Evidence for inactivation of cysteine proteases by reactive carbonyls via glycation of active site thiols

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Hyperglycaemia, triose phosphate decomposition and oxidation reactions generate reactive aldehydes *in vivo*. These compounds react non-enzymatically with protein side chains and N-terminal amino groups to give adducts and cross-links, and hence modified proteins. Previous studies have shown that free or protein-bound carbonyls inactivate glyceraldehyde-3-phosphate dehydrogenase with concomitant loss of thiol groups [Morgan, Dean and Davies (2002) *Arch. Biochem. Biophys.* **403**, 259–269]. It was therefore hypothesized that modification of lysosomal cysteine proteases (and the structurally related enzyme papain) by free and protein-bound carbonyls may modulate the activity of these components of the cellular proteolytic machinery responsible for the removal of modified proteins and thereby contribute to a decreased removal of modified proteins from cells. It is shown that MGX (methylglyoxal), GO (glyoxal) and glycolaldehyde, but not hydroxyacetone and glucose, inhibit catB (cathepsin B), catL (cathepsin L) and catS (cathepsin S) activity in macrophage cell lysates, in a concentration-dependent manner. Protein-bound

carbonyls produced similar inhibition with both cell lysates and intact macrophage cells. Inhibition was also observed with papain, with this paralleled by loss of the active site cysteine residue and formation of the adduct species *S*-carboxymethylcysteine, from GO, in a concentration-dependent manner. Inhibition of autolysis of papain by MGX, along with cross-link formation, was detected by SDS/PAGE. Treatment of papain and catS with the dialdehyde *o*-phthalaldehyde resulted in enzyme inactivation and an intramolecular active site cysteine–lysine cross-link. These results demonstrate that reactive aldehydes inhibit cysteine proteases by modification of the active site cysteine residue. This process may contribute to the accumulation of modified proteins in tissues of people with diabetes and age-related pathologies, including atherosclerosis, cataract and Alzheimer's disease.

Key words: aldehyde, cathepsin, cysteine protease, hyperglycaemia, protein glycation, thiol.

INTRODUCTION

Oxidation of amino acids, proteins, nucleic acids and lipids, treatment with elevated glucose concentrations (as observed in people with diabetes), thiamin deficiency and decomposition of triose phosphates can give rise to elevated *in vivo* levels of a range of reactive aldehydes, including GO (glyoxal), MGX (methylglyoxal) and GA (glycolaldehyde) [1–3]. These compounds react with proteins, lipids and nucleic acids to give adducts and cross-links (glycated materials) via the Maillard reaction; these materials are often termed AGEs (advanced glycation end-products), although they can arise via multiple pathways, not all of which involve glycation. These products have been shown to accumulate in tissues and plasma of people with diabetes and a number of age-related diseases, including atherosclerosis, cataract and Alzheimer's disease (reviewed in [4]).

Modification of proteins by reactive aldehydes is orders of magnitude more rapid than that induced by glucose alone [5], suggesting that these materials may play a major role in the formation of AGE, despite the lower tissue and plasma concentrations of these materials [6]. Consistent with these results, dicarbonyl-derived products have been detected at elevated levels in tissues treated with hyperglycaemia *in vivo* compared with controls (see, e.g., [7]). Reaction of proteins with both glucose (although slow) and reactive carbonyls, and a range of radicals

and other oxidants, can form protein-bound carbonyls, with these materials contributing to AGE formation (reviewed in [8,9]). The accumulation of these modified proteins in tissues with disease progression, and/or age, may occur as a result of enhanced generation, and/or inefficient removal of these species once formed. Both free and protein-bound carbonyls can also directly affect cellular function, with this occurring via multiple pathways, including enzyme inhibition (see, e.g., [10,11]).

Previously, we have shown that incubation of glyceraldehyde-3-phosphate dehydrogenase with reactive aldehydes (MGX, GO and GA) and protein-bound carbonyls (i.e. proteins preglycated with MGX) results in enzyme inactivation, with concomitant loss of thiol groups [12]. This loss of activity has been ascribed to direct adduction of the free or protein-bound carbonyls with the thiol groups of the enzyme [12]. In the present investigation, we have extended this work to cysteine proteases of the cathepsin/papain family: catB (cathepsin B), catL (cathepsin L), catS (cathepsin S) and papain. Cysteine proteases are proteolytic enzymes with a low- pK_a cysteine residue present at their active site [13]. The cysteine protease superfamily consists of papain and related plant proteases, cruzipain and related parasite proteases and some mammalian lysosomal cathepsins [14]. Lysosomal cathepsins play a key role, together with the proteasome, in the degradation of intracellular and extracellular proteins and have been shown to be involved in multiple physiological processes,

Abbreviations used: AGE, advanced glycation end-product; AMC, 7-amino-4-methylcoumarin; catB/catL/catS, cathepsin B/L/S; CMC, *S*-carboxymethylcysteine; DMEM, Dulbecco's modified Eagle's medium; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; FCS, fetal calf serum; GA, glycolaldehyde; GO, glyoxal; HA, hydroxyacetone; 4-HNE, 4-hydroxynonenal; LDL, low-density lipoprotein; MGX, methylglyoxal; NEM, *N*-ethylmaleimide; OPA, *o*-phthalaldehyde; THF, tetrahydrofuran; Z, benzyloxycarbonyl.

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including antigen presentation, extracellular matrix degradation, bone remodelling, hormone processing, cell growth, apoptosis and inflammation (reviewed in [15,16]).

