

Research Article

Non-cross-linking advanced glycation end products affect prohormone processing

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Advanced glycation end products (AGEs) are non-enzymatic post-translational modifications of amino acids and are associated with diabetic complications. One proposed pathomechanism is the impaired processing of AGE-modified proteins or peptides including prohormones. Two approaches were applied to investigate whether substrate modification with AGEs affects the processing of substrates like prohormones to the active hormones. First, we employed solid-phase peptide synthesis to generate unmodified as well as AGE-modified protease substrates. Activity of proteases towards these substrates was quantified. Second, we tested the effect of AGE-modified proinsulin on the processing to insulin. Proteases showed the expected activity towards the unmodified peptide substrates containing arginine or lysine at the C-terminal cleavage site. Indeed, modification with *N*^ε-carboxymethyllysine (CML) or methylglyoxal-hydroimidazolone 1 (MG-H1) affected all proteases tested. Cysteine cathepsins displayed a reduction in activity by ~50% towards CML and MG-H1 modified substrates. The specific proteases trypsin, proprotein convertases subtilisin-kexins (PCSKs) type proteases, and carboxypeptidase E (CPE) were completely inactive towards modified substrates. Proinsulin incubation with methylglyoxal at physiological concentrations for 24 h resulted in the formation of MG-modified proinsulin. The formation of insulin was reduced by up to 80% in a concentration-dependent manner. Here, we demonstrate the inhibitory effect of substrate-AGE modifications on proteases. The finding that PCSKs and CPE, which are essential for prohormone processing, are inactive towards modified substrates could point to a yet unrecognized pathomechanism resulting from AGE modification relevant for the etiopathogenesis of diabetes and the development of obesity.

Introduction

The modification of amino acids by hexoses and dicarbonyls can result in the formation of protein-bound advanced glycation end products (AGEs) [1]. Such protein-bound AGEs are implicated in the etiopathogenesis of aging-related diseases including diabetes mellitus and associated complications [2]. One potential pathomechanism is the impact of AGE modification of specific substrates such as prohormones on protease activity. The pathophysiological effects of altered proteolysis upon the formation of large, cross-linking AGEs in the extracellular matrix has been investigated in previous studies [3–5]. Glycation-altered proteolysis of short-lived intracellular proteins has also been proposed as a pathomechanism in diabetes and aging [6]. Such alterations are particularly interesting with regard to small non-cross-linking AGEs as these can form quickly. Reactions of the dicarbonyls methylglyoxal (MG) and glyoxal take place within minutes to hours rather than days and weeks which it takes for glucose-dependent modifications of amino acids to form [7,8].

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The majority of protein or peptide modifications with AGEs take place at side chains of arginine and lysine [7,9]. In addition, cysteine and histidine residues can be modified by dicarbonyls but the adducts are not stable [10]. Several groups of proteases cleave substrates C-terminal of these two basic amino acids and could thus be affected. This includes cysteine cathepsins (CTS) which are involved in the general protein turnover and show the lowest substrate specificity for arginine and lysine [11]. Neuroendocrine prohormones are processed by endoproteolytic cleavage at arginine or lysine with high specificity. This is carried out by proprotein convertase subtilisin–kexin (PCSK) followed by carboxy-terminal processing via carboxypeptidase E (CPE) [12–14]. This applies to the activation of proinsulin [15] to insulin, POMC to adrenocorticotrophic hormone, melanocyte stimulating hormone and beta-endorphin [16,17], and the activation of growth hormone releasing hormone and glucagon [17]. Trypsin is another endopeptidase with high specificity for the cleavage C-terminal of arginine and lysine [14]. The protease is of interest due to the extensive use for proteomics applications.

For the current study, we chose to investigate the effect of specific substrate-AGE modifications on above mentioned proteases. Peptide substrates with AGE modifications for activity measurements are not available. Thus, we aimed to synthesize such substrates with unmodified and modified versions of C-terminal arginine and lysine, namely methylglyoxal-hydroimidazolone 1 (MG-H1) and *N*^ε-carboxymethyllysine (CML) [18–22]. These were to be used to determine protease activity in a quantitative fashion by fluorometry or LC–MS/MS.

For the second part of the study, we wanted to test the effects of a non-cross-linking AGE modification on the processing of a prohormone, namely insulin. Proinsulin in humans is processed exclusively by the endopeptidase PCSK1 which removes the C-peptide followed by the removal of the C-terminal basic amino acids of the B-chain by CPE. This process was to be replicated *in vitro* for unmodified proinsulin and MG-modified proinsulin. The effect on the generation of insulin as well as the formation of modified proinsulin was to be quantified by LC–MS.

We hypothesize that substrate modifications even with non-cross-linking AGEs can have a significant effect on protease processing. Potential consequences of non-cross-linking AGEs could be an impaired protein turnover or a disturbed formation of neuroendocrine hormones.

Results

Synthesis of unmodified and modified peptides for activity assays

Peptides were synthesized by solid-phase peptide synthesis (SPPS). The structure was based on previously reported peptides suitable for activity assays of the proteases of interest. To test the effect of modification of the amino acid C-terminal of the fluorophore of the peptide substrates the methylglyoxal modified arginine, MG-H1, was incorporated via a protected amino acid. The N-terminus of the amino acid was modified with fluorenylmethoxycarbonyl (Fmoc) while the side chain was protected with bis(4-methoxyphenyl)methyl (Dod) resulting in Fmoc-MG-H1(Dod)-OH. This enabled us to incorporate MG-H1 selectively at the desired position. While the protecting group Dod could not always be removed to the full extent, free peptides were easily separated from those with a protecting group by preparative high-performance liquid chromatography (HPLC).

