerosis [8,9] and rheumatoid arthritis [10]. The primary mechanism for the detoxification of oxidized proteins is complete catabolism to their constituent amino acids; however, this process can be inefficient, as is evident from their accumulation. Despite extensive investigation into the pathways involved in the intracellular degradation of oxidized proteins, the precise mechanisms have yet to be completely elucidated.

Protein oxidation in vivo commonly results in the in situ modification of amino acid side chains, generating new oxidized amino acid residues within proteins. We have demonstrated that certain oxidized amino acids can be (mis)incorporated into proteins by protein synthesis, providing us with a unique way to generate biologically relevant proteins which contain oxidized amino acid residues [11,12]. This approach has since been adopted by others [13], and, unlike other more commonly used approaches for generating oxidatively modified proteins in vitro, many of which utilize strong oxidants [14,15], this approach minimizes potential damage to peripheral biomolecules such as lipids and DNA [16]. In addition, it allows us to accurately control the level of modification to the proteins and provides us with a unique opportunity to determine the relative contribution of individual oxidized species to impaired protein degradation and accumulation.
**EXPERIMENTAL**

**Materials**

L-[3-14C]-dopa and L-[U-14C]-leucine were purchased from Amersham Biosciences (GE Healthcare). L-Dopa, D-dopa, D,L-m-tyrosine, D,L-o-tyrosine, epoxomicin, p-NCS (p-nitrocatechol sulfate), N-Suc-Leu-Leu-Val-Tyr-AMC (where Suc is succinyl and AMC is 7-amino-4-methylcoumarin) and Boc-Leu-Ser-Thr-Arg-AMC (where Boc is t-butoxycarbonyl), Z-Phe-Arg-AMC and Ac-Nle-Pro-Nle-Asp-AMC (where Ac is acetyl and Nle is norleucine) were from Bachem AG. Digitonin was purchased from Merck. Custom primers were manufactured by Geneworks. iScript and IQ™ SYBR® Green Supermix were purchased from Bio-Rad Laboratories. DMEM (Dulbecco’s modified Eagle’s medium) and EEM (Eagle’s modified Eagle’s medium) deficient in tyrosine and phenylalanine were from JRH Biosciences. J774.A1 cells were from the A.T.C.C. (Manassas, VA, U.S.A.).

All aqueous solutions and buffers were prepared using water filtered through a four-stage Milli-Q system (Millipore). Other chemicals, solvents and chromatographic materials were analytic reagent, HPLC or molecular-biology grade.

**Protein degradation studies**

The cells used in these studies were a murine macrophage cell line (J774A.1). The cell culture methods used to generate oxidized-amino-acid-containing proteins were as described previously [12]. Cells used for degradation studies were plated in six-well plates at a density of 10^6 cells/well in complete DMEM [DMEM supplemented with 10% FCS (fetal calf serum), 2 mM L-glutamine, 100 units/ml L-penicillin and 100 µg/ml L-streptomycin] and incubated overnight. The following day, the medium was replenished with EMEM deficient in phenylalanine, tyrosine and leucine, but supplemented with 1 µM L-[14C]-leucine as well as 0, 150 or 500 µM L-dopa, D,L-m-tyrosine or D,L-o-tyrosine. When labelling with L-[3,4,5-3H]-dopa, the medium contained 2.5 µM L-[3-14C]-dopa and unlabelled dopa to the desired concentration. After 24 h of incubation, the medium was removed and replaced with 1 ml of DMEM containing 10% FCS and 10 mM each of leucine, phenylalanine and tyrosine as a ‘chase’. To determine the rate of degradation of 3H-labelled proteins, cultures were harvested at specific times, and the medium was subjected to 5% TCA (trichloroacetic acid) precipitation so that the relative amount of label present as free amino acid (TCA-precipitable) could be quantified via liquid-scintillation counting. Intact cells were lysed in 0.1% Triton X-100, and radiolabelled counts were quantified as free and protein-bound using TCA precipitation. The percentage degradation at each time point was represented as the amount of the free radioactivity in the medium as a percentage of the total radioactivity in the system. The amount of radiolabelled protein in the culture medium was used as a measure of cell viability. In all of the experiments reported, cell viability was >90%.

