



## Deleterious effects of reactive aldehydes and glycated proteins on macrophage proteasomal function: Possible links between diabetes and atherosclerosis

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### ABSTRACT

People with diabetes experience chronic hyperglycemia and are at a high risk of developing atherosclerosis and microvascular disease. Reactions of glucose, or aldehydes derived from glucose (e.g. methylglyoxal, glyoxal, or glycolaldehyde), with proteins result in glycation that ultimately yield advanced glycation end products (AGE). AGE are present at elevated levels in plasma and atherosclerotic lesions from people with diabetes, and previous *in vitro* studies have postulated that the presence of these materials is deleterious to cell function. This accumulation of AGE and glycated proteins within cells may arise from either increased formation and/or ineffective removal by cellular proteolytic systems, such as the proteasomes, the major multi-enzyme complex that removes proteins within cells. In this study it is shown that whilst high glucose concentrations fail to modify proteasome enzyme activities in J774A.1 macrophage-like cell extracts, reactive aldehydes enhanced proteasomal enzyme activities. In contrast BSA, pre-treated with high glucose for 8 weeks, inhibited both the chymotrypsin-like and caspase-like activities. BSA glycated using methylglyoxal or glycolaldehyde, also inhibited proteasomal activity though to differing extents. This suppression of proteasome activity by glycated proteins may result in further intracellular accumulation of glycated proteins with subsequent deleterious effects on cellular function.

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The continuous synthesis and degradation of proteins plays an essential role in the maintenance of cellular homeostasis and regulation of multiple cellular functions [1,2]. This continuous protein turnover minimises protein exposure to oxidants or other damaging agents, and therefore reduces the potential accumulation of materials with a modified structure or function [1]. However, despite this continuous turnover of proteins in most cells and tissues, evidence has been presented for an accumulation of modified proteins in a wide range of age-related diseases including Alzheimer's disease, Parkinson's disease, cataractogenesis, atherosclerosis and diabetes [3,4]. This accumulation implies either an increased rate of formation of damaged proteins or a decreased rate of removal, or both.

People with diabetes have elevated levels of modified proteins arising from glycation and glycoxidation reactions [5]. Glycated proteins arise from the nonenzymatic reaction of glucose, and reactive aldehydes derived from glucose, with proteins, lipids and nucleic acids. These reactions ultimately yield advanced glycation end

products (AGE) [6,7]. In a normal healthy population the level of glycated products on plasma proteins (e.g. albumin) is <3%, whilst in people with diabetes this proportion can be increased very dramatically [8,9]. Elevated levels of a number of low molecular mass aldehydes, including glyoxal (GO), methylglyoxal (MGO), 3-deoxyglucosone and glycolaldehyde (GA) have also been detected in people with diabetes [6]. GA can be generated from glucose and amino acid oxidation [10], GO from glucose oxidation [11] and MGO from the degradation of glucose, glycero phosphate, glyceraldehyde-3-phosphate, acetone, aminoacetone and amino acid oxidation [12–14]. Glycation reactions occur more rapidly with reactive aldehydes, such as GA, GO and MGO, than with glucose [6,15]. The elevated levels of these reactive aldehydes can be due to an increase in the rate of generation of these aldehydes [13] and/or deficiencies in their removal systems [13,16,17]. Previous studies have reported an upregulation of systems that detoxify these reactive aldehydes, including glyoxalases (glyoxalase I and II) [13,16,17] and aldose reductase [16]. However, these systems do not appear able to compensate adequately for an increased formation of reactive aldehydes in people with diabetes, and particularly those with complications [13,16,17].

When primary cellular defences, such as protein disulfide isomerases (which reduce disulfide bonds), methioine sulfoxide

**Abbreviations:** AMC, 7-amino-4-methylcoumarin; BSA, bovine serum albumin; DTT, dithiothreitol; GA, glycolaldehyde; GO, glyoxal; MGO, methylglyoxal; PBS, phosphate-buffered saline

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reductases (which reduce the sulfoxide back to the thio ether) or molecular chaperones (which refold partially denatured proteins) are unable to repair damage to proteins, the altered proteins are targeted for degradation by cellular protein removal systems [1]. The two major systems in eukaryotic cells responsible for this action are the endo-lysosomal system and proteasomes [3]. The latter, which are the major proteolytic pathway for intracellular proteins, are members of a class of proteases known as the *N*-terminal nucleophile hydrolases [18,19]. The 26S proteasomal complex appears to account for the majority of the ATP- and ubiquitin-stimulated proteolytic activity, and consists of a 20S proteolytic core and one or two 19S regulatory units [18,20,21]. There are three different types of active sites; two chymotrypsin-like ( $\beta 5$  subunit), two trypsin-like ( $\beta 2$  subunit) and two caspase-like (or PGPH) ( $\beta 1$  subunit). The activities of each of these three sites need to be evaluated in order to obtain a complete picture of the state of the proteasomal system inside cells, or the potency of inhibitors [21]. Previous studies have reported an age-related decline in the peptidase activities of the 20S and 26S proteasome in humans, with this decrease postulated to give rise to an accumulation of damaged proteins [22].

Reactive aldehydes (MGO, GO and GA) and pre-glycated proteins have been reported to alter cellular function via suppression of the activity of key cellular enzymes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a key glycolytic enzyme), glutathione reductase (GR, an important antioxidant enzyme), and lactate dehydrogenase (LDH) [23]. These aldehydes have also been demonstrated to inhibit cysteine proteases (cathepsins B, L and S) of the lysosomal cathepsin family in macrophage cell lysates in a concentration-dependent manner [7]. Under the same conditions glucose, or proteins pre-glycated with glucose had no effect [7,23]. This inhibition has been suggested to arise from aldehyde-induced modification of the thiol groups of these enzymes [7,23]. It has also been suggested that elevated levels of free low molecular mass aldehydes (GA, GO and MGO) play an essential role in diabetes-associated atherosclerosis via the formation of glycated low-density lipoprotein particles which may contribute to lipid-loading of macrophage cells in the artery wall [24]. The aim of the current study was therefore to test the hypothesis that elevated levels of glucose and reactive aldehydes, and pre-glycated proteins (generated by glucose and reactive aldehydes) may modulate the activity of the proteasome system, as this system appears to play an essential role in degradation of these modified proteins [1].

## 1. Materials and methods

### 1.1. Materials

Proteasomal enzyme activity was quantified using the pro-fluorescent peptides *N*-Suc-Leu-Leu-Val-Tyr-AMC (chymotrypsin-like activity), and Boc-Leu-Ser-Thr-Arg-AMC (trypsin-like activity) purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Caspase-like activity was examined using Ac-Nle-Pro-Nle-Asp-AMC (Auspep, Parkville, VIC, Australia). All substrates were prepared as 100 $\times$  stocks in DMSO (10 mM for chymotrypsin and caspase substrates, 60 mM for trypsin) [21] and stored at  $-20^{\circ}\text{C}$ . Glucose and sodium dihydrogen orthophosphate monohydrate ( $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$ ) were obtained from Merck (Kilsyth, VIC, Australia), digitonin from Calbiochem (La Jolla, CA, USA), disodium hydrogen orthophosphate dodecahydrate ( $\text{Na}_2\text{HPO}_4\cdot 12\text{H}_2\text{O}$ ) from Univar (Seven Hills, NSW, Australia), dithiothreitol was from Astral Scientific (Gymer, NSW, Australia), and Tris hydrochloride from Amresco (Solon, OH, USA). All other chemicals were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise noted. All solutions were prepared using nanopure water (Milli Q system, Millipore-Waters, Lane Cove, NSW, Australia) and treated with washed Chelex-100 resin (Bio-Rad, Regents Park, NSW, Australia) to remove trace metal ions.