The lysine modification CML was synthesized by selective alkylation of the lysine side chain on the solid phase. The lysine to be modified was incorporated with an allyloxycarbonyl (Alloc) protected side chain as Fmoc-Lys(Alloc)-OH. After Alloc removal, the amino group of the side chain was alkylated. While a portion of the peptide was alkylated twice at the lysine side chain, this byproduct could be easily separated from singly alkylated CML by preparative reverse phase HPLC.

A list with details of all peptides used in this study is given in Table 1. All peptides were characterized by LC–MS after purification by HPLC. Total ion chromatograms of the LC–MS analysis are supplied in the supplements (Supplementary Figures S1–S14). The chemical structure of the probes employed for the CTSL assay is shown exemplary for the rest (Figure 1). Reaction arrows indicate potential pathways of formation of the arginine or lysine adducts *in vivo*. These pathways differ from the SPPS-based pathway which was employed here to create the peptide substrates.

Proteases with intermediate specificity: cysteine cathepsins

As expected, both CTSL (Figure 2A) and CTSB (Figure 2B) showed activity towards peptides with arginine as well as the alternative peptides containing lysine. When arginine was replaced with the methylglyoxal-arginine adduct, MG-H1 a reduction in activity of ~50% was seen for both proteases. A similar effect was present when lysine was replaced with the glyoxal lysine adduct, CML, for CTSL and CTSB.

Table 1. Peptide sequence, corresponding average mass as well as theoretical m/z (m/z_{theor}) and m/z determined by LC–MS (m/z_{exp}) are given for the peptides employed for the activity assays

No.	Peptide sequence	Activity assay	Avg. mass	m/z_{theor}	m/z_{exp}	z	RT (min)
1	Ac-His-Arg-Tyr- Arg -ACC	CTSL, trypsin	872.92	873.41	873.39	2	8.71
2	Ac-His-Arg-Tyr- MG-H1 -ACC	CTSL, trypsin	926.98	927.42	927.39	2	8.94
3	Ac-His-Arg-Tyr- Lys -ACC	CTSL, trypsin	844.92	845.41	845.39	1	7.48
4	Ac-His-Arg-Tyr- CML -ACC	CTSL, trypsin	902.95	903.41	903.40	1	7.73
5	Cbz-Phe- Arg -ACC	CTSB, trypsin	655.70	656.28	656.27	1	13.72
6	Cbz-Phe- MG-H1 -ACC	CTSB, trypsin	709.75	710.29	710.27	1	13.46
7	Cbz-Phe- Lys -ACC	CTSB, trypsin	627.68	628.28	628.26	1	13.47
8	Cbz-Phe- CML -ACC	CTSB, trypsin	685.72	686.28	686.29	1	13.51
9	pGlu-Arg-Thr-Lys- Arg -ACC	PCSK1, -3, -7	870.96	871.45	871.44	1	6.99
10	pGlu-Arg-Thr-Lys- MG-H1 -ACC	PCSK1, -3, -7	925.00	463.24	463.22	2	7.91
11	Bz-Ala- Arg -OH	CPE	349.39	350.18	350.18	1	8.98
12	Bz-Ala- MG-H1 -OH	CPE	403.43	404.19	404.19	1	9.67
13	Bz-Ala- Lys -OH	CPE	321.37	322.18	322.18	1	8.45
14	Bz-Ala- CML -OH	CPE	379.40	380.18	380.18	1	7.96

Charge state (z) and retention times (RT) of the peptides are indicated for the LC–MS measurement. Amino acids are given as three letter code. Positions where amino acids were exchanged are shown in bold. Non-standard abbreviations are: Ac, Acetyl; ACC, 7-amino-4-carbamoylmethylcoumarin; Bz, benzoyl; Cbz, benzyloxycarbonyl; CML, carboxymethyllysine; MG-H1, methylglyoxal-hydroimidazolone 1; pGlu, pyroglutamic acid.

Proteases with high specificity: trypsin, PCSKs and CPE

Next, we tested proteases with high specificity towards arginine and/or lysine residues. The endopeptidase trypsin has great specificity towards arginine and lysine residues in the C-terminal position. We tested the effect of MG-H1 and CML substitution on this protease using the peptide family employed for the CTSL assay (Figure 3A) as well as the peptide family employed for the CTSB assay (Figure 3B). While activity was present towards arginine and lysine-containing peptide substrates, no activity was detectable towards those containing MG-H1 and CML.

Another set of proteases which require basic amino acids C-terminal of the cleavage site are the endopeptidases of the PCSK family. With the exception of subtilisin/kexin-isozyme-1 and PCSK9, the family is specific for dibasic cleavage sites with a strong preference for cleavage C-terminal of arginine. These proteases process a whole range of essential neuroendocrine peptide hormones.

The previously published peptide substrate suitable for activity measurement of PCSKs was synthesized by SPPS alongside the version containing the modified amino acid MG-H1. Similar to trypsin, activity was present as expected towards the arginine-containing substrate. Activity was completely absent towards the substrate containing MG-H1 in the C-terminal position for all three of the PCSKs tested (Figure 3C–E).

PCSKs act in concert with CPE. The carboxypeptidase CPE has great specificity for arginine and lysine and removes the C-terminal basic amino acids after endoproteolytic cleavage by PCSK to release the active peptide hormone. Activity of CPE was determined by LC–MS/MS towards previously published peptides containing either C-terminal arginine or lysine or towards one of the modified amino acids CML or MG-H1. Synthesis of this peptide was carried out by SPPS as described above. Activity was present for the substrate containing C-terminal arginine or lysine but not for either of the modified substrates in the C-terminal position (Figure 3F).