Altered expression or activity of this enzyme superfamily has been implicated in a number of pathological processes, including atherosclerosis, tumour invasion and metastasis, rheumatoid arthritis, osteoporosis and periodontitis (reviewed in [15,16]). In many of these pathologies, increased cathepsin expression has been observed (see, e.g., [17]), and a range of cathepsin inhibitors, including peptide aldehydes, have been investigated as potential therapeutic agents (see, e.g., [18,19]). Although evidence has been obtained for enzyme inhibition via covalent modification of the active site cysteine residue by aldehydes [20], little attention has been paid to the potential inhibition of cathepsins by structurally related free and protein-bound carbonyls generated by metabolic processes, and in particular in diabetes, where enhanced protein glycation and cathepsin inactivation may contribute to the accumulation of modified (glycated and/or oxidized) proteins. Glycation has been shown to decrease total cellular proteasome activity in human fibroblasts [21] and keratinocytes [22]. Glycated proteins have also been shown to accumulate in the lysosomes of diabetic cardiomyocytes [23]. It was therefore hypothesized that inhibition of protein catabolism by glycation of the enzymes responsible for the removal of modified proteins may contribute to a vicious cycle where the accumulation of glycated proteins increasingly inactivates the cellular proteolytic machinery (lysosomes and proteasomes) responsible for their removal, with consequent deleterious effects on normal cellular metabolism.

It is shown here that free MGX, GO and GA, as well as pre-glycated proteins, inhibit the activity of catB, catL and catS in isolated form and in J774A.1 cell lysates, in a concentration-dependent manner. Furthermore, proteins preglycated with GO or MGX inhibit these activities in intact cells. This inhibition is shown, with the structurally related cysteine protease papain, to be associated with loss of the active site cysteine residue and the formation of an adduct species, CMC (*S*-carboxymethylcysteine), from reaction with GO, in a dose-dependent manner. Furthermore, it is shown that a related dialdehyde, OPA (*o*-phthalaldehyde), can induce inactivation by cross-linking the active site cysteine to a neighbouring lysine residue, in a process that could be prevented by chemical blocking of the cysteine residue. Overall, these results demonstrate that reactive aldehydes, and protein-bound carbonyls, may contribute to the accumulation of modified proteins in cells under oxidative and carbonyl stress, by inhibiting enzymes that are designed to remove such proteins, thereby potentially contributing to cellular dysfunction.

MATERIALS AND METHODS

Materials

CatB and catL (both from bovine liver) and recombinant human catS (from *Escherichia coli*) were from Calbiochem (La Jolla, CA, U.S.A.). Papain was from Sigma or Roche (Castle Hill, NSW, Australia). Z-Phe-Arg-AMC (where Z is benzyloxycarbonyl and AMC is 7-amino-4-methylcoumarin), Z-Arg-Arg-AMC and Z-Val-Val-Arg-AMC were from Bachem AG (Bubendorf, Switzerland). All other chemicals were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise noted. HPLC solvents were obtained from EMD Chemicals (Merck, Kilsyth, VIC, Australia), and filtered before use through VacuCap 90 filter units with 0.2 µm Super membranes (Pall Corporation, Cheltenham, VIC, Australia). Water used in all experiments had been passed through a four-stage Milli Q system. Phosphate

buffer (0.2 M Na₂HPO₄ · 12H₂O and NaH₂PO₄ · H₂O) solutions were pretreated with Chelex 100 resin (Bio-Rad, Hercules, CA, U.S.A.) to remove trace metal ions.

Cell culture and preparation of cell lysates

J774A.1 cells (a murine macrophage-like cell line) were purchased from A.T.C.C. (Manassas, VA, U.S.A.) and cultured to approx. 80 % confluence at 37 °C under sterile conditions and an atmosphere of humidified 5 % CO₂ in DMEM (Dulbecco's modified Eagle's medium; Sigma), with added 10 % (v/v) heat-inactivated FCS (fetal calf serum; Gibco BRL, Life Technologies, Melbourne, VIC, Australia), 2 mM L-glutamine (Trace Scientific, Melbourne, VIC, Australia), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Sigma). Prior to lysis, cells were washed twice with PBS and then centrifuged at 1200 g for 5 min. The cells were resuspended and lysed in 0.1 % Triton X-100. The lysate was centrifuged at 13 000 g for 10 min, with the resulting supernatant stored in 0.5 ml aliquots at -80 °C until use. Cell protein concentration was determined by using the Bio-Rad protein assay (Bio-Rad, Regents Park, NSW, Australia), with BSA as a standard.

Preparation of glycated BSA and ovalbumin

Glycated BSA was prepared by incubation of BSA (50 mg/ml) with D-glucose (1 M) in 0.2 M phosphate buffer (pH 7.4) at 37 °C for 8 weeks under sterile conditions and an atmosphere of humidified 5 % CO₂ as described previously [12]. Control BSA samples were incubated in an identical manner in the absence of added glucose. MGX-, GO- and glucose-modified BSA and ovalbumin were generated by incubating the proteins (80 mg/ml) with MGX, GO or glucose (all 200 mM) respectively for 17 h at 37 °C in 50 mM phosphate buffer (pH 7.4). Control proteins were incubated in the absence of MGX, GO and glucose. Immediately after incubation, all protein samples were centrifuged at 1000 g for 1 h through Amicon Ultra-15 filter units with a 10 kDa molecular-mass cut-off membrane (Millipore, Australia) using nanopure water to remove unbound MGX, GO or glucose. This methodology results in the extensive formation of protein-bound carbonyl groups, with the exception of BSA or ovalbumin incubated with glucose for 17 h, where few protein-bound carbonyls are generated [24,25]. Further characterization of these materials was not attempted.