### 1.2. Preparation of glycated BSA

Extensively glycated BSA was prepared by incubation of BSA (50 mg mL $^{-1}$ ) with D-glucose (1 M) in 200 mM phosphate buffer (0.2 M  $\text{Na}_2\text{HPO}_4\cdot 12\text{H}_2\text{O}$  and  $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$ , pH 7.4, pre-treated with Chelex 100 resin to remove trace metal ions) at  $37^{\circ}\text{C}$  for 8 weeks under sterile conditions and an atmosphere of humidified 5%  $\text{CO}_2$ . Control BSA samples were incubated in a similar manner in the absence of glucose [7]. BSA modified by methylglyoxal (MGO), glycolaldehyde (GA) or glucose was generated by incubating BSA (80 mg mL $^{-1}$ ) with MGO or glucose at 10 and 200 mM, or GA at 10 mM, for 17 h at  $37^{\circ}\text{C}$  in 50 mM phosphate buffer (pH 7.4). Control BSA was incubated in a similar manner in the absence of MGO, GA or glucose. Immediately after incubation, protein samples were passed through 2 successive PD10 columns (Amersham Bioscience, Castle Hill, NSW, Australia) using chelexed-treated 50 mM phosphate buffer, pH 7.4, as the eluting buffer to remove unbound MGO, GA or glucose. With the exception of BSA incubated with glucose for 17 h where little adduction occurs, these incubations result in the formation of BSA with multiple covalently-bound sugar molecules or aldehyde groups [25–27] (see also below). Protein concentrations were determined using the Pierce (Rockford, IL, USA) bicinchoninic acid (BCA) assay with commercial BSA used as a standard [28].

### 1.3. Characterization of glycated BSA

The nature and extent of BSA glycation was assessed for each condition by HCl hydrolysis of triplicate samples as described previously [29], with modifications according to Zeng and Davies [30]. Briefly, vials containing 0.4 mg of protein were placed in Pico-Tag reaction vials (Alltech, Baulkham Hills, NSW, Australia) containing 1 mL of 6 M HCl and 50  $\mu\text{L}$  of thioglycolic acid. The reaction vials were subsequently evacuated and flushed with nitrogen gas three times, before hydrolysis under vacuum for 17 h at  $110^{\circ}\text{C}$ . Following drying down using a vacuum concentrator (Christ RVC 2-33; John Morris Scientific, Chatswood, NSW, Australia), samples were resuspended in 400  $\mu\text{L}$  of 0.2 M borate buffer, pH 9.0, and filtered through 0.2  $\mu\text{m}$  centrifugal filter units (Pall Nanosep MF; 500  $\mu\text{L}$  capacity). Samples were kept at  $4^{\circ}\text{C}$  prior to HPLC analysis.

Separation and quantification of AGEs was carried out by gradient HPLC using a Shimadzu HPLC system (Shimadzu Oceania, Rydalmere, NSW, Australia) consisting of a SIL-10ADVP auto injector (capable of pre-column derivatization), two LC-10ATVP pumps, DGU-20A<sub>3</sub> degasser, SCL-10-10AVP system controller, CTO-10ASVP column oven (set at  $30^{\circ}\text{C}$ ) and RF-10AXL fluorescence detector. The column used was a Beckman Coulter Ultrasphere ODS column (250  $\times$  4.6 mm, 5  $\mu\text{m}$  pore size) with corresponding guard column (45  $\times$  4.6 mm). Buffer A was composed of 4.8% (v/v) 1 M sodium acetate pH 6.5 in water, 4% (v/v) methanol, and water, whilst buffer B consisted of 100% methanol. A solvent gradient was utilized at 1 mL/min, which consisted of 15% buffer B for 40 min, 15–37% B over 36 min, 37–90% B over 2 min, 90% B for 7 min, 90–5% B over 1 min, and re-equilibration at 15% B for 9 min (total run time 95 min).

AGEs were quantified using authentic standards, which were used to construct standard curves over the range of 0–100 pmol per injection: *N*<sup>ε</sup>-carboxymethyllysine (CML; Sigma); *N*<sup>ε</sup>-carboxyethyllysine (CEL; Toronto Research Chemicals); (2S)-2-amino-5-(5-methyl-4-oxo-4,5-dihydro-1H-imidazol-2-ylamino)-pentanoic acid (MG-H1; PolyPeptide Laboratories); (2S)-2-amino-5-(2-amino-5-methyl-4-oxo-4,5-dihydro-imidazol-1-yl)-pentanoic acid (MG-H2; two isomers can be detected for this product, designated 2a and 2b; PolyPeptide Laboratories). One minute prior to injection, 40  $\mu\text{L}$  of each sample/standard was derivatized by the addition and mixing of 20  $\mu\text{L}$  of o-phthalaldehyde (OPA) containing 0.5% (v/v) 2-mercaptoethanol. Forty microliters of each sample was injected, and 30  $\mu\text{L}$  of each standard. The fluorescent derivatives were detected by fluorescence

using  $\lambda_{\text{ex}}$  340 nm and  $\lambda_{\text{em}}$  440 nm. Samples were diluted as required to ensure that all analytes were within the range of the relevant standard curves. Limits of detection for the AGEs were approx. 0.2 nmol AGE per mg of protein. All samples were standardized to their Gly content, as quantified by HPLC, thus serving as their own internal standards. Samples were diluted 100-fold with water, and separated using the same HPLC system and pre-column derivatization as for AGE detection, but with different buffers and gradient. Buffer A consisted of 20% (v/v) methanol, 5% (v/v) tetrahydrofuran, 10% aqueous 1 M sodium acetate pH 5.0, and water; buffer B was identical except it contained 80% (v/v) methanol in place of the 20% (v/v) methanol in buffer A. The following gradient was used: 5% B for 7 min, 5–25% B over 10 min, 25–50% B over 10 min, 50% B for 8 min, 50–100% B over 5 min, 100% B for 5 min, 100–5% B over 1 min, and re-equilibration at 5% B for 9 min (total run time 54 min). Fifteen microliters of each derivatized sample/standard was injected. The standards used were commercial products (Sigma), with standard curves prepared over the range 0–50 pmol per injection; this also allowed quantification of Arg in each sample.

#### 1.4. Cell culture

J774A.1 mouse macrophage-like cells (TIB-67, ATCC, Manassas, VA, USA) were grown in 175 cm<sup>2</sup> Falcon flasks (Becton Dickinson, Franklin Lakes, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM) with added heat-inactivated foetal calf serum (FCS) (10% (v/v); JRH Biosciences, Lenexa, Kansas, USA), 2 mM L-glutamine (Thermo Electron, Melbourne, VIC, Australia) and penicillin/streptomycin (100 U mL<sup>-1</sup> and 0.1 mg mL<sup>-1</sup>, respectively; BioWhittaker (Lonza, Walkersville, MD, USA)). Cells were harvested by scraping using a disposable cell scraper (Greiner Bio-One, Frickenhausen, Germany) and the cell suspension replated at 1:16 dilution every 3–4 days. Cell extracts for proteasome measurement activity, were prepared from confluent cultures of J774A.1 cells contained in 75 cm<sup>2</sup> Falcon flasks. The media was replaced with cold PBS, the cells removed by scraping, and subsequently washed three times in cold PBS by centrifugation (Beckman, Palo Alto, CA, USA) at 524 g for 5 min at 4 °C. Cell pellets were snap frozen in liquid nitrogen and stored at –80 °C.

#### 1.5. Preparation of cell extracts for proteasome activity measurement

Cell extracts containing proteasomes were prepared as described previously [21]. Briefly, cell pellets were resuspended in 500  $\mu$ L of homogenisation buffer (50 mM Tris–HCl, pH 7.5, 250 mM sucrose, 5 mM MgCl<sub>2</sub>, 2 mM ATP, 1 mM DTT and 0.5 mM EDTA) containing 0.025% (w/v) digitonin. ATP was employed to prevent dissociation of the 26S proteasome into its components and to ensure that 26S, and not 20S activity, was measured [21]. Resuspended cells were incubated on ice for 5 min to allow permeabilisation by digitonin, and were then transferred to glass tubes (8  $\times$  34 mm; Beckman, Palo Alto, CA, USA). The cytosol was forced out by centrifugation at 20,000g for 15 min at 4 °C using a Beckman centrifuge (120/1, Palo Alto, CA, USA). The optimal protein concentration for the proteasome activity assay was determined to be 11–12  $\mu$ g protein/reaction, with dilutions made using homogenisation buffer.