Effect of prohormone modification on active hormone formation

Next, we tested whether dicarbonyl modification of a physiologically important substrate also affects its processing via proteases. We used proinsulin as a model substrate and modified it with MG *in vitro* (Figure 4) [23]. Arginine and lysine residues are highlighted as these amino acids can become modified with MG. The

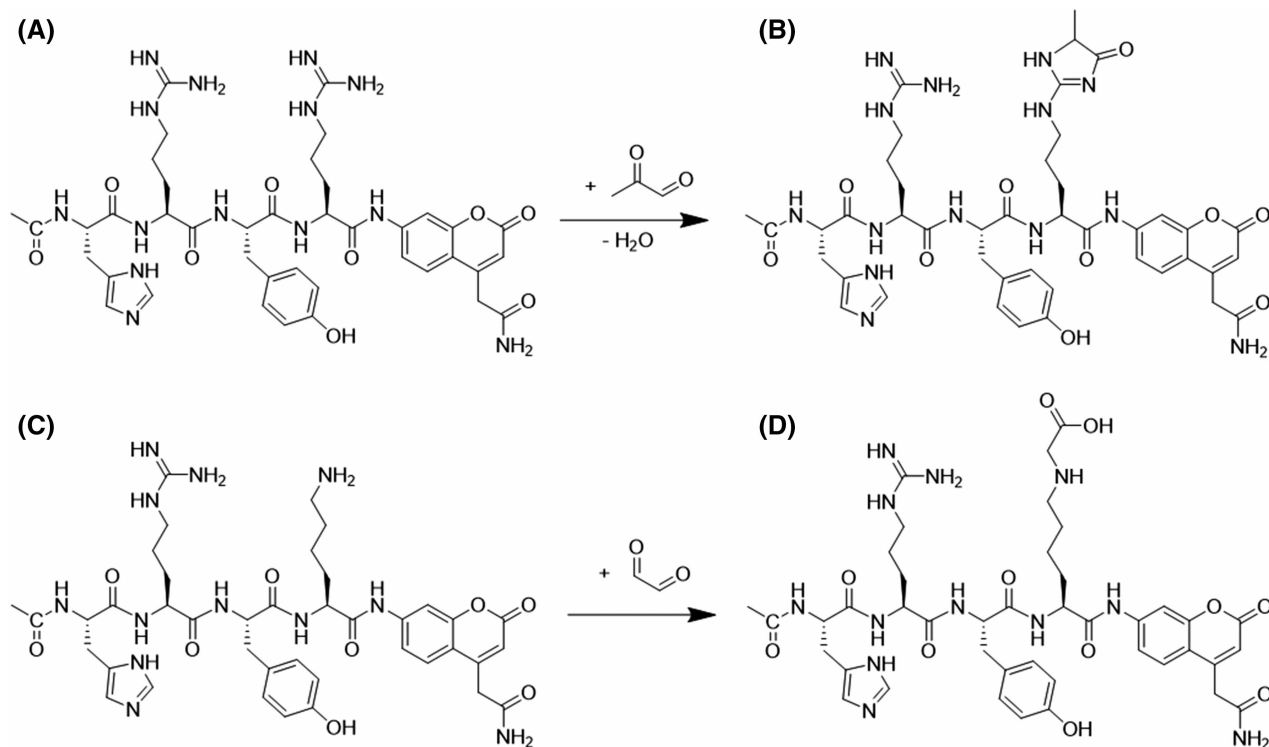


Figure 1. Exemplary chemical structure and formation pathway *in vivo*.

Chemical structure of the fluorescent peptide probes Ac-His-Arg-Tyr-X-ACC used for the CTSL activity assay are shown as an example. Peptides were synthesized by solid phase peptide chemistry allowing for selective incorporation of unmodified or modified amino acids in position P1. The variable X in position P1 represents unmodified amino acids Arg (A) or Lys (C) or modified amino acids MG-H1 (B) and CML (D). Potential pathways of formation of modified amino acids *in vivo* are indicated: Arginine forms MG-H1 upon modification with methylglyoxal and (A,B) lysine forms CML upon modification with glyoxal (C,D).

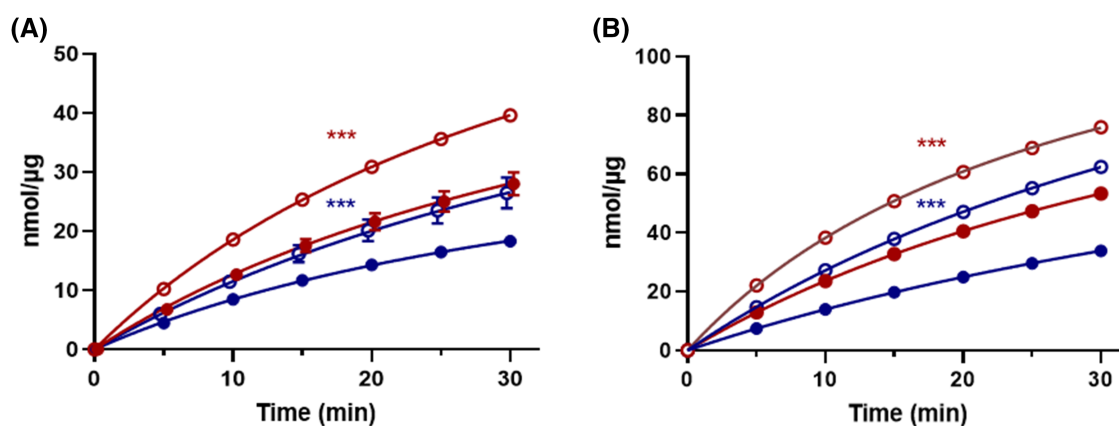


Figure 2. Kinetic measurement of protease activity of cysteine cathepsins CTSL and CTSB.

Protease activity was quantified by fluorimetry over 30 min. Activity measured is given for CTSL (A) with substrates Ac-His-Arg-Tyr-X-ACC and CTSB (B) with substrate Cbz-Phe-X-ACC. The variable X in position P1 represents unmodified amino acids Arg (open circle, red) or Lys (open circle, blue) or modified amino acids MG-H1 (closed circle, red) and CML (closed circle, blue). Data are $\pm$ SEM, $n = 3$. AUC of the curves was compared by two-tailed Student's *t*-test for modified vs. unmodified amino acid. *** $P < 0.001$.