Treatment of J774A.1 cells with preglycated proteins and cathepsin activity measurements

J774A.1 mouse macrophage-like cells were plated at 1 × 10⁶ cells per well in 6-well plates and allowed to adhere overnight. BSA glycated with MGX or GO was prepared as described above. The concentrate from the Amicon filter units was resuspended in 50 mM phosphate buffer (pH 7.4) and loaded on to cell cultures in complete medium (DMEM plus 10 % FCS) at either 1 mg/ml or 3 mg/ml glycated protein. After incubation for 24 h at 37 °C, cultures were lysed in water containing 1 M DTT (dithiothreitol), using three freeze-thaw cycles (-80 and 37 °C). The resultant supernatant was immediately used for cathepsin assays as outlined below. Incubations were conducted for 24 h at 37 °C in 6-well plates in triplicate. Cell viability was assessed by lactate dehydrogenase release and was > 80 % for all experiments described.

Papain activation

Commercial papain was activated as described previously [26]. Briefly, 1 ml of 0.4 mM papain (10 mg/ml; Roche) was incubated

with 32 μl of sodium borohydride (100 mM) and 568 μl of nanopure water for 10 min at 21 °C. Acetone (0.4 ml) was then added to destroy residual sodium borohydride, and the resulting solution was kept at 4 °C until use (within 2 h).

Assays of enzymatic activity of papain, catB, catL and catS

The activity of papain and catL was assayed by monitoring the time-dependent increase in fluorescence of AMC released from the substrate Z-Phe-Arg-AMC. catB and catS were assayed in a similar manner using the substrates Z-Arg-Arg-AMC and Z-Val-Val-Arg-AMC respectively [27]. Assays were carried out in triplicate in microtitre plates with fluorescence changes ($\lambda_{\text{ex}} = 360 \text{ nm}$, $\lambda_{\text{em}} = 460 \text{ nm}$, with 40 nm bandwidth) measured at 25 °C, using a Cytofluor II plate reader. Substrate stocks (40 mM) were prepared in DMSO and stored at -20 °C, before dilution with nanopure water for assays; the final DMSO concentration was $\leq 0.25\%$, with a total assay volume of 200 μl .

Papain (10 μl of 50 nM stock) was incubated with or without reactive carbonyls or other reagents in 0.1 M phosphate buffer (pH 6) containing 0.005 % Brij 35, 2.5 mM EDTA and 2.5 mM DTT for 5 min at 21 °C before addition of substrate solution. For assay of papain activity after OPA treatment, 10 μl of 0.2 μM papain activated with NaBH_4 as described above was incubated with or without OPA or other reagents in 0.1 M phosphate buffer (pH 6) containing 0.005 % Brij 35, 2.5 mM EDTA and 2.5 mM DTT for 5 min at 21 °C.

catB and catL (isolated enzymes or cell lysates, diluted as necessary) were activated for 5 min at 21 °C in 0.1 M phosphate buffer [pH 6 (catB) or pH 5.5 (catL)], containing 0.005 % Brij 35, 2.5 mM EDTA and 2.5 mM DTT. The samples were then treated with reactive aldehydes, or glycosylated BSA or ovalbumin in the case of inactivation study, brought to 150 μl in the aforementioned buffer without DTT, but containing protease inhibitors (5 mM benzamidine, 5 mM EDTA and 1 μM pepstatin A), and incubated for 5 min at 21 °C.

For assay of catS activity in cell lysates, samples were incubated for 1 h at 40 °C in 0.2 M phosphate buffer (pH 7.5) containing 5 mM DTT, 5 mM benzamidine, 5 mM EDTA, 1 μM pepstatin A, 1 mM PMSF and 0.01 % Triton X-100, to inactivate other cathepsins and activate catS. The samples were then treated with reactive aldehydes or glycosylated BSA or ovalbumin in the case of inactivation studies, brought to 150 μl in the same buffer and incubated for 5 min at 21 °C. For assay of isolated recombinant human catS, samples were activated in the above-mentioned buffer for 5 min at 21 °C, then treated with aldehydes, OPA or other reagents, and incubated for 5 min at 21 °C. Measurement of fluorescence changes was performed immediately after addition of 50 μl of 0.2 mM substrate. The rates of substrate cleavage were determined from the initial linear portions of plots of fluorescence against time (0–50 min). Enzyme activity is expressed in all cases as a percentage of control incubations.

For the cells treated with preglycosylated BSA, catB activity was measured as above in 0.1 M phosphate buffer (pH 6) containing 0.005 % Brij 35, 5 mM EDTA, 2.5 mM DTT, 5 mM benzamidine and 1 μM pepstatin A. catL activity was measured in the same buffer but at pH 5.5, with Z-Phe-Arg-AMC. catS activity was measured by monitoring the release of AMC from Z-Val-Val-Arg-AMC in 0.2 M phosphate buffer (pH 7.5) containing 5 mM DTT, 5 mM benzamidine, 5 mM EDTA, 1 μM pepstatin A, 1 mM PMSF and 0.01 % Triton X-100 after incubation at 40 °C for 60 min to inactivate other cathepsins and activate catS. Activity measurements were carried out in microtitre plates in triplicate as described above, with changes in fluorescence measured every 2 min for 30 min (catB and catL) or every 5 min for 50 min

(catS). Data were normalized to protein concentration [$\Delta \text{fluorescence} \cdot \text{min}^{-1} \cdot (\mu\text{g of protein})^{-1}$] and expressed as a percentage of control values, with cell protein concentrations determined with the Bio-Rad protein assay using BSA as a standard.

Thiol assay

Thiol concentrations were assayed spectrophotometrically using DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] [28]. Briefly, using 96-well plates, 50 μl of freshly prepared DTNB (3 mM) in 0.2 M phosphate buffer (pH 7.4) was added to 100 μl of diluted sample or buffer. Release of the 5-thiobenzoate anion was quantified at 412 nm using a SUNRISE plate reader (Tecan) and converted into thiol concentrations using standard curves prepared using GSH (0–0.5 mM).