#### 1.6. Treatment of cell extracts with free aldehydes, glucose and glycated BSA

The effect of aldehydes and glucose on proteasome activity, was examined using cell extracts incubated with MGO (0.25, 0.5, 1, 10 and 20 mM), GO or GA (1, 10 and 20 mM) at 21 °C for 5 min. Free glucose (5 and 20 mM) was investigated in a similar manner. The effects of glycated BSA on proteasome activity were examined using 0.4, 1 or 4 mg mL<sup>-1</sup> pre-glycated BSA prepared as described above.

#### 1.7. Proteolytic activity measurement and analysis

Immediately after completion of the incubations with the required materials (free aldehyde, glycated BSA, epoxomicin, or no additives), proteolysis was measured by a continuous assay of peptidase activity using fluorescence detection [21]. Samples were prepared in reaction buffer (50 mM Tris–HCl, pH 7.5, 40 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM ATP, 1 mM DTT, 0.5 mg/mL BSA) with 100  $\mu$ M for the chymotryptic and caspase substrates, or 600  $\mu$ M tryptic substrate. Fluorescence ( $\lambda_{\text{ex}}$  360 nm,  $\lambda_{\text{em}}$  460 nm) was measured using a Cytofluor II plate reader every minute for 30 min. Potential contributions from non-proteasomal enzyme activity were examined using the proteasome-specific inhibitor epoxomicin with proteasome activity considered to be the fraction of fluorescence increase that was sensitive to this agent; fluorescent change due to proteasomal activity were therefore calculated as the difference in the rate of fluorescence change over time ( $\Delta$ FU/min) between determinations made in the presence (pre-incubation with 20  $\mu$ M reagent at 21 °C for 30 min) and absence of this agent [21].

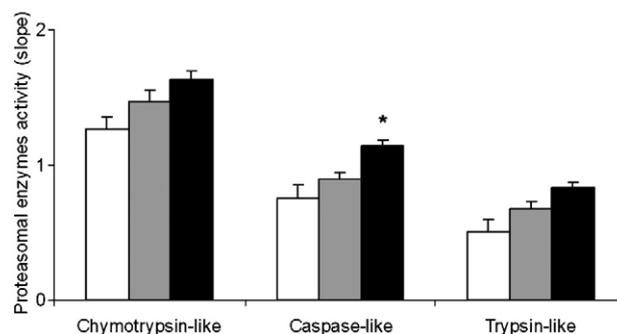
#### 1.8. Statistical analyses

Data from all enzymatic activity measurements are expressed as mean  $\pm$  SEM from 3 or more separate experiments, each assayed in triplicate. HPLC samples were analysed from triplicate experiments. Statistics analysis was performed using Prism 4.03 (GraphPad software, San Diego, CA, USA). For pair comparisons, paired *t*-tests were employed. For multiple comparisons, one-way analysis of variance (ANOVA) with Newman–Keuls Multiple Comparison test was employed. For the AGE and amino acid analyses one-tailed unpaired *t*-tests were employed comparing the glycated BSA samples to the corresponding control. Results were considered statistically significant at the *P* < 0.05 level.

## 2. Results

### 2.1. Effect of glucose on proteasomal enzyme activities in J774A.1 cell extracts

Incubation of cell extracts with 5 or 20 mM glucose at 21 °C for 5 min had no effect on chymotrypsin- and trypsin-like activities as compared with glucose-free controls, although a dose-dependent trend towards an enhancement in activity was observed (Fig. 1). With 20 mM glucose a significant increase (ca. 1.5-fold) in caspase-like activity was observed compared to the no glucose condition, but this was not significant when compared to the 5 mM glucose system.



**Fig. 1.** Effect of free glucose on proteasomal enzyme activities in J774A.1 cell extracts. Cell extracts were incubated with glucose (0, 5 or 20 mM) for 5 min at 21 °C. Then the chymotrypsin-, caspase- or trypsin-like activities were assessed and analysed as described in the Materials and methods. White, grey and black bars represent proteasomal activity of cell extracts incubated with 0, 5 and 20 mM glucose, respectively. \**P* < 0.05 compared to 0 mM glucose condition using one-way ANOVA and Newman–Keuls multiple comparison test. Data are mean  $\pm$  SEM of three or more experiments.

## 2.2. Effect of reactive aldehydes on proteasomal enzyme activities in J774A.1 cell extracts

The effect of reactive aldehydes on the proteasomal enzyme activities were investigated by incubation of cell extracts of J774A.1 cells with GA, GO or MGO over the concentration range 0.25–20 mM, at 21 °C for 5 min. Proteasomal activities were then quantified as described above. Treatment of the proteasomal preparations with all three reactive aldehydes resulted in a dose-dependent increase in chymotrypsin-like activity (Fig. 2A). This increase was statistically significant at 10 mM for each aldehyde. However, at the highest concentration examined there was attenuation in activity found with GA treatment, whereas no further increase was observed with GO, and the activity was further enhanced with MGO. Thus the chymotrypsin-like activity of the cell extracts of J774A.1 cells differentially responsive to MGO, GO and GA.

Differences in the response to aldehyde treatment were also observed with the caspase-like activity (Fig. 2B). This activity was relatively insensitive to MGO treatment with the exception of the highest concentration examined. In the cases of GA- and GO-treatment, a dose-dependent enhancement in caspase-like activity was apparent with a significant increase detected at 10 mM. Interestingly, a trend towards a suppression of caspase-like activity was observed at very high aldehyde levels (20 mM GO, 10 mM MGO) when compared to control, but this did not reach statistical significance.

Trypsin-like activity was significantly modulated by MGO at 0.5 mM and above, resulting in a significant enhancement of activity, with this stimulation being concentration-dependent (Fig. 2C). In contrast, GO and GA only increased trypsin-like activity at high concentrations (10 and 20 mM for GA and GO, respectively).

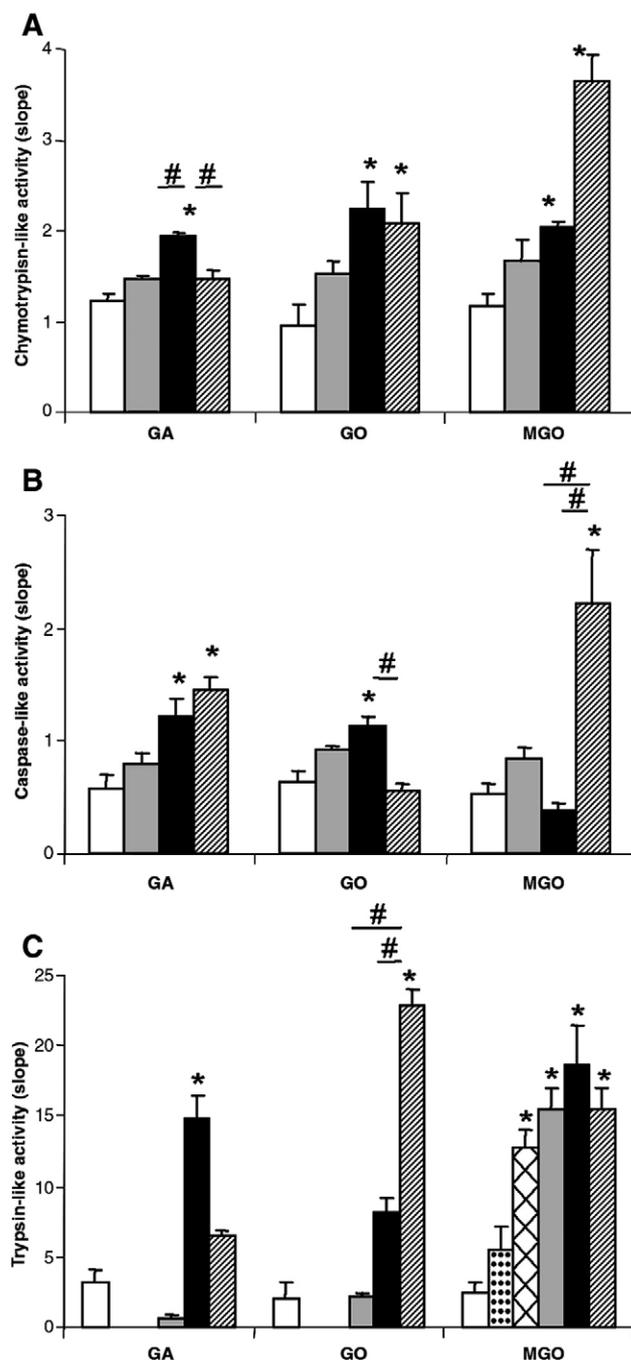
## 2.3. Characterization of BSA modification by glucose and reactive aldehydes