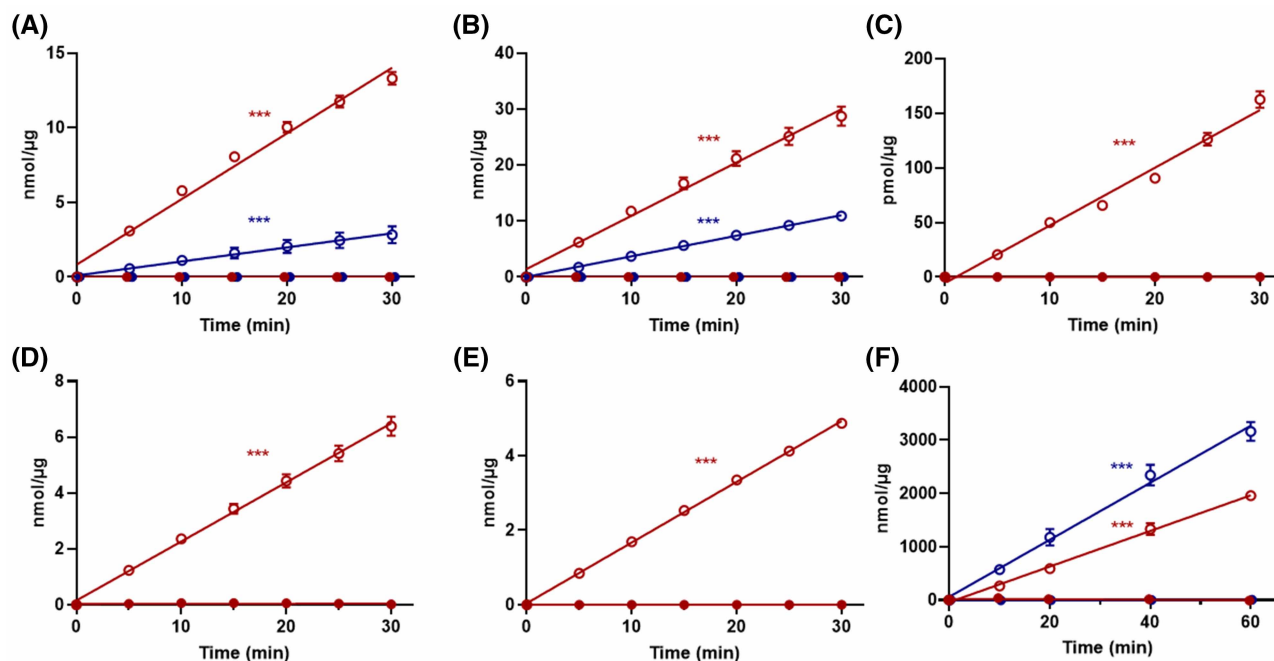


Figure 3. Kinetic measurement of protease activity of trypsin, preproteinconvertases and carboxypeptidases.

Protease activity was quantified by fluorimetry (A–E) or LC–MS/MS (F). Trypsin activity was determined for substrates Ac-His-Arg-Tyr-X-ACC (A) and Cbz-Phe-X-ACC (B). Activity of PCSK1 (C), -3 (D) and -7 (E) was quantified towards substrates pGlu-Arg-Thr-Lys-X-ACC. Protease activity for CPE was quantified towards substrates Bz-Ala-X-OH (F). The variable X in position P1 represents unmodified amino acids Arg (open circle, red) or Lys (open circle, blue) or modified amino acids MG-H1 (closed circle, red) and CML (closed circle, blue). Data are $\pm$ SEM, $n = 3$, AUC of the curves was compared by two-tailed Student's t -test for modified vs. unmodified amino acid. *** $P < 0.001$.

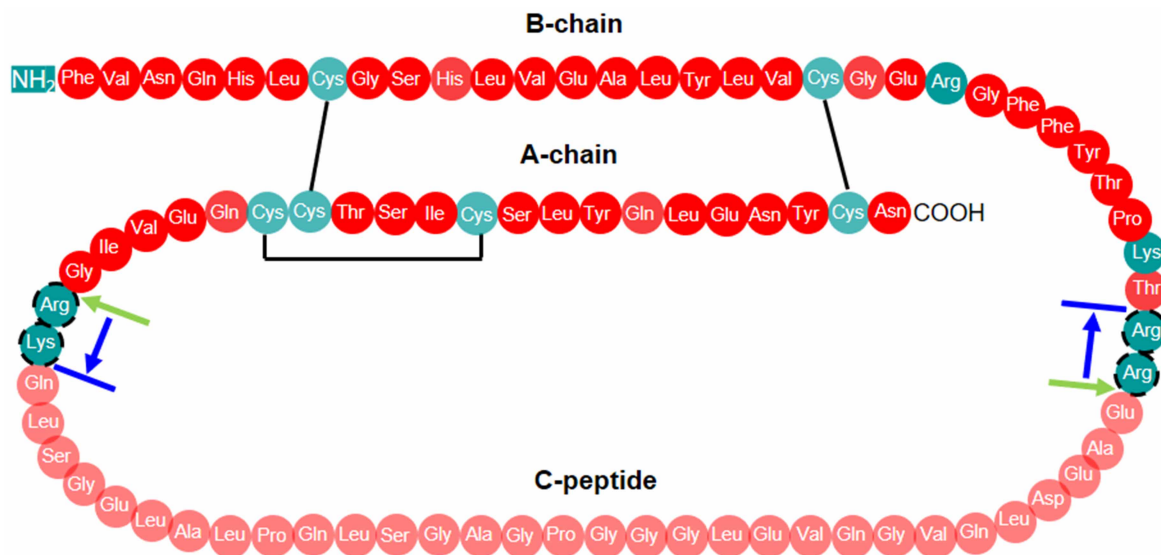


Figure 4. Processing and potential sites of modification of human proinsulin.