**SDS/PAGE and redox staining**

Cells were plated in 75 cm² flasks at a density of 3.25 x 10^6 cells/flask and incubated with either 150 or 500 µM L- or D-dopa for 24 h in the presence and absence of 0.5 µg/ml cycloheximide. After 24 h, cells were harvested, washed three times in PBS, pelleted, then snap-frozen and stored at −80°C until required. The pellet was lysed in 0.1% Triton X-100, the lysate was centrifuged at 13 000 g for 30 min, and the protein concentration of the supernatant measured using the Bio-Rad Coomassie Blue protein assay with BSA as a standard. A total of 100 µg of protein was separated using one-dimensional Tris/glycine SDS/PAGE (10% gels) [25], then the proteins were transferred on to PVDF membranes overnight [26]. Membranes were developed in 0.24 mM Nitro Blue Tetrazolium in alkaline glycinate as described previously [27]. Membranes were scanned using a Umax Powerlook 1120 flatbed scanner. The colour was then completely stripped with 100% methanol and the membranes were re-stained with Amido Black protein stain (0.1% in 40% methanol and 10% acetic acid) and scanned again.

For the detection of high-molecular-mass SDS-stable aggregates, SDS/PAGE was performed using Novex NuPAGE™ Tris/acetate 3–8% (Invitrogen) gels. Gels were stained for protein with colloidal Coomassie Blue. For redox staining, proteins were transferred overnight at 4°C on to PVDF membranes using Towbin transfer buffer [26] (without methanol), then redox- and protein-stained as described above.

**HPLC analysis**

For quantification of protein-incorporated oxidized amino acids, protein pellets from cells treated with 0, 150 or 500 µM oxidized amino acids were washed and delipidated by resuspending the pellet twice in 5% TCA containing 0.02% sodium deoxycholate and then washing once in ice-cold acetone. The resultant pellet was hydrolysed under anaerobic conditions using a standard gas-phase acid-catalysed method (HCl containing mercaptoacetic acid) [21]. For quantification of free dopa, cells were washed four times in PBS, lysed in 0.1% Triton X-100 and incubated in 10 mg/ml sodium borohydride for 10 min. Proteins were then precipitated with 5% TCA, and samples were centrifuged at 14 000 g for 10 min. The resulting supernatant was loaded on to the column for chromatography as described below.

HPLC analysis for dopa, m-tyrosine, o-tyrosine and p-tyrosine levels in proteins was performed using an LC-10A HPLC system (Shimadzu) equipped with a column oven (Millipore) set to 30°C and using a modification of methods developed in our laboratory [4]. System operation was automated by Class LC-10 software. Chromatography was performed on a Zorbax ODS column with a PeliGuard guard column (LC-18). The mobile phase was a gradient of solvent A (10 mM sodium phosphate buffer, pH 2.5, with 100 mM sodium perchlorate) and solvent B [80% (v/v) methanol]. Separation was performed at a flow rate of 1 ml/min with the following gradient: isocratic elution with 100% solvent A for 12 min, then decrease to 80% solvent A over 8 min; elution at 80% solvent A for 3 min before changing to 50% solvent A for 3 min; isocratic elution at 50% solvent A for a further 3 min, then re-equilibration with 100% solvent A for 10 min. The eluate was monitored by UV (Shimadzu), fluorescence (Hitachi F-1080) and EC (electrochemical) (Antec Leyden) detectors in series. The UV detector was set at a wavelength of 280 nm, fluorescence at λ_em of 320 nm, and the electrode potential in the EC detector was set at 1.2 V. When unmodified p-tyrosine displayed an off-scale response in the fluorescence channel, quantification was undertaken by either UV or EC detection. Retention times were defined with reference to known concentration standards, and identities were confirmed by UV absorption, fluorescence and EC detection. Standards were run after every three samples, and standard curves were constructed to determine the concentration of unknowns. Results were expressed as a ratio of µmol of oxidized amino acid to mol of the parent amino acid, tyrosine.
Real-time RT (reverse transcription)–PCR