Modification of the glycosylated and control BSA was characterized by HPLC analysis. Incubation of BSA with 200 mM MGO resulted in the detection of significant levels of the AGEs CML, CEL, MG-H1, MG-H2a and MG-H2b (Fig. 3) and 58% loss of parent Arg (Fig. 3). Lys residues were also depleted but this was not quantified (see below). Significant Arg modification was also evident upon incubation with 10 mM MGO, as evidenced by elevated levels of CML, MG-H1, MG-H2a and MG-H2b, and loss of 23% of the parent Arg (Fig. 3). Reaction at Lys residues was less marked in this case as judged by the extent of CEL formation (Fig. 3). With GA as the modifying agents, elevated levels of CML were detected together with a small, but significant, loss of Arg (Fig. 3).

Incubation of BSA with glucose for 8 weeks resulted in the formation of very high levels of CML (over 30-fold greater than 10 mM GA, and over 300-fold greater than MGO), as well as significant amounts of CEL. A significant (22%) loss of Arg was also detected, which is not accounted for by the concentration of AGEs detected by HPLC, consistent with the formation of additional products such as *N*<sup>ω</sup>-carboxymethylarginine (CMA), which is known to be formed upon incubation of protein with glucose for prolonged periods, but which is susceptible to acid hydrolysis [31]. Parent Lys was also depleted in these samples consistent with the formation of Lys-derived AGEs (CML, CEL), but this was not quantified absolutely, due to variability in Lys derivatization by OPA. No significant AGE formation, or parent Arg, was observed upon incubation of BSA with 10 mM or 200 mM glucose for 17 h (Fig. 3).

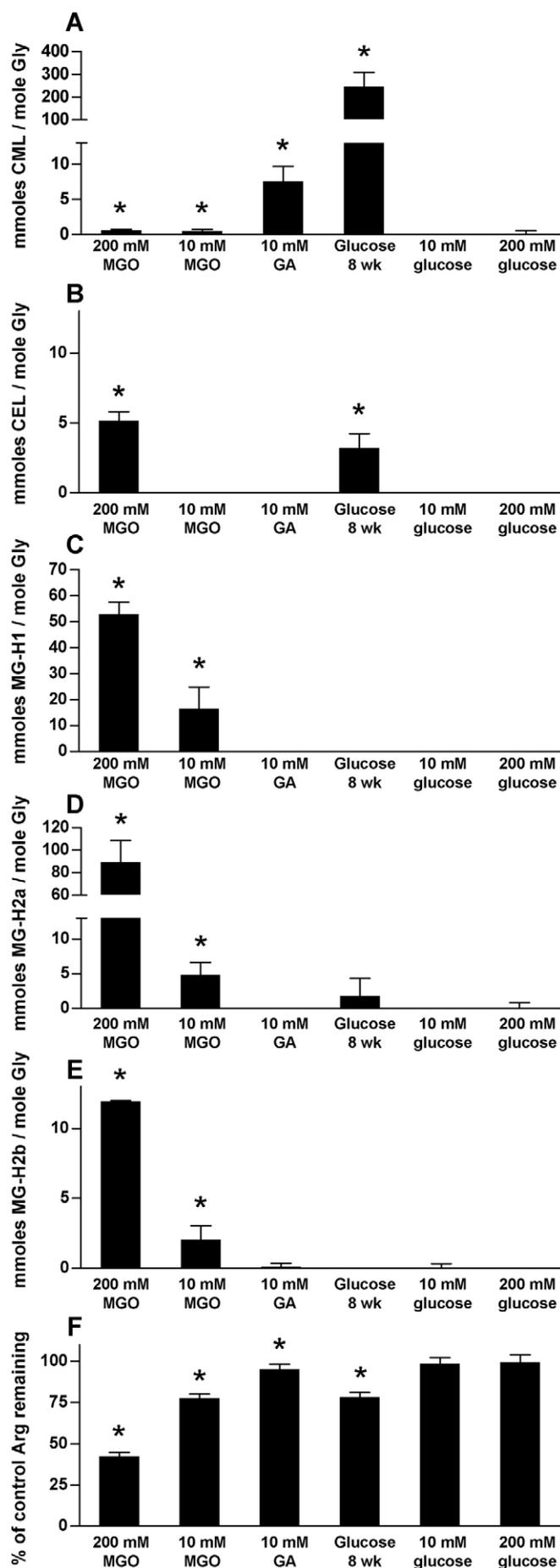
## 2.4. Effects of native and glycosylated proteins on proteasomal enzyme activity

The effects of the glycosylated and control BSA on proteasome activity were examined by incubating J774A.1 cell extracts with three



**Fig. 2.** Free reactive aldehydes enhance proteasomal enzyme activities. Cell extracts were incubated with GA and GO (0, 1, 10 and 20 mM), and MGO (0, 0.25, 0.5, 1, 10 and 20 mM) for 5 min at 21 °C. The (A) chymotrypsin-, (B) caspase-, and (C) trypsin-like activities were measured as described in the Materials and methods. White bars represent control, dotted bars show 0.25 mM (MGO, trypsin-like activity only), cross hatched bars represent 0.5 mM (MGO, trypsin-like activity only), grey bars show 1 mM, black bars represent 10 mM and hatched bars represent 20 mM treatments. \**P*<0.05 or #*P*<0.05 for results compared to control or to other treatments, respectively, using one-way ANOVA and Newman-Keuls multiple comparison test. Data are mean ± SEM of three or more experiments.

different concentrations of native or modified protein (0.4, 1 and 4 mg protein mL<sup>-1</sup>) for 5 min at 21 °C. Initial studies examined control (native) incubated BSA (Fig. 4). Chymotrypsin activity was sensitive to inhibition by the highest concentration of BSA incubated overnight, or for up to 8 weeks at 37 °C (Fig. 4A). Caspase-like activity was decreased significantly on incubation with the highest concentration of native BSA incubated for 8 weeks, but not at shorter times or with lower concentrations (Fig. 4B). Trypsin-like activity was



unaffected (Fig. 4C). In the light of this data, subsequent comparisons were made versus the corresponding samples incubated with native BSA for the same period.

BSA pre-glycated with glucose for 17 h had no significant effects on proteasomal activity compared to the incubated protein control (data not shown). In contrast, BSA glycated by incubation with 1 M glucose for 8 weeks, gave rise to a significant inhibition of both the chymotrypsin- and caspase-like activities at the highest concentration of BSA used compared to the incubated protein control. The trypsin-like activity was not affected (Fig. 5). With the highest protein concentration (4 mg/mL) an apparent negative gradient was observed; this arises from the subtraction of the fluorescence changes observed with the epoxomicin controls from the glycated protein data, and is believed to reflect inhibitory effects of the native or glycated BSA on other proteolytic systems present in the cell extracts (e.g. calpains and lysosomal cathepsins) [21].