Shown is the sequence of human proinsulin adapted from Oyer et al. The cleavage site of PCSK-1 (green arrow) and amino acids removed by CPE (blue arrow) are indicated. Processing releases C-peptide (light red) and the intact insulin molecule consisting of A- and B-chain connected by disulfide bridges (black line). Basic amino acids arginine and lysine and the N-terminus which are potential modification sites for dicarbonyls are labeled in green. Cysteines, which are reversible sites of modification are labeled in light green. Figure 4 has been reproduced from the Figure 8 of the publication by Oyer et al. [23].

prohormone was incubated with equimolar concentrations 10 μ M, 50 μ M or without MG for 24 h. The MG concentrations were chosen based on previously reported — MG levels in tissue and cells. Due to the reactivity of the molecule determination of MG levels is a difficult task and levels are not universally agreed upon. Reported levels span a wide range from 0.3 to 174 μ M in mouse brain and from 14 to 25 μ M in HeLa-, COS-1- and 3T3 cells [24–26]. Proinsulin was then processed with PCSK1 followed by CPE in order to yield insulin. MG-H1 modified proinsulin and processed insulin were quantified by LC–MS (Table 2).

Incubation of proinsulin with MG resulted in the formation of methylglyoxal-modified proinsulin in a dose-dependent manner which remained even after PCSK1 processing. Two different methylglyoxal-dependent modifications of proinsulin were seen with a mass shift of +54.0 and +72.0. These correspond to shifts seen upon the formation of methylglyoxal-hydroimidazolone proinsulin (MG-H-proinsulin, +54.0, Figure 5A) and carboxyethyl proinsulin (CE-proinsulin, +72.0, Figure 5B). In parallel to the increasing amounts of MG-H-proinsulin and CE-proinsulin, lower amounts of fully processed insulin were detectable after the addition of CPE (Figure 5C).

Discussion

Here, we provide proof that non-cross-linking AGE modifications of substrates have a significant inhibitory effect on protease processing. To our knowledge, this is the first time that this was investigated by employing chemically defined AGE-modified protease substrates. Specifically, we investigated the effect when arginine is modified to MG-H1 and lysine is modified to CML. In both cases, we see a partial loss of activity of CTSB and CTSL and a complete loss of activity by trypsin, PCSKs and CPE towards the substrates. We also show exemplary for proinsulin activation that such an inhibition is present for physiologically relevant processes. The formation of MG-H-proinsulin and CE-proinsulin shown here is associated with a strong reduction in the formation of active insulin via processing by the physiologically relevant enzymes PCSK1 and CPE.

The altered cleavage of lysine after modification to CML is likely due to the newly formed carboxy group. This modification reverses the charge of the side chain. Trypsin features a glutamic acid while PCSK3 features both glutamic acid and aspartic acid in the active site, both of which would lead to electrostatic repulsion of CML [27,28]. Similarly, duck carboxypeptidase D which is structurally related to CPE contains an aspartic acid residue in the active site [29]. The modification of the guanidine group of the arginine side chain by methylglyoxal to MG-H1 results in a drop of the pK_a from 12 to 4.6 resulting in the loss of the positively charged side chain [18]. This will lead to altered electrostatic attraction of MG-H1 as compared with arginine in the active sites. In addition, the hydroimidazolone ring of MG-H1 may present a steric hindrance.

The molecular basis for the preference of cathepsin B and cathepsin L towards arginine and lysine is less clear. Histidine, cysteine and serine are proposed to be essential for the active site of the endopeptidases [11,30]. This could explain the less stringent charge selectivity resulting in the capacity to degrade the substrates despite the modification to CML or MG-H1.

We demonstrate that modification of proinsulin with MG leads to an altered processing via PCSK1 and CPE. Incubation of proinsulin with MG *in vitro* leads to an accumulation of MG-H and CE-modified proinsulin and a decreased production of active insulin. This illustrates the sensitivity of PCSK1 and CPE towards modifications of the prohormone. It is likely that this lack of processing is due to the modification of the dibasic cleavage sites. However, modification of arginine or lysine of the B-chain or the N-terminal primary

Table 2. Average mass, theoretical m/z (m/z_{theor}) and m/z determined by LC–MS (m/z_{exp}) are given for the prohormones and hormones

(Pro)-hormone	Avg. mass	m/z_{theor}	m/z_{exp}	z	RT (min)
CE-proinsulin	10 548.86	1758.02	1758.01; 1760.50	6	13.3
MG-H-proinsulin	10 530.84	1755.02	1755.00; 1757.50	6	13.3
Proinsulin (human rec.)	10 476.79	1746.01	1746.00; 1748.50	6	13.4
Insulin	6889.78	1378.03	1378.03; 1381.22	5	13.9
Porcine insulin	5777.55	1444.41	1444.41; 1447.41	4	14.1

In addition, the charge state and the retention time of the molecules are shown.

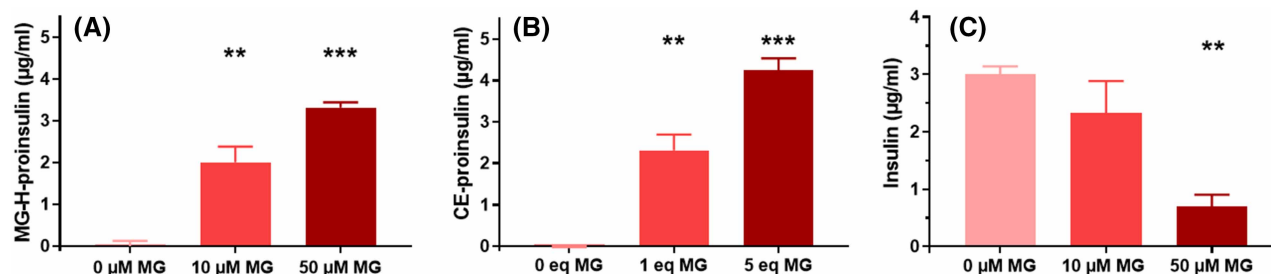


Figure 5. Effect of MG modification of proinsulin on the processing.