SDS/PAGE analysis of papain glycosylated with MGX

Papain (0.1 mM) was incubated with or without 100, 300 and 500 mM MGX in 50 mM phosphate buffer (pH 7.4) at 37 °C for 4 h. SDS/PAGE was performed using the method of Laemmli with 12 % (w/v) polyacrylamide gels [29]. Prior to loading, samples were mixed with equal volumes of loading buffer containing 10 % (w/v) SDS, 10 % (v/v) glycerol, 2.5 % (v/v) saturated Bromophenol Blue and 5 % (v/v) 2-mercaptoethanol in 300 mM Tris/HCl (pH 6.8). The samples were then heated at 95 °C for 5 min. After cooling, 46 μg of protein was loaded into each well of the gel and separated using a Bio-Rad system. Bands were visualized with Coomassie Blue staining and digitized using a Bio-Rad Gel Doc 1000 system.

Fluorescence detection of cross-link formation induced by OPA

In individual wells of a 96-well plate, 20 μl of papain (10 mg/ml), or recombinant human catS (16 $\mu\text{g}/\text{ml}$), was incubated with or without 20 μl of NEM (*N*-ethylmaleimide; 10 mM) in 133 or 153 μl of 0.2 M borate buffer (0.2 M $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$; pH 9) respectively, at 21 °C for 5 min. OPA (27 μl of 7.4 mM incomplete reagent; Sigma) was then added to the appropriate wells. After mixing and incubating for 10 min at 21 °C, fluorescence ($\lambda_{\text{ex}} = 360 \text{ nm}$ and $\lambda_{\text{em}} = 460 \text{ nm}$) was measured at 25 °C, using a Cytofluor II plate reader.

HPLC analysis of CMC on glycosylated papain

HPLC quantification of CMC formed on GO-treated papain was carried out essentially as described previously [30]. Briefly, 0.1 mM papain was incubated with various concentrations of GO in 50 mM phosphate buffer (pH 7.4) at 37 °C for 2 h. The samples were then dried in a Speed-vac, re-dissolved in 6 M HCl and transferred to hydrolysis vials. The vials were then placed in Pico-Tag reaction vessels containing 1 ml of 6 M HCl (BDH-Merck) and 50 μl of mercaptoacetic acid, subjected to repeated rounds of nitrogen flushing and evacuation and then hydrolysed at 110 °C for 22 h. The hydrolysed samples were dried in a Speed-vac, re-dissolved in 400 μl of sodium borate buffer (0.2 M, pH 9) and filtered through 0.45 μm Nanosep MF GHP centrifugal devices. Samples were kept at 4 °C prior to HPLC analysis.

CMC was quantified by reversed-phase HPLC using a Zorbax C-18 ODS column (5 μm particle size, 4.6 mm $\times$ 250 mm; Agilent, Forest Hill, VIC, Australia) fitted with a Supelco Pelliguard LC-18 guard column (4.6 mm $\times$ 20 mm; Sigma-Aldrich). Samples were derivatized with OPA prior to injection using an autosampler method employing a Shimadzu SIL-10A auto injector (Shimadzu, Rydalmere, NS, Australia), to which were added 20 μl of aq. 5 % 2-mercaptoethanol solution and 40 μl of OPA to

Figure 1 Inactivation of isolated cathepsins (catB, catL and catS) and papain, and cathepsin (catB, catL and catS) activity in J774A.1 cell lysates, by reactive aldehydes and glucose

(A–D) After activation with DTT, all four proteases catB (A; 2.5 m-units/ml), catL (B; 1.8 nM), catS (C; 8 ng/ml) and papain (D; 2.5 nM) were treated with or without various concentrations (0, 0.5, 1, 2, 5, 10 and 20 mM) of MGX (■), GO (▲), GA (◆), HA (△) and D-glucose (□) at 21 °C for 5 min before assessment of residual enzymatic activity as described in the Materials and methods section. (E–G) Diluted J774A.1 cell lysates were treated with or without various concentrations of aldehydes and glucose (concentrations and conditions as in A–D) after activation of the cysteine proteases with DTT. The lysate activities of catB (E), catL (F) and catS (G) were assayed as described in the Materials and methods section. For assay of each enzyme activity, equal amounts of cell lysate were used for both control and treatment with various concentrations of carbonyls. Results were expressed as percentage of control (Ctrl). Results shown are means $\pm$ S.D. for three or more determinations.

40 μ l of sample. The solutions were then mixed, and after 2 min, 80 μ l of the derivatized sample was injected on to the column. Samples were separated (flow rate 1 ml/min) using the following gradient: 100% buffer A for 2 min; then 0–30% buffer B over 18 min; 30–60% buffer B over 10 min; 60–100% buffer B over 5 min; 100% buffer B for 5 min; 100–0% buffer B over 7 min; and re-equilibration at 100% buffer A for 5 min. Buffer A consisted of 20% (v/v) methanol and 2.5% (v/v) THF (tetrahydrofuran) in 20 mM sodium acetate (pH 5.4; BDH–Merck). Buffer B consisted of 80% methanol and 2.5% THF in 20 mM sodium acetate (pH 5.4). Buffers were degassed by sonication under vacuum and sparged with helium during chromatography. Derivatized amino acids were detected by fluorescence (λ_{ex} = 340 nm and λ_{em} = 440 nm; Shimadzu RF-10Ax1 detector), and

CMC was quantified by use of a standard curve constructed using authentic material.