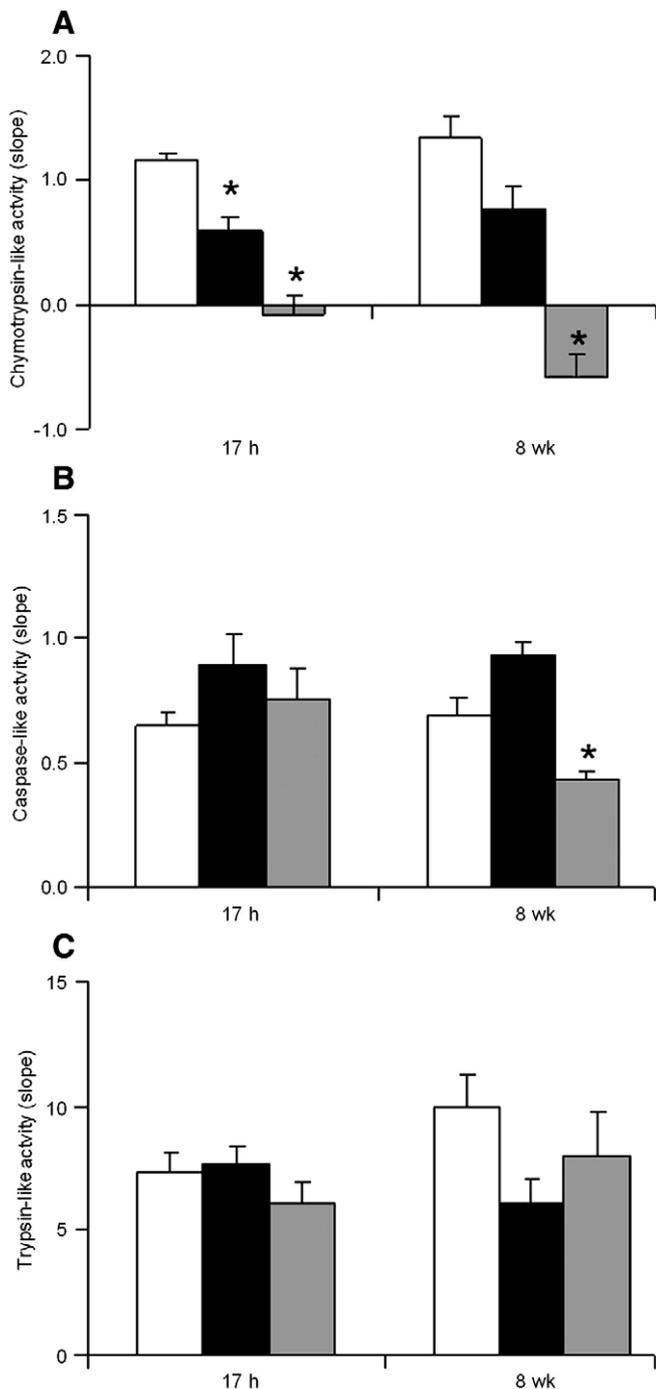
No significant inhibitory effects on the trypsin-like activity of the proteasomal extracts was observed with BSA pre-glycated with glucose over the range of BSA and glucose concentrations, and glycation conditions, examined (Fig. 5). In contrast to the other activities (see above) a significant increase in trypsin-like activity was observed with the highest concentration of BSA modified with either 10 mM or 200 mM glucose for 17 h (Fig. 5F).

The effects of BSA glycated by reactive aldehydes on proteasomal enzyme activities were examined in a similar manner to that outlined above, with the BSA (0.4, 1 or 4 mg protein mL<sup>-1</sup>) pre-incubated 10 mM GA (GA (10 mM)-BSA), 10 or 200 mM MGO (MGO (10 mM)-BSA and MGO (200 mM)-BSA, respectively) for 5 min at 21 °C. Whilst low concentrations of GA-modified BSA (0.4 (data not shown) and 1 mg protein mL<sup>-1</sup>) had no significant effects upon any of the proteasomal activities, high concentrations of this material (4 mg protein mL<sup>-1</sup>) significantly inhibited both the chymotrypsin- and caspase-like activities (Fig. 6A–D). In contrast, this material stimulated trypsin-like activity (Fig. 6E–F). BSA modified with 10 mM MGO had no effect upon any of the measured proteasomal activities at any of the protein levels examined. With BSA modified with 200 mM MGO, an inhibition of caspase-like activity was observed at all the protein concentrations examined (Fig. 6C–D). In addition, inhibition of both the chymotrypsin and trypsin activities were detected with the highest concentrations (4 mg protein mL<sup>-1</sup>) of this modified protein (Fig. 6B and F).

### 3. Discussion

This study has examined the effects of short-term exposure to glucose, reactive aldehydes and pre-glycated proteins on the activities of one of the major components of the intracellular protein removal systems, the proteasome. One of the defining features of diabetes is hyperglycaemia, with elevation of blood glucose levels from 5 mM to up to ca. 30 mM in extreme cases [32]. This elevation in glucose is associated with elevations in the levels of reactive aldehydes derived from glucose and other metabolic processes, and the generation of glycated/glycoxidized proteins [32]. Elevated levels of these materials

**Fig. 3.** Characterization of AGE formation and parent amino acid loss on glycated BSA samples. Control and glycated BSA samples, prepared as indicated in the text, were analysed by HPLC after fluorescent tagging. Concentrations were determined by comparison with authentic standards of AGE or parent amino acids derivatized under identical conditions. Data for AGE are expressed as millimoles of product relative to moles of parent Gly, an amino acid not expected to be modified by glycation; this comparative measure accounts for any protein loss during processing. Values for control BSA samples taken through identical protocols have been subtracted from the treatment data; in all cases these control data were typically <0.2 mmol AGE/mol Gly. Data for parent amino acid are expressed as a % of the levels detected in control BSA. Absolute levels of the amino acids quantified were in agreement with the expected amino acid composition from protein databases. Data are mean ± SD for triplicate determinations from separate experiments. \**P* < 0.05 relative to the corresponding BSA control for each condition, using 2-tailed unpaired *t*-tests.