MG modified proinsulin was quantified after digestion with PCSK1. Two mass shifts after MG modification were observed. These correspond to previously reported mass change of methylglyoxal-derived-hydroimidazolone modified arginine (A, MG-H-proinsulin, +54.0) and carboxyethyl adduct to arginine or lysine (B, CE-proinsulin, +72.0). Formation of insulin was quantified after sequential digestion with PCSK1 and CPE (C). MG-H1 modified proinsulin is also given relative to the total amount of active insulin formed (C). Data are $\pm$ SEM and $n = 3$; ** $P < 0.01$, *** $P < 0.001$; Student's t -test for 10 μM MG or 50 μM MG vs. 0 μM MG.

amine cannot be excluded. Modification of cysteine side chains in the current experiment is highly unlikely to interfere with the digestion process. Aminoguanidine was added prior to the digestion so that any MG bound reversibly to cysteine would have reacted covalently with aminoguanidine [10].

There are limitations of the current data set with regard to the relevance on prohormone processing *in vivo*. While we used MG levels in the range previously reported in cells and tissue, additional proteins are present *in vivo* and these would function as alternative targets for MG binding, lowering the MG stress towards the prohormone [24–26]. Also, the modification of proteins with each individual AGE *in vivo* is low [31,32]. In support of a potential relevance *in vivo*, it needs to be considered that there are many different types of precursors, several of which have not been investigated thoroughly [33]. Thus, even if the concentration of a single precursor and modification is low, the sum of different types of modifications could result in a significant pathophysiological effect. Furthermore, protein-bound AGEs only represent one part of the burden caused by non-enzymatic glycation. There is evidence that free AGEs are elevated more strongly than protein-bound AGEs which at least in part stem from the hydrolysis of protein-bound AGEs [34]. These free AGEs are also associated with the development of diabetic complications [35]. Thus, studies investigating protein-bound and free AGEs in parallel to protein turnover are needed. It would also be important to obtain more data for protein-bound AGEs for such tissues which are prone to the development of complications in diabetes. In addition to the modification of substrates, reactive dicarbonyls may also bind to proteases directly modifying their activity [36,37]. Thus, in situations of elevated dicarbonyl levels, there are two mechanisms affecting proteolysis, namely the modification of substrates as well as the modification of the protease itself.

An undisturbed prohormone processing is essential and changes in PCSK1 and CPE activity are associated with obesity, diabetes and hyperproinsulinemia [38–40]. A disturbed prohormone processing has been shown for patients with type 2 diabetes as well as type 1 diabetes where pro islet amyloid polypeptide (proIAPP) processing was affected in addition [41–43]. An altered processing of POMC which is also carried out by PCSK1 and CPE is associated with obesity [44]. Considering the more recent subclassification of patients with type 2 diabetes it would be those with insulin deficiency which are most likely affected by the altered prohormone processing described here [45]. In addition, we show that proteases which are involved in the bulk degradation of proteins via the lysosomes, such as CTSL and CTSB, are capable of degrading substrates modified with MG-H1 and CML but at a decreased rate. Such an impairment may be pathophysiologically relevant in diseases associated with aging where proteostasis is affected like Alzheimer's and Parkinson's disease [6,46,47].

One essential finding for proteomics analysis is the complete lack of processing of MG-H1-modified arginine and CML-modified lysine residues by trypsin. There is the possibility to look for missed cleavage sites at the post-analytical stage when employing trypsin digestion prior to LC–MS/MS analysis. Nevertheless, the peptides present after a missed cleavage will in many cases be too large for sensitive detection. Thus, a proteomics approach for the detection of AGE-modified sites will ideally employ additional proteases complementing trypsin.

In summary, we show that there are strong changes in proteolytic activity towards non-cross-linking AGE-modified amino acids. The altered proteolytic behavior can be explained by changes in electrostatic

interactions as well as steric changes of the modified amino acids. Whether the demonstrated effect of altered proteolysis upon AGE modification of prohormones is a potential pathomechanism for diabetes, obesity and related endocrine disorders needs to be investigated in future studies.

Experimental procedures

Solvents and chemicals were obtained from Merck (Darmstadt, Germany) unless specified otherwise. Protected amino acids were obtained from Orpegen Peptide Chemicals GmbH (Heidelberg, Germany) unless stated otherwise.

Solid-phase peptide synthesis

Peptides for the fluorophore-based assay were synthesized with the C-terminal fluorophore 7--amino-4-carbamoylmethylcoumarin (ACC) [48]. Blocking of the N-terminus was carried out by acetylation (Ac), pyroglutamic acid (pGlu) or benzyloxycarbonyl (Cbz) in line with the previous synthesis of the respective substrates. Amino acids in P1 are indicated with X and were replaced with Arg, MG-H1, Lys or CML. The following three groups of peptides were synthesized for the fluorimetry-based assay: Ac-His-Arg-Tyr-X-ACC, pGlu-Arg-Thr-Lys-X-ACC and Cbz-Phe-X-ACC.

Peptides were prepared as described previously [19,48]. The fluorescent leaving group *N*-Fmoc-ACC (2 eq, ABCR, Germany) was coupled to Rink Amide resin (1 eq, 50 μ mol, Tentagel S RAM, RAPP Polymere GmbH, Germany) with HOBt (2 eq) and DICl (2 eq) in DMF after Fmoc removal from the resin with 20% piperidine/DMF.