RESULTS

Inactivation of isolated catB, catL and catS and papain by free carbonyls

The effects of reactive carbonyls on the activities of cysteine proteases were investigated by incubation of isolated catB, catL and catS and papain with or without glucose or carbonyl compounds [MGX, GO, GA and HA (hydroxyacetone)] at 21 °C for 5 min before assay of residual enzyme activity. MGX, GO and GA each inhibited all four of the enzymes in a concentration-dependent manner (Figures 1A–1D). In contrast, glucose and

HA had little or no inhibitory effect (Figures 1A–1D). For all four enzymes, the inhibitory efficacy of the carbonyls decreased in the order $\text{MGX} > \text{GO} > \text{GA} > \text{HA} \approx \text{glucose}$, consistent with structure-dependent enzyme inhibition.

Inactivation of isolated catL by protein-bound carbonyls

Glycosylated proteins were prepared by incubation of BSA or ovalbumin with MGX, GO and glucose at 37 °C in phosphate buffer (pH 7.4), for 17 h, and 8 weeks in the case of glucose-modified BSA, followed by removal of excess unchanged modifying agent. Control proteins were incubated in the absence of carbonyls or glucose. Isolated catL was treated with the modified proteins for 5 min at 21 °C before residual enzyme activity was assessed. Treatment of catL with non-glycosylated BSA controls caused a decrease in enzyme activity (to $60 \pm 0\%$ of initial control values when 4 mg/ml protein was employed) when compared with untreated enzyme, probably as a result of competitive digestion of BSA and substrate Z-Phe-Arg-AMC (results not shown). BSA pretreated with MGX and GO induced significantly greater loss of catL activity than was observed with unmodified protein (to $33 \pm 4\%$ for MGX-treated BSA, and $50 \pm 3\%$ for GO-treated BSA, with 4 mg/ml protein). BSA incubated with glucose for 17 h did not give any additional inhibition compared with untreated BSA (results not shown). In contrast, BSA treated with glucose for 8 weeks induced a much greater decrease in catL activity (to $38 \pm 0\%$ of initial control activity with 4 mg/ml protein) than the incubated BSA control (to $51 \pm 1\%$), or BSA treated with glucose for 17 h ($60 \pm 0\%$). Control BSA incubated for 8 weeks gave rise to a greater loss of enzyme activity than either the 17 h BSA control or BSA incubated with glucose for 17 h.

Non-glycosylated ovalbumin also competed with Z-Phe-Arg-AMC for enzymatic digestion, resulting in a decrease in catL activity (to $89 \pm 1\%$ of initial control activity with 4 mg/ml protein). Incubation of the enzyme with ovalbumin pretreated with MGX and GO caused a greater loss of catL activity than control protein samples (to $70 \pm 2\%$ for MGX-treated, and $74 \pm 1\%$ for GO-treated, with 4 mg/ml protein), whereas ovalbumin treated with glucose for 17 h had no additional inhibitory effects over ovalbumin controls ($90 \pm 1\%$ for glucose-treated, versus $89 \pm 1\%$ for untreated, with 4 mg/ml protein). In each case, protein treatment with MGX resulted in a greater loss of enzyme activity compared with protein treated with GO or glucose, and the extent of inhibition was dependent on the concentration of modified protein employed (results not shown).

Inactivation of catB, catL and catS in J774A.1 cell lysates by low-molecular-mass carbonyls

The effects of low-molecular-mass carbonyls on the activities of the cysteine proteases in cell lysates were examined by treatment of J774A.1 cell lysates with or without MGX, GO, GA, HA and glucose at 21 °C for 5 min, followed by assessment of residual catB, catL and catS activity. MGX, GO and GA all inhibited each of the enzymes in a dose-dependent manner, whereas HA and glucose were ineffective (Figures 1E–1G). As with the isolated enzymes, the efficiency of inhibition decreased in the order $\text{MGX} > \text{GO} > \text{GA} > \text{HA} \approx \text{glucose}$.

Inactivation of catB, catL and catS in J774A.1 cell lysates by protein-bound carbonyls

MGX- and GO-modified BSA inhibited the activity of each of the cathepsin enzymes in J774A.1 cell lysates in a concentration-dependent manner (Figure 2). BSA modified by glucose for 17 h

Figure 2 Inactivation of catB, catL and catS in J774A.1 cell lysates by protein-bound carbonyls

Diluted J774A.1 cell lysates were treated at 21 °C for 5 min with or without various concentrations (0, 0.5, 1, 2, 3 and 4 mg/ml) of BSA and ovalbumin preglycosylated with MGX, GO and glucose, after activation of the cysteine proteases with DTT. The activities of catB, catL and catS were assayed as described in the Materials and methods section. For assay of each enzyme, equal amount of cell lysates was used for both control and treatment samples, with various concentrations of glycosylated or non-glycosylated proteins. The results are expressed as a percentage of control (Ctrl) (cell lysate alone). (A–C) Residual enzymatic activities of catB, catL and catS respectively, after treatment of cell lysates with BSA preglycosylated for 17 h with MGX (■), GO (▲) and glucose (△) and BSA incubation control (□). Results shown are means $\pm$ S.D. for three or more determinations.

did not induce any greater effect than incubated BSA control (Figure 2). The effects of MGX- and GO-modified ovalbumin on catL activity in J774A.1 cell lysates were similar to those observed with the isolated enzyme, with MGX- or GO-treated ovalbumin inhibiting catL activity in J774 cell lysates in a dose-dependent manner (results not shown). Incubated control protein samples gave rise to a decrease in catB and catL activity in the lysates, but these samples had no effect on catS activity (Figure 2C), probably due to the different substrate specificities of these enzymes.