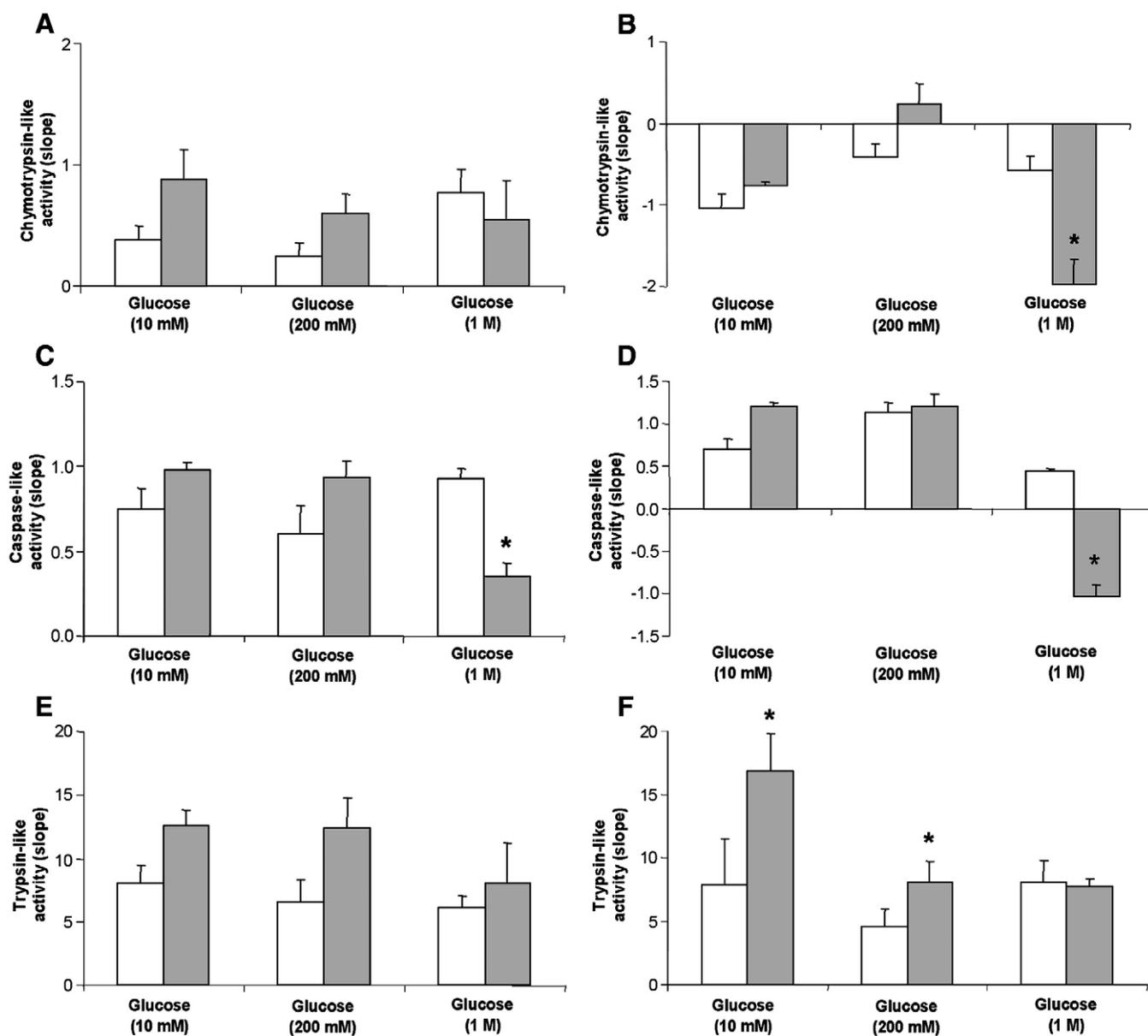
**Fig. 4.** Effect of native incubated BSA on proteasomal enzyme activities of J774A.1 cell extracts. White, black and grey columns represent 5 min incubation of cell extracts at 21 °C with 0, 1 and 4 mg/mL of control BSA that was incubated for 17 h or 8 weeks. Proteasomal enzyme activities of J774A.1 cell extracts after treatment were determined using (A) chymotryptic, (B) caspase or (C) tryptic substrate and measurement of specific activities as described in the Materials and methods. \* $P < 0.05$ , using one-way ANOVA and Newman–Keuls multiple comparison test to relevant control. Each column represents mean  $\pm$  SEM of 3 or more experiments.

have been detected in people with diabetes and correlate with the extent and duration of disease [33–37]. Thus increases in the level of glycated haemoglobin, a commonly used marker of hyperglycaemia, have been shown to both correlate with, and predict future cardiovascular disease [35–37]. The effects of these materials on proteasome function have been studied here in cell extracts from a macrophage-like cell line, as such cells play a key role in the development of atherosclerosis – the major macrovascular compli-

cation of diabetes, and the most important contributing factor to mortality and morbidity in people with diabetes [38]. Partially-purified cell extracts were used in these studies as modulation of the proteasomal system in intact cells by these agents is difficult to assess without accompanying perturbation of other aspects of cell metabolism. In their role as scavenging cells of the immune system, macrophages are likely to be exposed to glycated proteins as well as elevated glucose and reactive aldehydes [6,39]. However the effects of glucose, reactive aldehydes and glycated proteins upon cellular metabolism and proteasomal function are not yet fully understood, though there is considerable current interest in this area, with previous studies having examined, for example, the rates of degradation of glycated/glycosidized proteins in other cell types and tissues [3,22,40–42], and the effect of reactive aldehydes and glycated proteins on the other major proteolytic system of cells, the endo-lysosomal system (e.g. [3,7,43]).

The data reported here demonstrate that the effects of glucose, reactive aldehydes and glycated proteins on the three major activities of the proteasome are diverse and complex, with both stimulation and inhibition detected under different conditions. The data obtained indicate that free reactive aldehydes induce more pronounced effects on proteasomal enzyme activities than glucose itself under the same conditions. This is consistent with previous data that have indicated that reactive aldehydes are considerably more reactive (up to 20,000-fold) than glucose itself [15,25], though these aldehydes are present at much lower levels (see below). Glucose is the most abundant, but is also considered to be the least deleterious of the common sugars present in humans [3]. *In vivo* studies have suggested that elevated levels of glucose alone are insufficient to accelerate diabetes-associated atherosclerosis [44]. The results of the current study are also consistent with previous studies that have reported that elevated levels of glucose did not inhibit some cytosolic (e.g. glyceraldehyde-3-phosphate dehydrogenase and glutathione reductase) and lysosomal enzymes (e.g. the cysteine-dependent cathepsins, B and L), *in vitro*, whereas reactive aldehydes showed marked inhibitory effects [7,23].

In contrast to these previous reports of enzyme inhibition induced by reactive aldehydes (see above), the major effect observed in the current study was a stimulation of proteasomal enzymatic activity. A similar enhancement of nuclear proteasomal activity has been detected in intact cells treated with GO [45], though the exact mechanism by which this occurs has not been identified. A number of treatments, such as incubation with basic polypeptides, SDS and fatty acids have previously been shown to activate the 20S proteasome by a mechanism that involves changes in protein conformation [20]. Thus alterations in the conformation of the  $\beta$  subunits have been proposed to enhance catalytic activity, and changes in the  $\alpha$  ring may facilitate substrate entry to the catalytic core [20]. In the case of the 26S proteasome examined here, reactive aldehyde-induced alteration to either the lid or base of the 19S proteasome that delivers substrates to the proteolytic core may also play a role. As the species used to assess enzymatic activity in the current study were small peptides, rather than large proteins, changes in the conformation of the  $\beta$  subunits may be the most likely cause of the observed stimulation of activity, as the entry of these (unstructured/unfolded) peptides into the protein core are less likely to be affected by steric factors, though this obviously requires further study. It has also been reported that post-translational modifications of the proteasome complex (or its subunits) including phosphorylation and acetylation, can modulate proteasomal activities [19,46,47]. Thus phosphorylation of the  $\alpha$  subunits appears to enhance activity of the proteasome in mammalian cells, possibly by inducing conformational changes that modulate substrate accessibility [46]. Modification of specific Arg or Lys residues on proteasomal subunits by the reactive aldehydes under study (cf. data in Fig. 3) may induce similar conformation changes, and it is therefore of interest to note that enhanced levels of glycated ( $N^F$ -