Cells were treated with 0 or 500 μM L-dopa, m-tyrosine or o-tyrosine for 24 h in six-well plates at a density of 10^4 cells/well, and total RNA was isolated from using TRI Reagent (Sigma–Aldrich). A total of 500 ng of RNA was reverse-transcribed using the one-step iScript cDNA synthesis kit (Bio-Rad Laboratories) according to the manufacturer’s instructions. Primer pairs were designed with Beacon Design v4.0 (Bio-Rad Laboratories) using sequence data from the NCBI database (http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi). Sequences for the forward and reverse primers were as follows: Cat (cathepsin) B forward, 5′-gtgctactgtgtgtgattac-3′; CatB reverse, 5′-aggaggaggtgtgttagtga-3′; cystatin B forward, 5′-gggtagggggagagataac-3′; cystatin B reverse, 5′-tctggctctttgtgctgtgata-3′; 18S RNA forward, 5′-cggctacatcatacaggagaaag-3′; 18S RNA reverse, 5′-gcggtaattacgcgctgc-3′; 20S proteasome forward, 5′-ttcggtctgtgctgtggag-3′; 20S proteasome reverse, 5′-gccagccgcaagggcagatg-3′; 26S proteasome forward, 5′-tcggctacacatcaggatt-3′; 26S proteasome reverse, 5′-gcggcgccggaagggcagatg-3′. Custom oligonucleotides were manufactured by Geneworks. Real-time PCR was conducted with the Bio-Rad IQ™ SYBR® Green Supermix according to the manufacturer’s instructions. In all cases, reaction conditions were: denaturation at 94°C for 30 s, annealing at 55°C for 1 min and extension at 72°C for 1 min, with the reaction completed in 30 cycles. Fold changes were calculated with the comparative method [28] and expressed as fold change relative to 18S RNA.

Activity studies

Cells were plated in 75 cm^2 flasks at a density of 3.25 × 10^6 cells/flask and incubated with 0 or 500 μM L-dopa, D-dopa, m-tyrosine or o-tyrosine. After 24 h, cells were harvested, washed three times in PBS and pelleted. All assays were conducted in triplicate in 96-well microplates. Protein concentrations were calculated using the Bio-Rad Coomassie Blue protein assay adapted for microplates. Protein absorbance was measured at 595 nm and was converted into absolute values using BSA standards (0–0.083 mg/ml).

Measurement of CatB and CatL activities

CatB activity was measured by the initial linear increase in fluorescence following the cleavage of AMC from the peptide substrate Z-Arg-Arg-AMC. A 10 μl aliquot of lysate was diluted into 0.1 M phosphate buffer (pH 6) containing 2.5 mM EDTA, 2.5 mM DTT (dithiothreitol), 0.005 % Brij 35, 1 μM peptatin A and 5 mM benzamidine. Following the addition of 50 μM substrate, the plate was activated for 5 min at room temperature (22°C) on a plate shaker, then changes in fluorescence (λex 360 nm, λem 460 nm) were measured every 2 min for 30 min using a fluorescent plate reader (CytFluor II, PerSeptive Biosystems). Results were normalized to protein concentration, and activity was expressed as a percentage of control. CatL activity was measured in the same buffer as CatB, but at pH 5.5 and with Z-Phe-Arg-AMC as the substrate.

Measurement of 26S proteasome activity

Proteasome chymotryptic, trypic and PGPH (peptidylglutaminyl hydrolysing) activities were measured by the initial linear rates of cleavage of the fluorescently labelled peptide substrates N-Suc-Leu-Leu-Val-Tyr-AMC, Boc-Leu-Ser-Thr-Arg-AMC and Ac-Nle-Pro-Nle-Asp-Arg-AMC respectively (based on the method of [29]). Following harvesting, J774 cell pellets were resuspended in homogenization buffer (50 mM Tris/HCl, pH 7.5, 250 mM sucrose, 5 mM MgCl2, 2 mM ATP, 1 mM DTT, 0.5 mM EDTA and 0.025% digitonin) and incubated on ice for 5 min to allow permeabilization by digitonin. The proteasome was extracted by centrifugation at 20000 g for 15 min at 4°C. The supernatant was transferred to a fresh tube, diluted to give equal protein concentrations in each sample and then incubated in the presence or absence of the proteasome inhibitor epoxomicin (20 μM) for 30 min at room temperature. Proteasome activity was measured in reaction buffer (50 mM Tris/HCl, pH 7.5, 40 mM KCl, 5 mM MgCl2, 0.5 mM ATP, 1 mM DTT and 0.2 mg/ml BSA) with 100 μM chymotryptic/PGPH or 600 μM trypic substrates by measuring the change in fluorescence (λex 360 nm, λem 460 nm) over 30 min using a fluorescent plate reader. Rates of substrate cleavage were normalized to protein concentrations (ΔFU/min per μg of protein, where ΔFU is change in fluorescence units) determined using the Bio-Rad protein assay, and activity was expressed as a percentage of control.