Inactivation of catB, catL and catS in intact J774A.1 cells by protein-bound carbonyls

To confirm that the above inactivation of cathepsin enzymes also occurs in intact cells, MGX- and GO-modified BSAs were generated as described above and subsequently incubated with J774A.1 cells for 24 h at 37 °C, before assay of cathepsin activity as described in the Materials and methods section. Cell viability

Figure 3 Inactivation of catB, catL and catS in intact J774A.1 cells by BSA preglycated with GO or MGX

J774A.1 mouse macrophage-like cells were treated for 24 h with either 1 or 3 mg/ml of untreated (control) or preglycated BSA generated by treated with GO or MGX as outlined in the Materials and methods section. The residual cellular activity of catB (A, B), catL (C) or catS (D) after treatment was measured as described in the Materials and methods section and normalized to protein concentration. * $P < 0.05$; ** $P < 0.001$, *** $P < 0.0001$ using Student's unpaired t test against control samples (CTRL).

was examined in all cases to confirm that treatment with the modified proteins did not give rise to significant decreases with the concentrations and incubation times employed. With catB activity, GO-modified BSA at a modified protein concentration of 1 mg/ml gave rise to a statistically significant inhibition of activity (Figure 3). With higher levels of GO-modified BSA (3 mg/ml), complete inhibition of catB activity was observed (Figure 3B). In contrast, MGX-modified BSA did not give rise to significant inhibition at either protein concentration. With catL activity, GO-modified BSA at 1 mg/ml again caused significant loss of activity, but MGX-modified BSA did not (Figure 3C). In contrast, with catS activity and identical levels of modified protein, MGX-modified BSA gave rise to a significant loss of activity (Figure 3D); GO-modified BSA showed a trend towards loss of activity, but this failed to reach significance. BSA modified by glucose was not examined due to the negative results obtained with the cell lysates.

Formation of cross-links and inhibition of autolysis of papain upon glycation with MGX

Previous studies have shown that papain and related enzymes can undergo autolysis (self-proteolysis) [31] in the absence of substrate. The effect of low-molecular-mass carbonyls on this proteolytic activity was examined by SDS/PAGE. Due to the limited availability of the isolated cathepsins, only papain was examined in detail. Papain (0.1 mM) was incubated with or without various concentrations of MGX in 50 mM phosphate buffer (pH 7.4) at 37 °C for 4 h and then analysed on reducing gels. In control samples, both the parent protein (band at ~23 kDa) and fragmented materials (≤ 6.5 kDa) arising from autolysis were detected (Figure 4). With increasing MGX concentrations the intensities of the fragment bands decreased, whereas those of parent protein

Figure 4 Inhibition of autolysis and formation of cross-links on glycation of papain with MGX

Papain (0.1 mM) was incubated with or without various concentrations (0, 100, 300 and 500 mM) of MGX in 50 mM phosphate buffer at 37 °C for 4 h. SDS/PAGE analysis was performed as described in the Materials and methods section. Papain (46 μ g) was loaded on to each well of the gel. Lane 1, 0.1 mM papain control; lanes 2–4, 0.1 mM papain glycosylated with 100, 300 and 500 mM MGX respectively; lane 5, molecular mass markers. MW, molecular mass (sizes given in kDa). The protein bands assigned to parent protein (23 kDa), cross-linked (46 kDa) and fragmented (≤ 6.5 kDa) materials are indicated by arrows on the left-hand side of the gel. A representative gel is shown.

bands increased, consistent with significant inhibition of the self-digestion reaction in the MGX-treated samples (Figure 4, lanes 1–4). This loss of activity is ascribed to enzyme inhibition. MGX treatment also gave rise to weak bands at higher molecular mass, consistent with the formation of cross-linked materials. These bands increased in intensity with higher MGX concentrations, but accounted for only a small fraction of the protein.

Loss of thiol groups on papain upon reaction with low-molecular-mass carbonyls

As thiol groups on proteins are known targets of glycation [30], it was hypothesized that the observed inhibition of cysteine protease activity might arise from reaction of the low-molecular-mass-, or protein-bound, carbonyls with the active site cysteine residues of these enzymes. To test this hypothesis, the loss of thiols on activated papain was quantified, using DTNB, after incubation with, or without, various concentrations of MGX, GO and glucose for 5 min at 21 °C in 0.2 M phosphate buffer (pH 7.5). Reaction with MGX and GO resulted in a dose-dependent decrease in protein thiol concentrations, whereas glucose had no detectable effect under these conditions (Figure 5). MGX incurred a more marked loss than GO, consistent with the enzyme inhibition data. As papain contains only a single free cysteine residue, with this present at its active site (SWISS-PROT Protein Sequence database entry P00784), this result is consistent with direct carbonyl-induced modification of this residue.

Formation of CMC on papain upon treatment with GO

Confirmation of the modification of the active site cysteine residue of papain was obtained by quantifying the formation of CMC, an adduct species generated on reaction of GO with cysteine residues. Incubation of papain (0.1 mM) with various concentrations of GO (0–25 mM, in 50 mM phosphate buffer, pH 7.4), at 37 °C for 2 h, gave rise to increasing yields of CMC in the GO-treated, but

Figure 5 Loss of thiol groups on papain upon reaction with free carbonyls

Papain (0.1 mM), activated with NaBH₄ as described in the Materials and methods section, was treated with or without various concentrations (0, 50, 100, 150, 200 and 250 mM) of MGX (■), GO (▲) and glucose (□) for 5 min at 21 °C in 0.2 M phosphate buffer (pH 7.5), before assessment of residual thiol concentrations using DTNB as described in the Materials and methods section. The results were expressed as percentage of control (ctr) (untreated papain). Results shown are means ± S.D. for three determinations.