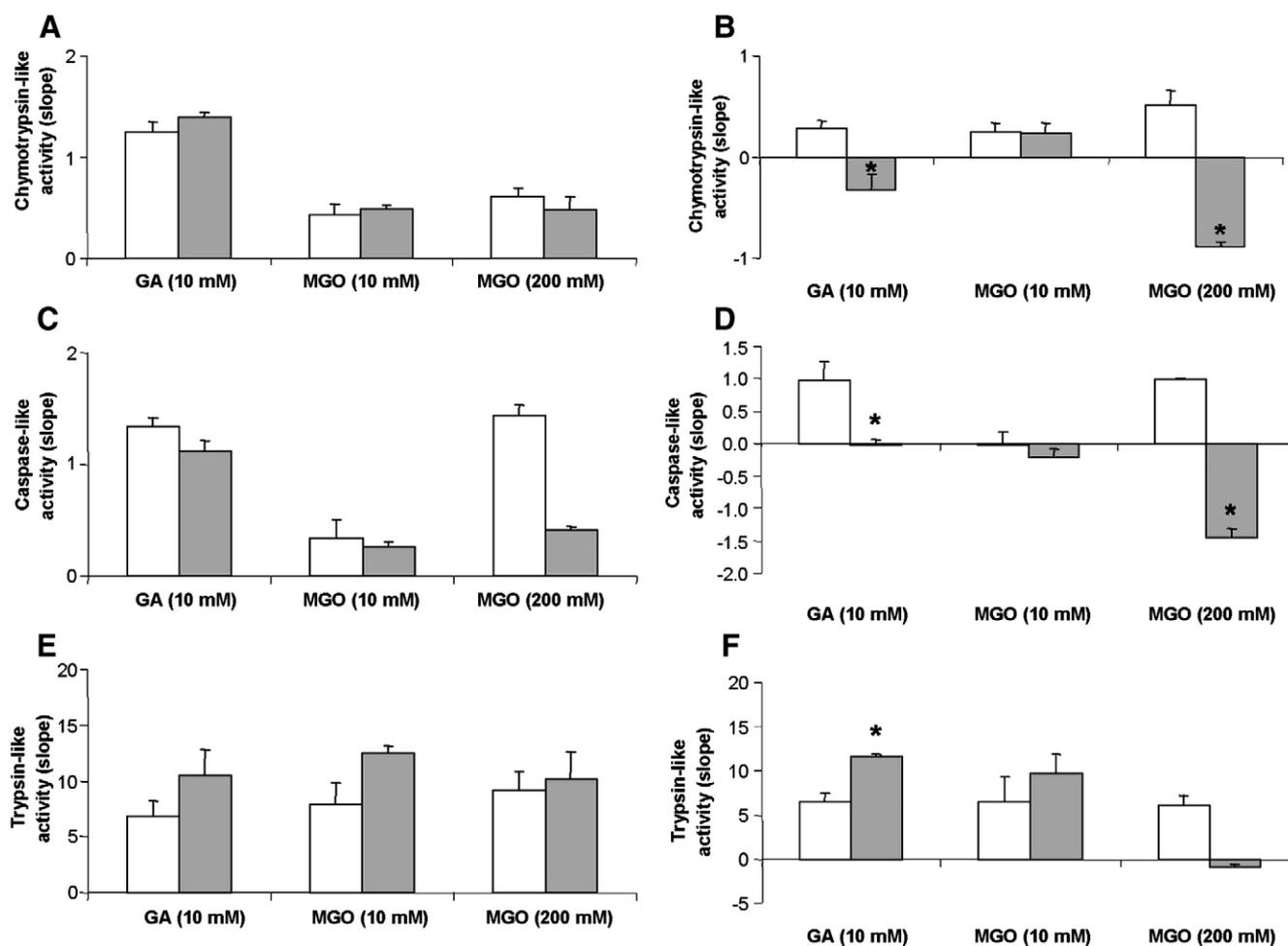
**Fig. 5.** Effect of BSA, pre-glycated with glucose, on proteasomal enzyme activity of J774A.1 cell extracts. BSA was pre-glycated with 10 or 200 mM glucose for 17 h or 1 M glucose for 8 weeks as described in Materials and methods. White and grey bars represent control BSA and BSA glycated with glucose, respectively. Cell extracts were incubated with 1 mg/mL (A, C and E) or 4 mg/mL (B, D and F) of glycated BSA or control BSA for 5 min at 21 °C, then the specific proteasome substrate was added and the activities were measured as described in the text. \* $P < 0.05$ , using two-tailed paired  $t$ -tests. Each column represents mean  $\pm$  SEM of 3 experiments.

carboxymethyllysine containing) proteasome protein have been detected in aging cells [48]. In contrast, acetylation of the  $N$ -terminal Thr residue of the active  $\beta$  subunits and the  $\alpha$  subunits results in inactivation of 20S proteasome [19,47]. A number of oxidants have also been shown to alter proteasome activity, with both inhibition [49,50] and activation reported [51]. Upregulation of proteasome activity may be of considerable biological relevance in light of the key role that this complex plays in the degradation of inhibitory proteins, such as  $\text{I}\kappa\text{B}$ , which prevents NF- $\kappa\text{B}$  activation and signalling [52], as has been reported in atherosclerotic plaques from people with diabetes [53].

The data reported here are in contrast to a previous study that reported an inhibitory effect of GO on proteasomal enzyme activities *in vitro* [22]. There are however, a number of significant differences between these two studies, with this previous investigation showing that GO inhibited the 20S proteasome purified from human placenta after a 30 min incubation period [22]; this preparation lacks the regulatory subunits present on the 26S form, and hence effects of the

reactive aldehydes on substrate accessibility and the control of this process, would not have been detectable in this earlier study. The 20S form of the proteasome was not examined in the current study, as the majority of protein turnover in eukaryotic cells appears to occur via the 26S complex [21,54]. Furthermore, whilst the short incubation times employed in the current study may be sufficient to alter the conformational structure of the 26S complex in such a way as to allow greater substrate accessibility and thus enhanced activity, the longer incubation times, used by Bulteau et al. [22], may result in the accumulation of more severe damage to the 20S core and a suppression of enzyme activity.

In contrast to the absence of effects induced by free glucose, significant changes in activity were detected with BSA pre-treated with glucose. With low (pathologically-relevant) concentrations of glucose (10 mM, 17 h), and short incubation times with high glucose (200 mM glucose, 17 h), a small but significant stimulation of the trypsin-like activity was detected with the highest concentrations of glycated protein examined. However with heavily glycated BSA (1 M



**Fig. 6.** Effects of BSA pre-glycated with reactive aldehydes on proteasomal enzyme activities of J774A.1 cell extracts. Cell extracts were incubated with 1 mg/mL (A, C and E) or 4 mg/mL (B, D and F) of control BSA, or BSA modified using 10 mM GA, 10 mM MGO or 200 mM MGO, for 5 min at 21 °C. Chymotrypsin-, caspase- or trypsin-like activity was measured as described in Materials and methods. White and grey bars represent control BSA and modified BSA by reactive aldehydes, respectively. \* $P < 0.05$ , using a two-tailed paired  $t$ -test relative to the corresponding control BSA. Each column represents mean  $\pm$  SEM of three experiments.

glucose, 8 weeks) marked inhibition of both the chymotrypsin- and caspase-like activities were observed and the stimulation of trypsin-like activity was reversed. With the short incubation times (17 h) only low levels of early (reversible) glycated adducts would be expected to be formed with glucose (10 and 200 mM), as a chemical relaxation time of about 2.5 h has been reported for glucose and human serum albumin (HSA) in blood plasma to give the Schiff's base under physiological conditions [55]. This low level of modification has been confirmed here (Fig. 3). Subsequent rearrangement of this species to fructosamine or an Amadori product occurs over a much longer time-scale with equilibrium reached by ca. 38 h [55]. Thus it would appear that the formation of low levels of early (reversible) adducts on BSA do not significantly inhibit proteasomal enzymatic activities, whereas the advanced glycation products that are formed in high yield as a consequence of very high glucose concentrations and long-term incubation (Fig. 3), have marked inhibitory effects. An absence of marked inhibitory effects with the combination of BSA and short-term glucose incubation has also been reported for lysosomal cysteine-dependent cathepsins [7]. The inhibition observed with high glucose/long-term incubation are consistent with those of Stolzing et al. [41] for isolated 20S proteasomes where inhibition of the chymotrypsin-, caspase- and trypsin-like activities was observed on incubation with AGE-BSA (produced by incubation of BSA with 0.25 and 1 M glucose for 6 weeks) for 1 h. In both the current study and this previous work [41] the caspase-like activity appears to be the most markedly

affected, and it is clear from the current data that this inhibition is rapid and can occur in <5 min.