Measurement of arylsulfatase activity

Arylsulfatase activity was determined by measuring the enzymatic hydrolysis of the ester sulfate bond of p-NCS based on the methods of Orange and Moore [30]. A 10 μl volume of cell lysate (as used in the cathepsin activity studies) was diluted into 100 μl of 0.1 M sodium acetate buffer (pH 5) containing 0.1 % Triton X-100 and 75 μM p-NCS and incubated at 37°C for 30 min. The reaction was stopped by the addition of 100 μl of freshly prepared stop buffer [2 ml of 0.1 M HCl containing 4 % (w/v) hydroquinone mixed with 40 ml of 2.5 % NaOH containing 5 % (w/v) Na2SO4]. Absorbance was measured at 540 nm on a Sunrise remote-control plate reader (Tecan) to determine the free nitrocatechol released by the arylsulfatase enzyme. Results were normalized to protein concentration and expressed as a percentage of control.

Fluorescence microscopy

J774 cells were plated in six-well plates at a density of 10^6 cells/well. After 24 h the medium was changed to EMEM deficient in phenylalanine and tyrosine, but supplemented with 0 or 500 μM L-dopa, D-dopa, D,L-o-tyrosine or D,L-m-tyrosine, and the cultures were incubated for a further 24 h. The medium was replaced with HBSS (Hanks buffered salt solution) and cellular autofluorescence was detected in the UV (λex 360–370 nm, λem 420–460), blue range (λex 470–490, λem 510–500 nm) and green (λex 520–550, λem 580) ranges of the spectrum using an Olympus X171 inverted microscope.

RESULTS

Incorporation of oxidized amino acids into J774 cell proteins is protein-synthesis-dependent

Proteins containing incorporated dopa, m-tyrosine or o-tyrosine were synthesized by J774 cells when the oxidized amino acids were supplied to the cells in tyrosine- and phenylalanine-deficient medium. The level of oxidized amino acid incorporation was measured by HPLC after TCA precipitation and acid-phase hydrolysis of the proteins. Levels of oxidized amino acid incorporation increased with increasing concentrations of oxidized amino acids supplied to the cells in the culture medium (Figure 1). The levels of incorporation of o-tyrosine and m-tyrosine were approx. 2-fold higher than that of dopa. Incorporation was dependent on protein synthesis, as was evident by an...
Cells were incubated in medium deficient in L-phenylalanine and L-tyrosine, but supplemented with 0, 150 or 500 µM (A) L-dopa, (B) D,L-o-tyrosine or (C) D,L-m-tyrosine for 24 h. TCA-precipitated proteins were subjected to acid-phase hydrolysis, and the oxidized amino acids and tyrosine content were quantified by HPLC. Results are expressed as a ratio of oxidized amino acids to tyrosine and are means ± S.E.M. of three or more determinations. *P < 0.05, **P < 0.01, ***P < 0.001, compared with control using one-way ANOVA with Dunnett’s post-hoc test.

absence of dopa-containing proteins in lysates from cells treated with L-dopa plus the protein synthesis inhibitor cycloheximide (Figure 2, lanes 4 and 6) and in cells treated with D-dopa (Figure 2, lanes 7 and 8) as detected by redox staining. This was due to the stereoselectivity of the tRNA synthetase, since D-dopa was actively transported into the cytosol (Figure 3). Less dopa was detected in the cytosol of cells incubated with L-dopa than in those incubated with D-dopa (Figure 3).

The effects of oxidized amino acid incorporation on the rate of protein turnover

The effects of oxidized amino acid incorporation into cell proteins on the rate of total protein turnover by J774 cells were measured. Cells were allowed to incorporate L-[14C]leucine (1 µM) and

Figure 1 Incorporation of oxidized amino acids into nascent polypeptides by J774 cells

Cells were incubated in medium deficient in L-phenylalanine and L-tyrosine, but supplemented with 0, 150 or 500 µM (A) L-dopa, (B) D,L-o-tyrosine or (C) D,L-m-tyrosine for 24 h. TCA-precipitated proteins were subjected to acid-phase hydrolysis, and the oxidized amino acids and tyrosine content were quantified by HPLC. Results are expressed as a ratio of oxidized amino acids to tyrosine and are means ± S.E.M. of three or more determinations. *P < 0.05, **P < 0.01, ***P < 0.001, compared with control using one-way ANOVA with Dunnett’s post-hoc test.

either 0 or 1 mM D,L-o-tyrosine, D,L-m-tyrosine or 150 or 500 µM L-dopa for 24 h, and the turnover of L-[14C]leucine-labelled proteins was measured over the following 24 h. This method
Degradation of proteins containing oxidized amino acids