Figure 6 Formation of CMC on papain upon treatment with GO

Papain (0.1 mM) was incubated with or without various concentrations (0, 1.88, 5, 12.5, 20 and 25 mM) of GO in 50 mM phosphate buffer (pH 7.4) at 37 °C for 2 h. The samples were then dried in a Speed-vac and hydrolysed in 6 M HCl at 110 °C for 22 h. CMC was quantified by reversed-phase HPLC as described in the Materials and methods section. Results shown are means ± S.D. from three determinations.

not control, samples, as determined by HPLC analysis (Figure 6). Related products formed by MGX have not yet been characterized.

Formation of intra-molecular cross-links in the active sites of papain and human catS

The potential formation of intra-molecular cross-links (cf. the above evidence for inter-molecular cross-links) within the active site of cysteine proteases was examined using papain and human catS, as both these proteins have at least one lysine residue near the active site cysteine residue (cf. SWISS-PROT Protein Sequence database entries P00784 and P25774 respectively). The dicarbonyl compound OPA was used for these studies, as cross-linked products of MGX and GO with cysteine and lysine residues have not been characterized in detail. OPA is known to react with this pair of residues to give an isoindole derivative that can be readily detected by fluorescence spectroscopy; the formation of this derivative has been employed previously to examine the proximity of cysteine and lysine residues on enzymes (see, e.g., [32]). Treatment of papain (10 nM) or recombinant human catS (3.4 nM) with various concentrations of OPA (0.185–0.74 mM)

resulted in a concentration-dependent loss of enzyme activity when compared with controls (Figures 7A and 7C). Incubation of papain (40 μM) or human catS (67 nM) with OPA (1 mM) in 0.2 M borate buffer (pH 9) for 10 min at 21 °C resulted in an increase in fluorescence ascribed to the isoindole derivative ($\lambda_{\text{ex}} = 337$ nm and $\lambda_{\text{em}} = 427$ nm) when compared with controls (Figures 7B and 7D). Pre-incubation of either enzyme with NEM (1 mM) to block the active site cysteine residue resulted in almost complete inhibition of this increase in fluorescence, consistent with the formation of an intra-molecular cross-link between the active site cysteine and a neighbouring residue (presumed to be the lysine residue) and the observed loss of enzyme activity. Similar experiments were not carried out with other isolated cathepsins due to limitations on enzyme availability.

DISCUSSION

In the present study, it has been shown that the low-molecular-mass carbonyl compounds MGX, GO and GA inhibit the cysteine proteases catB, catL and catS, both in isolated form and in J774A.1 cell lysates, in a concentration-dependent manner. In contrast, HA and glucose showed little or no inhibitory effects under the conditions employed. The extent of inhibition was structure-dependent, with this decreasing in the order MGX > GO > GA > HA ≈ glucose. Protein-bound carbonyls generated as a result of adduction of these reactive aldehydes to proteins also gave rise to significant inhibition of isolated catL, each of the cathepsin enzymes (catB, catL and catS) in J774A.1 cell lysates, and some of these enzyme activities (catB and catL with GO-modified BSA, and catS with MGX-modified BSA) in intact J774A.1 cells. Interestingly, the pattern of inhibition observed with the free carbonyls, where MGX gives a greater extent of inhibition than GO (see Figure 1), is somewhat different from that observed for intact cells treated with BSA preglycated with GO and MGX (see Figure 3). With the intact cells, the GO-treated BSA appears to be a much more potent inhibitor than MGX-treated BSA, with only the latter giving significant inhibition of catS activity, and not catB or catL. This difference may arise from the rate, or extent, of endocytosis and delivery to the lysosomes of the modified proteins. These processes might also be expected to be affected by the nature and extent of the modifications on the protein; this has not been examined further here.

As the free carbonyls examined in the present study are related in structure to peptide aldehydes, which are well-known inhibitors of cysteine proteases [18,19], it was hypothesized that these carbonyls, both free and protein-bound, inhibit cysteine proteases via direct reaction of the active site cysteine residue with the aldehyde moiety (i.e. through glycation of the thiol group). The low-molecular-mass carbonyls may also induce enzyme inactivation via cross-link formation (either inter- or intra-molecularly), as suggested previously [12].

Using papain as a model, it has been shown that MGX, GO and GA, but not HA and glucose, inhibit the activity of papain concomitantly with loss of the single, active site, cysteine residue. In the case of GO, a product arising from this reaction, CMC, has been detected and quantified. Evidence has also been presented for the generation of inter-molecular cross-links and the inhibition of enzyme autolysis in the presence of MGX. Results have also been obtained, with both papain and recombinant human catS, for the formation of intra-molecular cross-links on reaction with the related dialdehyde OPA. The formation of these cross-links parallels loss of the enzyme activity. All of these results are consistent with inhibition of cysteine proteases by both free and protein-bound carbonyls via adduction (glycation) of the

Figure 7 Inactivation of papain and catS by OPA

The effect of OPA on the activities of papain (**A**) and catS (**C**) was examined by incubating papain (10 nM; activated with NaBH_4) or recombinant human CatS (3.4 nM; activated with DTT) with or without various concentrations (0, 0.185, 0.37, 0.555 and 0.74 mM) of OPA or 1 mM iodoacetic acid (IAA; positive control for blocking the cysteine residue) in phosphate buffer for 5 min at 21 °C before assessment of residual enzymatic activity as detailed in the Materials and methods section. For fluorescence detection of proximal cysteine and lysine residues on papain (**B**) and catS (**D**) using OPA, both papain and catS were not pre-activated with DTT to avoid cleavage of disulphide bonds. Papain (40 μM) or human catS (67 nM) was incubated with or without 1 mM OPA in 0.2 M borate buffer (pH 9) for 10 min at 21 °C. For negative controls, papain or CatS was pre-incubated with 1 mM NEM to block the active site cysteine residue in both enzymes. Fluorescence was recorded as described in the Materials and methods section. Results shown are means $\pm$ S.D. for three determinations. Ctrl, control.

active site cysteine residue. A similar mechanism of inactivation of cathepsins and papain has also been observed with amino acids, peptides and proteins that have been subjected to oxidative modification and contain hydroperoxide groups [33].