Reactive aldehydes are known to be generated during glucose degradation (e.g. over a 3 week period at pH 7.4 and 37 °C), and that the presence of proteins, such as HSA, enhances their rate of formation [55]. Elevated levels of these aldehydes have also been detected in human plasma from people with diabetes compared to healthy controls, though there is considerable variation in the reported values [56–58]. The high reactivity of these aldehydes [55] with nucleophilic sites present on proteins (primarily Arg, Lys, His and Cys side-chains and the  $N$ -terminal amino group), limits the concentration of these materials in plasma, and these values represent steady-state concentrations at the time of sampling, rather than the overall fluxes of modifying agent to which a protein may be exposed over its lifetime. Whether these plasma levels reflect those present within cells is also unclear, and no specific measurements have been reported for cells. As many of the metabolic processes that generate reactive aldehydes are intracellular (e.g. decomposition of triose phosphates [13]) it is likely that cellular levels will be higher than those detected in plasma where the levels may reflect primarily glucose auto-oxidation and efflux of the reactive aldehydes from cells. Higher fluxes might also be expected at sites of inflammation (e.g. within the artery wall during the development of atherosclerosis) as a result of the localisation of enzymes that can generate reactive aldehydes at such sites (e.g. myeloperoxidase which generates GO and MGO from the amino acids

serine and threonine [14]). It is already established that other products of myeloperoxidase-mediated reactions accumulate on proteins to much higher levels in atherosclerotic lesions than are present in plasma/serum (up to 8-fold greater for apolipoprotein A-I, ca. 100-fold for apolipoprotein B-100 and ca. 115-fold for total protein [59,60]). In addition it is well established that there is a marked accumulation of lipid oxidation products, another potential source of reactive aldehydes, in atherosclerotic lesions, with these levels massively exceeding those in plasma [61]. These data suggest that higher levels of reactive aldehydes are likely to be present in atherosclerotic lesion and surrounding tissue, than might be detected in plasma. Thus measurements of steady-state levels of reactive aldehydes in plasma, may provide very limited information on the overall fluxes of reactive aldehydes to which proteins may be exposed within cells and tissues, and particularly at the sites of developing micro- and macrovascular complications in diabetes.

In the light of the above discussion, it is possible that the inhibitory effects observed with highly glycosylated BSA on the chymotrypsin- and caspase-like activities of the proteasome may arise from reactions of reactive aldehydes formed from glucose over the extended incubation period, rather than from direct glucose reactions (cf. the elevated levels of CML and CEL detected on the BSA incubated with glucose for 8 weeks). This conclusion is consistent with the observed inhibitory effect of BSA pre-glycosylated with the aldehydes (as opposed to the stimulation detected with the free aldehydes themselves), with this inhibition being most evident with the caspase- and chymotrypsin-like activities. Thus inhibition of both activities was detected with 4 mg mL<sup>-1</sup> of BSA pre-glycosylated with 10 mM GA (in contrast to a stimulation of trypsin-like activity). In contrast, with the same concentrations of MGO, no significant changes in activity were detected, though inhibition was again seen (with all three activities) with higher concentrations of MGO. These data suggest that the species generated by GA, such as CML (cf. Fig. 3) have a greater inhibitory effect than those generated by MGO (CEL and MG-H1/2), though whether this is a manifestation of the nature of these products, or their relative yields is unclear. These inhibitory effects of pre-glycosylated proteins mirror the suppressive effects of similar species on the activity of lysosomal cathepsins [7,62].

Previous studies have shown that incubation of MGO with BSA at a molar ratio of 10:1 results in a rapid increase in both irreversible and reversible binding of MGO to BSA and a rapid decrease in free MGO over 24 h [15]. The major sites of irreversible adduction under these conditions are Arg residues with these yielding imidazolones including MG-H1 and MG-H2a/b [15]. Incubation of GA with BSA at a molar ratio of 200:1, results in modification of Lys residues and generates C-2-imine cross-links and CML, with the maximum formation of these products detected after ca. 30 min [63]; these data are consistent with the results obtained in the current study (cf. Fig. 3), where the molar ratios of aldehyde:BSA were 8, 166 and 8 for the 10 and 200 mM MGO, and 10 mM GA conditions respectively. The AGE products formed on the BSA may be responsible for the observed inhibition of enzymatic activity, though it is also possible that this arises from loss of the parent amino acid side-chains. Whether, and exactly which, AGE species is/are responsible for inhibition, cannot be discerned from the present data due to the difficulty in generating specific individual modifications, in isolation, on this protein. However it is clear from the data obtained that the extent of proteasomal enzyme alteration by glycosylated proteins varies with: (1) the total amount of glycosylated proteins that the proteasomal preparations are exposed to, (2) the nature of the glycosylating agent, (3) the extent of glycosylation at a fixed incubation time and protein concentration, and (4) the nature of proteasomal active site, with the sensitivity to inhibition typically following the order caspase-like > chymotrypsin-like > trypsin-like.

The mechanism of inhibition may be any of the following: (1) glycosylation may result in the formation of protein cross-links and AGE

that stabilize the BSA structure [18,22,64] resulting in a slower rate of degradation by 26S proteasome, and hence slower cleavage of the peptide used to assess enzyme activity as a result of competition for active sites [22]; (2) the glycosylated BSA may react, or form an adduct, with the nucleophilic oxygen of the Thr-1 residues in the  $\beta$ -type subunits [65] thereby inhibiting activity; (3) the AGE (or oxidation) products may modulate the structure or function of the  $\alpha$  ring of catalytic core that facilitates the substrate entry [2,65] with this resulting in reduced access of the peptide substrate and hence a reduced rate of cleavage; or (4) the glycosylated protein may modulate (or block) the activity of 19S regulatory subunits, that control the access of substrates to the core [65]. Which of these mechanisms are operative in the current study cannot be determined from the current data, and further studies are required to elucidate this point.

One limitation of the current investigation is the use of cell extracts that contain the proteasome, rather than highly purified preparations. The former were employed due to difficulties in isolating sufficient active 26S complex from cells, without significant inactivation and/or loss of the 19S subunits that would compromise data analysis. However the cruder cell extracts are likely to contain other non-proteasomal proteases, such as calpains and possibly lysosomal cathepsins [21]. Possible contributions of these non-proteasomal enzymes to the observed proteolytic activity was minimised by quantifying activity at pH 7.4, where most lysosomal enzymes show little activity [21], and by including parallel epoxomicin-treated samples. Subtraction of residual activity obtained in the presence of this specific proteasomal inhibitor [66], from the total activity, provided data that has been ascribed to the proteasome [21]. However, in some experiments this resulted in an apparent “negative” activity which is consistent with the glucose/free aldehyde or glycosylated proteins also inhibiting non-proteasomal activity in the samples, resulting in activities lower than those of the epoxomicin controls. Potential circumvention of this effect by either pre- or post-treating the incubations with epoxomicin was considered but not attempted as this would result in different overall incubation periods and potentially misleading data. Blockade of other contributing proteolytic activities by use of inhibitors was not attempted due to the diverse and unknown nature of all of these activities and the non-availability of specific inhibitors.

In conclusion, glycosylated proteins generated by either reactive aldehydes or long-term exposure to high glucose concentrations has been shown to inhibit the chymotrypsin-, caspase- and to a lesser extent the trypsin-like activities of proteasomal fractions from J774A.1 cells. This suppression of proteasome activity by glycosylated proteins, in conjunction with the previously reported inhibition of lysosomal cathepsin enzymes by the same species [7] may contribute to the intracellular accumulation of glycosylated and otherwise damaged proteins in people with diabetes. A decreased rate of intracellular catabolism of these glycosylated proteins is likely to result in a higher concentration (accumulation) and increased residence time of these materials within cells resulting in an enhanced level of further secondary reactions and advanced glycation products. A longer half-life for these modified proteins within cells would also be expected to increase the likelihood of protein cross-linking and aggregation, with deleterious consequences on cellular function, and result in a further inhibition of protein catabolism. Thus a vicious, self-sustaining, cycle of ever-increasing cellular dysfunction may result in cells exposed to these agents and material, with this contributing to the cell and tissue alterations observed in diabetes-associated atherosclerosis.

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