Figure 4 Turnover of bulk (L-[14C]leucine-labelled) and L-[3-14C-alanine]dopa-labelled proteins by J774 cells

Newly synthesized proteins were metabolically labelled with 1 µM L-[14C]leucine and (A) 1 mM D,L-o-tyrosine, (B) 1 mM D,L-m-tyrosine or (C) 150 µM or 500 µM L-dopa in medium deficient in phenylalanine, tyrosine and leucine. (D) Newly synthesized cell proteins were metabolically labelled with 5.75 µM L-[3-14C-alanine]dopa and unlabelled L-dopa (final concentrations as indicated) in medium deficient in phenylalanine and tyrosine. After 24 h, the medium was replaced with a ‘chase’ medium containing excess phenylalanine, tyrosine and leucine (for leucine label) and the cells were harvested at the times indicated. Protein degradation was calculated from a ratio of free radiolabel in the medium to total radioactivity in the system. Results are means ± S.D. of three or more determinations. **P < 0.01, ***P < 0.001 using Student’s two-tailed t test (A, B and C) or one-way ANOVA with Tukey’s post-hoc test compared with 150 µM L-dopa (D).

measures the turnover of newly synthesized ‘bulk’ proteins, i.e. proteins containing L-[14C]leucine, some of which also contain the oxidized amino acid as part of the amino acid sequence. There was an increased turnover of bulk proteins in cells synthesizing proteins containing o-tyrosine and m-tyrosine (Figures 4A and 4B). The turnover rate of m-tyrosine-containing proteins was greater than that of o-tyrosine-containing proteins. No change in the rate of bulk protein turnover was found in cells synthesizing proteins containing incorporated dopa at any of the time points examined when compared with cells synthesizing only native proteins (Figure 4C).

In order to specifically examine the turnover of the proteins containing incorporated dopa rather than bulk proteins, cells were allowed to incorporate L-[3-14C-alanine]dopa (150–1000 µM), and the degradation of the 14C-labelled proteins was measured. A concentration-dependent decrease in the turnover rate of proteins containing incorporated dopa was found at the higher levels of dopa incorporation (Figure 4D).

The impact of proteins containing incorporated oxidized amino acids on the expression and activity of proteasomes

The ubiquitin–proteasome system is the primary pathway for the degradation of damaged or misfolded proteins (reviewed in [31]). To investigate the impact of the modified proteins on the expression and function of proteasomes, we conducted quantitative real-time RT–PCR of the major isoforms: the 20S and the 26S proteasome. We found only modest differences in mRNA levels for the 26S proteasome, where primers were designed against the 19S regulatory subunit (Figure 5A). The expression of the 20S proteasome, as measured by changes in the level of the α-type 1 subunit, was marginally increased with dopa incorporation and decreased with m-tyrosine incorporation (Figure 5B).

To determine whether proteins containing oxidized amino acids had an impact on the function of the proteasome, we measured the tryptic, chymotryptic and PGPH activities of the proteasome. As shown in Table 1, no significant changes to the function of the 26S proteasome were found under any of the conditions examined.

The impact of proteins containing incorporated oxidized amino acids on the expression and activity of lysosomal enzymes

The impact of proteins containing oxidized amino acids on the expression of CatB, a key proteolytic enzyme from the lysosomal pathway, was examined. We found a significant increase in CatB expression when proteins containing o-tyrosine, m-tyrosine or L-dopa were present in cultures. L-Dopa had the greatest effect, increasing mRNA levels 3.7-fold (Figure 6A). Levels of mRNA for the major cysteolysin inhibitor of cathepsins, cystatin B, were also increased, with m-tyrosine having the greatest effect, increasing mRNA 10-fold (Figure 6B).

The activity of the major cysteine proteases in the lysosomes, CatB and CatL, were dramatically increased in lysates from cells synthesizing proteins containing incorporated L-dopa, but not in those synthesizing proteins containing m-tyrosine or o-tyrosine (Table 2). To ascertain whether the increased cathepsin activity observed with L-dopa resulted from a selective increase in protease activity or from a global increase in the total number of lysosomes, we measured the activity of a lysosomal enzyme not involved in proteolysis, arylsulfatase. There was no change in arylsulfatase activity under the conditions examined in the
R. A. Dunlop, R. T. Dean and K. J. Rodgers

Figure 5  Differential expression of the (A) 26S and (B) 20S proteasome in response to the synthesis of proteins containing incorporated oxidized amino acids

J774 cells were incubated with medium deficient in phenylalanine and tyrosine, but supplemented with either 0 or 500 µM oxidized amino acids as indicated. After 24 h, total RNA was extracted, reverse-transcribed to cDNA and quantified using real-time PCR. Changes were calculated with the comparative method according to Livak and Schmittgen [28] and expressed as a percentage of control (fold change). Results are from triplicate experiments and RNA extractions, reverse-transcribed in duplicate and subjected to real-time PCR. *P < 0.05 using one-way ANOVA with Newman-Keuls multiple comparison test.