The plasma levels of individual unsaturated aldehydes have been reported to be in the low micromolar range in people with diabetes, with these significantly elevated compared with controls (see, e.g., [34]). These values are, however, misleading, as they represent the steady-state concentrations of highly reactive species, and do not give an indication as to the flux of reactive aldehydes (i.e. total amount) to which proteins are exposed. This is exemplified by the much higher value for the plasma AGE burden reported for people with diabetes of approx. 170 μM [7]. This latter value is also likely, however, to be an underestimate of the true level of protein modification, as this value is derived from the quantification of a small number of well-characterized products, and these materials are unlikely to account for the total population of modified species generated. Furthermore, these values are again steady-state plasma values, and do not take into account turnover of these compounds, nor differences between plasma and tissue concentrations, which have been shown to be significantly different in some, but not all, cases (see, e.g., results in [7]). As such, the actual plasma concentration of reactive aldehydes to which plasma proteins are exposed with is likely to be considerably higher than suggested by these steady-state measurements. The millimolar concentrations of free aldehydes employed in the present study may therefore reasonably approximate to pathological levels, particularly at the lower end of the range examined.

To our knowledge, no previous studies have reported on the inhibitory effects of MGX, GO, GA and glycated proteins on the activity of cysteine proteases and identified the mechanism of inactivation, though similar results have been obtained using lipid-derived aldehydes. Thus 4-HNE (4-hydroxynonenal) and phospholipid hydroxyalkenals have been shown to inactivate catB

by forming Michael adducts with active site cysteine and histidine residues [35,36], and oxidized LDL (low-density lipoprotein) and 4-HNE-modified LDL, both of which are likely to contain reactive carbonyl groups at surface sites, have been shown to inactivate catB by cross-linking the aldehydic moiety of apolipoprotein B to the active site thiol of the enzyme [37].

Oxidized LDL and oxidized lipids have also been reported to reduce intracellular enzyme activities of catB [38,39] and catL [40] in murine macrophages. Similarly, BSA glycated with glucose or fructose has been shown to cause a decrease in lysosomal cathepsin activity in microglial cells [41], and decreased activities of catB and catL have been observed in rat mesangial cells [42] and periodontal-ligament-derived fibroblastic cells [43], on culture in high-glucose media. Incubation (24 or 48 h at 37 °C) of LLC-PK1 cells (a cell line derived from porcine proximal tubule cells) with BSA containing AGEs, but not control protein, has been reported to result in a decline in the activities of lysosomal catB, catL and catH in a time- and dose-dependent fashion [44], and reduced activities of catB and catL have been detected in skeletal muscles [45] and glomerular and tubular tissues of streptozotocin-induced diabetic rats (see, e.g., [46,47]). This reduction in lysosomal cathepsin activity in kidney may partly contribute to mesangial expansion and glomerular basement membrane thickening in diabetic renal hypertrophy [48].

It has been proposed that accumulation of oxidized LDL in macrophage foam cells in atherosclerotic lesions may be partly due to inactivation of lysosomal cathepsins induced by oxidized LDL and oxidized lipids [38–40]. However, this conclusion has been challenged, as no decrease in cathepsin activity was observed by other workers on treatment of J774 macrophages with oxidized LDL for 24 h [49]. Increased expression of these tightly regulated enzymes [50] has also been detected in atherosclerotic lesions, diabetes (see, e.g., [51]) and other tissues (reviewed in [52]). Nevertheless, the effect of oxidation and glycation on the activities of lysosomal cathepsins appears to be cell- and tissue-specific and

-regulated, as increased activities were observed in serum, skin, liver and spleen and decreased activities were observed in kidney of streptozotocin-induced diabetic rats [53]. A decrease in catB activity during early stages and subsequent increase in later stages of diabetes have also been observed in the visual cortex of diabetic rabbits [54]. In some diseased tissues, increased accumulation of modified proteins and concomitant increased cathepsin expression and activity can be accounted for in terms of a compensatory response of the cells to the accumulation of modified undegraded (or undegradable) proteins [27], potentially as a result of the inactivation of lysosomal cathepsins by the proteins targeted for degradation, and subsequent up-regulation of cathepsin gene and protein expression, and cathepsin activity.

In other pathological tissues where cellular defence systems may be deficient, the inactivation of lysosomal cathepsins by reactive carbonyls and glycated or oxidized proteins may not be adequately compensated for by up-regulation of cathepsin gene and protein expression and may contribute to a vicious cycle where glycated or oxidized proteins induce damage to the cellular proteolytic machinery, which, in turn, leads to increased accumulation of modified proteins.

In conclusion, the present study has shown that reactive aldehydes and protein-bound carbonyls inhibit lysosomal cysteine proteases through modification of the active site cysteine residue. This result provides a partial explanation for the accumulation of modified proteins, particularly in the lysosomes, in a wide range of cells treated with oxidative and carbonyl stress. It may also provide an explanation for the accumulation of damaged proteins in a large number of human pathologies, including aging, diabetes, Alzheimer's, Parkinson's and other neurodegenerative diseases [55,56].

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