Fmoc was removed and the first amino acid (10 eq) was coupled using HATU (10 eq) and 2,4,6-collidine (10 eq) in DMF for 24 h. The MG-modified amino acid MG-H1 was incorporated with the side chain protecting group bis(4-methoxyphenyl)methyl (Dod) resulting in the building block Fmoc-MG-H1(Dod)-OH. This building block was synthesized according to a previously published procedure [18]. The amino acid CML was incorporated via Fmoc-Lys(Alloc)-OH (ABCR, Germany). The selective modification of the lysine side chain to yield CML is described in detail later. Alternatively, arginine or lysine in position P1 were incorporated as Fmoc-Arg(Pbf)-OH and Fmoc-Lys(Boc)-OH, respectively. The following amino acids (10 eq) were coupled with HOBt (10 eq) and DICl (10 eq) in DMF: Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Tyr(OtBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Phe-OH, Fmoc-Thr(OtBu)-OH, Fmoc-Ala-OH.

For blocking of the peptides with the sequence Ac-His-Arg-Tyr-X-ACC, Fmoc was removed after the fourth amino acid. Acetic acid (10 eq) was coupled with, HOBt (10 eq) and DICl (10 eq) in DMF. For blocking of peptides containing pyroglutamic acid with the sequence pGlu-Arg-Thr-Lys-X-ACC Fmoc was removed after the fourth amino acid. The amino acid pGlu (10 eq) was coupled with HOBt (10 eq) and DICl (10 eq) in DMF. For the blocking of peptides with the sequence Cbz-Phe-X-ACC Benzyl chloroformate (5 eq) and DIPEA (10 eq) were added in DMF after Fmoc removal.

Introduction of the lysine side chain modification was carried out similarly to the procedure described previously [22]. After blocking of the peptides Alloc was removed using tetrakis-triphenylphosphine palladium (5 mg) and dimethylaminoborane (20 mg) in DCM for 20 min. The syringe was washed thoroughly with DCM and MeOH for three times followed by washing on a shaker in a mixture of 10% MeOH/DCM for 20 min. 2-chloro-trityl 3-bromoacetate (2CT3B) was prepared as described previously [22]. Bromoacetic acid (9.6 mmol) was incubated with equimolar amounts of 2-chlorotriptyl chloride and DIPEA in DCM (25 ml). The mixture was stirred for 1 h at room temperature, the solvent removed and the dry product was used without further purification. The peptide was incubated with 20 eq of 2CT3B and 20 eq of DIPEA in DCM overnight.

For the synthesis of benzoyl (BZ) protected peptides, Bz-Ala-X-OH for the LC-MS/MS based activity assay the same building blocks in position P1 as described above were used. The first amino acid (1 eq, 50 μ mol) was loaded onto the CTC resin (1.6 mmol/g, 100 mg; Iris Biotech, Germany) with 2 eq DIPEA in DCM for 1 h. Fmoc-Ala-OH was coupled with HBTU (5 eq each) and DIPEA (10 eq). Fmoc was removed and benzoyl chloride (5 eq) and DIPEA (10 eq) were added in DMF. Incorporation of modified amino acids was carried out as described above.

All peptides were de-protected using 2.5% TIS and 2.5% water in TFA with the exception of MG-H1-containing peptides. These were incubated with 5% para-Kresol, 5% Ethane-1,2-dithiol and 90% TFA for 24 h. Peptides were precipitated in diisopropylether and purified by preparative reverse phase HPLC.

Fluorescence activity measurements

CTSL and CTSB activity assays were carried out as described previously [19,49]. Assay was carried out in 200 μ l activity assay buffer (100 mM sodium acetate, 100 mM NaCl, 1 mM EDTA, 0.01% Brij-35, 10 mM DTT, pH 5.5) containing 5 μ M substrate Ac-His-Arg-Tyr-X-ACC (CTSL) or Cbz-Phe-X-ACC (CTSB) in black flat bottom 96 well plates. The assay was started by the addition of the respective enzyme to a concentration of 1 ng/ μ l. Fluorescence measurement (exc 380 nm, em 460 nm, 435 nm cut-off filter) was carried out at 25°C at the start of the incubation and every 10 min for 30 min. The amount of the released fluorophore was calculated via a 7-amino-4-carboxymethylcoumarin standard curve. The activity of the protease is reported as nmol per min and μ g of protease.

Enzyme activity for trypsin was monitored similar to the CTSL and CTSB activity with the following changes: Buffer for trypsin activity measurement was ammonium bicarbonate buffer (50 mM) [50]. Trypsin was added to the assay at a concentration of 0.25 ng/ μ l. Both Ac-His-Arg-Tyr-X-ACC and Cbz-Phe-X-ACC-based substrates were tested for trypsin activity. Fluorescence measurement and calculations to quantify activity were carried out as described above for CTSL and CTSB.

The activity assay for PCSKs was carried out as described previously [20]. In short, activity assay buffer (25 mM Tris, 25 mM Mes, 2.5 mM CaCl_2 , pH 7.4) containing peptide substrates pGlu-Arg-Thr-Lys-X-ACC (50 μ M) was prepared. The assay was started by the addition of the enzymes PCSK1 (2810-SE-010, R and D Systems, U.S.A.), PCSK3 (1503-SE010, R and D Systems, U.S.A.) or PCSK7 (2984-SE-010, R and D Systems, U.S.A.) to a final concentration of 2 ng/ μ l. Fluorescence measurement and calculations to quantify activity were carried out as described above for CTSL and CTSB.

Measurement of carboxypeptidase E activity

CPE activity was determined as described previously [21] with some modifications. In short, CPE (10 ng/ml) was incubated with the peptide substrates Bz-Ala-X-OH (500 μ M) in assay buffer (50 mM sodium acetate, 5 μ M ZnCl_2 , pH 5.5) for up to 60 min at room temperature in triplicates. Tubes were flash frozen in liquid nitrogen and stored at -80°C until analysis of free amino acids and free AGEs was carried out by LC–MS/MS.