Table 1  Proteasome activity in the presence of oxidized-amino-acid-containing proteins

J774 cells were incubated as described in the Experimental section, and the tryptic, chymotryptic and PGPH activities were measured with fluorescently labelled peptide substrates essentially as described by Kisselev and Goldberg [29]. Data were collected in triplicate as change in fluorescence units over time, normalized to protein concentration (ΔFU/min per µg of protein) and expressed as a percentage of control values. Results are means ± S.D. for at least four independent experiments.

<table>
<thead>
<tr>
<th>Specific activity (% of control)</th>
<th>Tryptic</th>
<th>Chymotryptic</th>
<th>PGPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Dopa</td>
<td>115 ± 16</td>
<td>115 ± 13</td>
<td>126 ± 45</td>
</tr>
<tr>
<td>D-Dopa</td>
<td>138 ± 27</td>
<td>102 ± 29</td>
<td>111 ± 19</td>
</tr>
<tr>
<td>o-Tyrosine</td>
<td>104 ± 12</td>
<td>111 ± 24</td>
<td>86 ± 10</td>
</tr>
<tr>
<td>m-Tyrosine</td>
<td>108 ± 66</td>
<td>121 ± 34</td>
<td>116 ± 35</td>
</tr>
</tbody>
</table>

Figure 6  The effect of oxidized-amino-acid-containing proteins on the expression of lysosomal enzymes

J774 macrophages were treated with 0 or 500 µM L-dopa, o-tyrosine or m-tyrosine for 24 h as outlined in the Experimental section. Gene expression for (A) CatB and (B) cystatin B was measured with real-time PCR. Fold changes were calculated according to the comparative method of Livak and Schmittgen [28]. Each point represents triplicate experiments and RNA extractions, reverse-transcribed in duplicate and subjected to real-time PCR. *P < 0.05, ***P < 0.001 using one-way ANOVA with Newman-Keuls multiple comparison test.

Evidence for protein aggregation in cells after incorporation of l-dopa into proteins

J774A.1 cells incubated with o-tyrosine, m-tyrosine, D-dopa or L-dopa for 24 h were examined via fluorescence microscopy in the UV, blue and green ranges of the spectrum. We did not observe any detectable autofluorescence in cells treated with o-tyrosine or m-tyrosine (results not shown); however, in cells incubated with 500 µM L-dopa for 24 h, autofluorescence was observed in both the UV (λex 360–370, λem 420–460) (Figure 7A) and blue (λex 470–490, λem 510–550) (Figure 7B) ranges of the spectrum. A present study (Table 2), suggesting that the activity of the cysteine proteases was selectively modified in response to proteins containing incorporated dopa. A small but significant decrease in the activity of both CatB and CatL was found in cells incubated with D-dopa.

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Table 2  Activity of the lysosomal enzymes CatB, CatL and arylsulfatase when J774 cells were incubated with oxidized amino acids

<table>
<thead>
<tr>
<th></th>
<th>CatB</th>
<th>CatL</th>
<th>Arylsulfatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-Dopa</td>
<td>403 ± 21 ***</td>
<td>360 ± 24 ***</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>o-Dopa</td>
<td>80 ± 15 ***</td>
<td>76 ± 16 ***</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>o-Tyrosine</td>
<td>98 ± 3</td>
<td>88 ± 3</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>m-Tyrosine</td>
<td>90 ± 3</td>
<td>115 ± 6</td>
<td>100 ± 6</td>
</tr>
</tbody>
</table>

Small amount of background fluorescence was observed in the non-incorporation control D-dopa, in both the UV (Figure 7C) and blue (Figure 7D) ranges of the spectrum.

To determine whether the autofluorescence observed in the cells treated with 500 µM L-dopa could be attributed to SDS-stable protein aggregates, we resolved the cell lysates on low-percentage (3–8 % acrylamide) gradient gels. Sample preparation was modified (from that used to generate the data in Figure 2) to retain any high-molecular-mass aggregates. A small amount of high-molecular-mass material was detected in the wells of samples treated with 500 µM L-dopa, but not under any of the other conditions (Figure 8A, indicated by the arrowheads). To determine whether these aggregates contained proteins with incorporated dopa, samples were re-run and transferred on to PVDF membranes. The material that was retained in the well was positive by redox-staining (Figure 8B), suggesting that it contained dopa, and also stained for protein with Amido Black (Figure 8C).

DISCUSSION

Analysis of proteins from a range of pathological tissues has provided evidence for the accumulation of proteins damaged by hydroxyl radicals [4–6,32–34]. Aromatic amino acid residues in proteins are among the major targets [32], and the present study highlights the diverse effects that hydroxyl radical addition to aromatic amino acid residues can have on protein turnover and on the proteolytic machinery of the cell.

Consistent with previous studies from our laboratory, the oxidized amino acids m-tyrosine and dopa [11,12] and o-tyrosine (the present study), when supplied to cells in medium deficient in the parent amino acid, were biosynthetically incorporated into the polypeptide chain of proteins, modelling proteins in which these modifications are generated in situ by oxidants. m-Tyrosine caused a much greater increase in protein catabolism than o-tyrosine, despite similar levels of incorporation into proteins. Protein unfolding and exposure of hydrophobic regions are considered to be a signal for protein degradation [35,36], leading us to speculate that m-tyrosine might interfere with protein conformation to a greater extent than o-tyrosine. The increase in bulk protein turnover after m-tyrosine incorporation is greater than would be predicted from the levels of substitution of m-tyrosine into proteins. This is a consistent finding [12] and suggests that other mechanisms might be involved, in addition to protein modification from m-tyrosine. In cells synthesizing o-tyrosine- and m-tyrosine-containing proteins, there was no evidence of protein aggregation or changes in cellular fluorescence, supporting the view that these proteins are efficiently degraded by the cell. Furthermore, we report modest increases in mRNA for the lysosomal cysteine proteinase CatB, as well as the cysteine proteinase inhibitor cystatin B, but no significant changes were found in CatL and CatB activity or in the activity of the proteasome.

The favoured position for hydroxylation of tyrosine residues is the 3 position on the aromatic ring [18], generating dopa.

Figure 7  Fluorescence microscopy of L- and D-dopa-treated cells

J774 cells were plated in six-well plates at a density of 10⁶ cells/well. After 24 h, the medium was changed to EMEM deficient in phenylalanine and tyrosine, but containing 500 µM l-dopa. After a further 24 h, the medium was replaced with HBSS and cellular autofluorescence was visualized using an inverted microscope. (A) and (B) Fluorescent images taken using (A) ultraviolet (λex 360–370 nm, λem 420–490 nm) and (B) blue (λex 470–490, λem 510–550) light of l-dopa-treated cells. (C) and (D) Equivalent images for d-dopa. Scale bar, 20 µm. Images are representative of three independent experiments.

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The addition of this new hydroxy group generates a reactive catechol species which can readily undergo an autodioxidative cascade without the need for enzymatic catalysis [16]. The reactivity of the dopa residue may promote protein cross-linking, since dopa can react with other amino acid side chains, including cysteine residues, to generate cross-linking species such as 5-cysteinyl-dopa [19]. The ability of protein-incorporated dopa to decrease protein turnover rates has been demonstrated in previous cellular studies [11,12] and also in a study using a cell-free transcription/translation system in which we demonstrated that proteins containing dopa readily misfold [37]. In the present study, we extend these studies and provide evidence for the presence of dopa-containing aggregates in cells after incubation with L-dopa. 

One weakness of the cell studies carried out previously was that it was not possible to determine which effects were due to free (non-incorporated) L-dopa, which is also reactive [20], and which were mediated by proteins containing incorporated dopa. In the present study, we utilized D-dopa, the stereoisomer of L-dopa, which possesses redox properties identical with those of L-dopa and is transported into the cell, but is not incorporated into nascent polypeptides via protein synthesis. D-Dopa therefore effectively provides a ‘non-incorporation’ control for L-dopa. When the same concentration of D-dopa and L-dopa was provided in the medium, the levels of D-dopa detected in the cytosol were much greater than those of L-dopa. Incorporation of L-dopa into nascent polypeptides would constitute a very modest contribution to this disparity, since dopa can react with other amino acid side chains, including tyrosine residues during protein hydrolysis before HPLC. Levels of protein-incorporated dopa were lower than in previous studies from our laboratory [11,12] and were more comparable with levels of dopa found in proteins in pathological tissues such as cataracts [6], making these findings of potentially greater pathological interest.