The release of MG-H1, arginine, CML and lysine from the respective peptide substrates was determined by isotope dilution, tandem mass spectroscopy, as previously described [51]. Briefly, an aliquot of reaction mixtures (40 μ l) was diluted 1:10 with water and filtered by microspin ultrafiltration (10 kDa cut-off) at 20 000g for 30 min at 4°C . The ultrafiltrate (~ 30 μ l) was then spiked with an equal volume of 0.2% TFA in water containing the isotopic standards (5–25 pmol). Normal and isotopic standards were either purchased (Cambridge Isotope, Polypeptide Laboratories, Iris Biotech) or prepared in-house, as described previously [24,31]. Samples were then analyzed by LC–MS/MS using an ACQUITY ultra-HPLC system with a Xevo-TQS LC–MS/MS spectrometer (Waters). Two 5 μ m Hypercarb columns (Thermo Scientific) in series were used: 2.1×50 mm, fitted with a 5×2.1 mm pre-column, and 2.1×250 mm. The mobile phases were 0.1% TFA in water and 0.1% TFA in 50% water. The column temperature and flow rate were 30°C and 0.2 ml/min, respectively. Analytes were eluted using a two-step gradient and the columns washed after each sample with 0.1% TFA in 50% THF, as described previously [51]. AGEs were detected by electrospray positive ionization with multiple reaction monitoring (MRM). The ionization source temperature was 150°C and the desolvation temperature was 500°C . The cone gas and desolvation gas flows were 150 and 1000 l/h, respectively. The capillary voltage was 0.5 kV. Molecular ion and fragment ion masses, as well as cone voltage and collision energies, were optimized to ± 0.1 Da and ± 1 eV for MRM detection of the analytes. Acquisition and quantification were completed with MassLynx 4.1 and TargetLynx 2.7 (Waters).

In vitro processing of proinsulin

Recombinant human proinsulin (10 μ M; 1336-PN-050, R and D Systems, U.S.A.) was modified with increasing amounts (0 eq, 10 μ M (1 eq), or 50 μ M (5 eq)) of MG in phosphate buffer (0.1 M, pH 7.4) for 24 h at 37°C . Remaining MG was scavenged by the addition of aminoguanidine to a final concentration of 1 mM followed by incubation for 1 h at 37°C . Concentrated sodium acetate buffer (0.25 volumes of 200 mM sodium acetate, 50 mM CaCl_2 , pH 5.5) was added and pH 5.5 was adjusted with HCl. Digestion was started by the addition of PCSK1 to a concentration of 10 ng/ μ l and carried out for 4 h at 37°C . A portion of the samples was frozen without further digestion. To the remaining samples, CPE was added to a concentration of 1 ng/ μ l and samples were incubated for 3 h at 37°C . Samples were flash frozen and stored at -80°C .

LC–MS analysis of proinsulin processing

Porcine insulin (0.5 µg; I0320000, Merck, Germany) was added to each sample as an internal standard. The sample was cleaned by solid-phase extraction (SOLA HRP, Thermo) the eluant was freeze dried and resuspended in 10% acetonitrile/water with 0.1% TFA. The amount of proinsulin and insulin (19278-5ML, Merck, Germany) were quantified via respective standard curves also including porcine insulin as internal standards by LC–MS (Exactive, Thermo Scientific, U.S.A.). Upon incubation of proinsulin with methylglyoxal two major new peaks formed. These were assigned to methylglyoxal-derived-hydroimidazolone modified proinsulin (MG-H-proinsulin) and carboxyethyl-modified proinsulin (CE-proinsulin) based on the mass shift of 54.0 and 72.0, respectively. While cysteine modification by MG to the hemithioacetal would yield a similar mass shift as the modification to carboxyethylarginine or carboxyethyllysine the modification is not stable and would reverse upon the addition of aminoguanidine [10]. The MG-modified proinsulin was quantified via the proinsulin standard curve. Separation was performed on an Agilent 1200 Series HPLC system fitted with a C18 column (Hypersil Gold aQ; Thermo Scientific, U.S.A.). Solvents used were phase A water (0.1% TFA) and phase B acetonitrile (0.1% TFA). A 20 min gradient with 0–100% acetonitrile with 0.1% TFA.

Statistical analysis

Data was analyzed by Graph Pad Prism and error bars indicate the standard error of the mean. Student's *t*-test was carried out and *P*-values are indicated for each figure.

Data availability

All data are contained within the manuscript.

Competing Interests

Sebastian Brings, Walter Mier, Peter P. Nawroth and Thomas Fleming are named as inventors in a pending patent that discloses the therapeutic use of MG scavenging peptides.

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CRediT Author Contribution

Sebastian Brings: Conceptualization, Formal analysis, Investigation, Visualization, Methodology, Writing — original draft, Writing — review and editing. **Walter Mier:** Conceptualization, Writing — review and editing. **Barbro Beijer:** Investigation, Methodology, Writing — review and editing. **Elisabeth Kliemank:** Investigation, Methodology, Writing — review and editing. **Stephan Herzig:** Conceptualization, Writing — review and editing. **Julia Szendroedi:** Conceptualization, Funding acquisition, Writing — review and editing. **Peter Nawroth:** Conceptualization, Funding acquisition, Writing — review and editing. **Thomas Fleming:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing — review and editing.

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Abbreviations

2CT3B, 2-chloro-trityl 3-bromoacetate; ACC, amino-4-carbamoylmethylcoumarin; AGE, advanced glycation end products; CML, carboxymethyllysine; CPE, carboxypeptidase E; HPLC, high-performance liquid chromatography; MRM, multiple reaction monitoring; SPPS, solid-phase peptide synthesis.

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