Although dopa incorporation at the levels used in the present study did not alter bulk protein (i.e. L-[14C]leucine-labelled proteins) turnover appreciably, we demonstrated, using L-[3-14C]-alaninedopa, that the dopa-containing proteins themselves were degraded less efficiently with increasing incorporation of dopa. At levels of L-dopa incorporation which resulted in a decreased rate of proteolysis, there were no changes in the activity of the proteasome, indicating no damage to the proteasome from either L-dopa incorporation into proteasomal subunit proteins or from the delivery of proteins containing dopa to the proteasome. The activity of the lysosomal proteinases CatB and CatL was increased almost 4-fold, suggesting that the dopa-containing proteins were being delivered to the lysosomes. It is possible that the formation of aggregates and delivery of these aggregates to the lysosomal system might protect the proteasome from damage.

The activity of the lysosomal enzyme arylsulfatase was unchanged, suggesting that cathepsin activity was selectively increased. Regulation would appear to occur at the level of gene expression, since there was a significant increase in the mRNA encoding CatB. These results are consistent with observations of increased lysosomal enzyme activity in pathologies characterized by protein deposition and accumulation such as Alzheimer’s disease [38,39] and motor neuron disease [40]. Furthermore, increased expression of a number of lysosomal proteinases (CatB, CatH, CatL and CatS) was demonstrated in the striatum of rats administered oral L-dopa for 6 months [41]. Further evidence for L-dopa incorporation in vivo was presented in a study from our laboratory showing increased levels of proteins containing L-dopa in peripheral lymphocytes isolated from Parkinson’s disease patients undergoing levodopa (L-dopa) therapy [24].

Fluorescent pigment can accumulate in cells as a result of aging (lipofuscin) or from a range of pathologies associated with impaired proteolysis (ceroid) [42–44]. Despite differing mechanisms of formation, the composition of these fluorochromes is virtually identical, consisting of cross-linked proteins, lipids, carbohydrates and trace transition metals [45]. Both are visible
in the UV and blue ranges of the spectrum and are defined by their spectral properties, which vary depending on the tissue type and method of detection. Lipofuscin has been defined as having an excitation wavelength between 320 and 480 nm and an emission wavelength between 460 and 630 nm [42]. Ceroid is similar, with excitation maxima of 350/360 nm and differing emission wavelengths, including 454 [43], 430 [44] and 539 [42] nm, depending on the model. Consistent with the overlapping spectral properties and composition of ceroid and lipofuscin, we report fluorescence in the both the UV and blue ranges of the spectrum in cells treated with L-dopa. The pattern of cellular distribution of fluorescence was more disperse in the blue channel, a feature which we are currently characterizing further with the use of localization tools for lysosomes (Acridine Orange) and nuclei [DAPI (4′,6-diamidino-2-phenylindole)]. Fluorescence was only slightly visible in cultures treated with D-dopa; however, this did not correlate with the generation of SDS-stable aggregates, supporting the view that incorporation of dopa into proteins was essential for aggregate formation. It should be noted that the free dopa moiety can itself fluoresce, and this probably accounts for the observed background fluorescence in cultures treated with D-dopa. Although we did not fully characterize the aggregates generated in the present study, they are likely to be much less complex than classic ceroid/lipofuscin, as detailed above. Unlike the aforementioned studies where aggregates accumulated in vivo over a long period of time, the experimentally generated aggregates in the present study were formed in 24 h and probably consist primarily of aggregated proteins. A short incubation time probably accounts for the small size of the bands reported in Figure 8. It was also possible to generate aggregates using lower concentrations of L-dopa over longer periods of time (results not shown), and, as such, we predict the appearance of larger bands with increasing incubation time.

In summary, the present study demonstrates that the nature of the oxidative modification to proteins can determine how efficiently proteins are removed from the cell by proteinolysis. Proteins containing dopa were inefficiently degraded because of their ability to form high-molecular-mass aggregates. When examining the turnover or accumulation of oxidized proteins in cells, the nature of the modifications should be considered. Protection against the generation of dopa or other species that promote resistance to proteinolysis may prove to be critical for prevention of the toxicity associated with oxidative stress in pathologies associated with protein deposition is implicated, such as resistance to proteolysis may prove to be critical for prevention against the generation of dopa or other species that promote resistance to proteinolysis. The Authors Journal compilation © 2008 Biochemical Society

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