Biochimica et Biophysica Acta 1863 (2017) 654–662

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

A scavenger peptide prevents methylglyoxal induced pain in mice

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ARTICLE INFO

Article history:
Received 26 July 2016
Received in revised form 4 November 2016
Accepted 4 December 2016
Available online 6 December 2016

Keywords:
Methylglyoxal
Scavenger
Peptide
Pain
Diabetic complications

ABSTRACT

The reactive metabolite methylglyoxal (MG) has been identified as mediator of pain. Scavenging of free MG and the prevention of MG-derived post-translational modifications may provide a useful therapeutic treatment. An arginine-rich, fatty acid coupled, cyclic peptide (CycK(Myr)R4E) with high proteolytic stability and prolonged circulation was developed for the scavenging of MG. It was shown to reduce the formation of albumin-MG adducts in vitro and prevented MG-induced pain by reducing plasma MG levels through the formation of peptide-MG adducts in vivo. CycK(Myr)R4E therefore presents a promising option for the treatment of pain and other diabetic complications associated with high MG levels.

1. Introduction

Diabetes is defined by high blood glucose levels. Despite the high efficacy of the modern standard therapies, diabetic patients develop serious complications. Diabetic complications are in part caused by reactive dicarbonyl compounds via the covalent modification of proteins resulting in advanced glycation end-products (AGEs) [1,2]. Methylglyoxal (MG) represents the most abundant reactive dicarbonyl compound in plasma of diabetic patients [3]. We have previously shown that MG is causative of hyperalgesia, an increased sensitivity towards pain, associated with diabetic neuropathy [4]. In independent studies it was shown that formation of the MG derived AGE methylglyoxal-hydroimidazolone 1 correlates with the development of diabetic nephropathy, diabetic neuropathy and diabetic retinopathy [5–7]. Thus, therapeutic lowering of MG levels is a promising approach to treat diabetic neuropathy and other diabetic complications associated with MG like diabetic nephropathy [5]. Strategies to target elevated MG levels include inducers of the MG detoxifying enzyme Glo1 and small molecule MG scavengers like aminoguanidine and alagebrium [8]. Whereas the former compounds are currently under investigation, the latter compounds failed in clinical trials due to side effects or lack of efficacy [9–11]. Alternative small molecule scavengers include creatine, arginine and pyridoxamine [12–15] but as aminoguanidine and alagebrium they suffer from a short half-life in vivo [16–19].

The scavenging reaction is a comparably slow process. Therefore the ideal scavenger has to have a long circulation time, combined with a reactivity which is specific to avoid aberrant activity [20,21]. The current first-line therapy for type 2 diabetes, metformin is a slow MG scavenger so that new scavengers must be faster than metformin. Herein, we report in vitro and in vivo data on a newly developed arginine-based MG scavenging peptide with such characteristics. This opens up new options for the treatment of pain and other diabetic complications associated with excessive MG formation.

2. Materials and methods

2.1. Animal studies

Mice were housed with a 12-hour/12-hour light/dark cycle and had free access to water and food. All procedures in this study were approved by the Animal Care and Use Committees at the Regierungspräsidium Tübingen and Karlsruhe, Germany (35-9185.81/G-3/15).

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http://dx.doi.org/10.1016/j.bbadis.2016.12.001

2.1.1. Hot plate assay and MG scavenging studies

C57BL/6 mice (Charles River, Boston, USA) were acclimatized for 1 week prior to testing. A total of 36 mice were randomly assigned to three groups. One group received the peptide CycK(Myr)R_4E (0.25 mg/mouse in 0.9% NaCl) ip while the two other groups received saline ip. One of the saline and the peptide treated group received MG (5 μg/g) iv 30 min after peptide/saline injection while the third group received saline iv. Pain response was tested using a hot plate analgesia meter (Columbus Instruments, Ohio, USA) with the plate set to 50 °C 3 h after MG injection. Mice were removed from the plate when hind pare lifting, licking, shaking or jumping occurred. Mice were removed after a maximal cut-off time of 60 s. Pain response for each animal was measured in triplicates. Plasma was collected after the hot plate assay by cardiac puncture and frozen at −80 °C.

To determine the effect of the peptide on the MG-plasma levels, mice were treated with peptide and MG as described for the pain response assay. Blood was collected through the submandibular vein into EDTA tubes 30 min after MG injection. Samples were spun for 5 min at 3000*g* at 4 °C, the supernatant frozen in liquid N_2 and stored at −80 °C until analysis. MG content was determined by LC-MS/MS analysis as described below.

2.1.2. PET analysis

DOTA coupled peptides were labeled with ^{68}Ga as described previously [22]. In short ^{68}Ga was eluted from the generator into a tube containing 20 nmol of the peptide and 0.5% ascorbic acid in 0.5 M Na-acetate buffer. The mixture was incubated at pH 3.5–4.0 for 10′ at 95 °C while stirring to allow for the ^{68}Ga-DOTA complex to form. Free ^{68}Ga was removed using C18 solid phase extraction cartridges (Thermo Scientific, USA) and the product was checked by HPLC (Agilent Technologies, USA) equipped with a radio flow detector. The labeled peptide was injected iv into NMRI mice. PET analysis was carried out for 0–1 h and for another 20 min after 2 h while cumulative images are shown for 0–20 min, 40–60 min and 120–140 min.

Data was normalized for animal weight and injected dose and pictures and data are given as standardized uptake value (SUV). For quantitative comparison the area under the curve for the SUV values of the different regions was determined and compared by Student's *t*-test.

2.2. Materials

Protected amino acids were purchased from Orpegen Peptide Chemicals (Heidelberg, Germany). All other chemicals were purchased from Sigma-Aldrich (Munich, Germany) unless indicated otherwise.

2.3. Peptide synthesis

Peptides were synthesized on solid phase, using Fmoc-chemistry. Coupling of amino acids (10 eq.) was carried out with HBTU (9.8 eq.) and DIPEA (20 eq.) in NMP and Fmoc was removed by incubation in 20% piperidine/NMP unless noted otherwise. After coupling and deprotection, resin was washed with NMP. For synthesis of CycR_4E, CycK(Myr)R_4E and CycK(DOTA)R_4E, CTC-resin (1.1 mmol/g, 200 mg) was loaded with 50 μmol Fmoc-Glu(OAll)-OH in the presence of DIPEA (2 eq.) in DCM for 1 h. Remaining active sites were capped by incubation with DCM/MeOH/DIPEA at a ratio of 17/2/1 for 30 min. Four Fmoc-Arg(Pbf)-OH were coupled and Fmoc of the fourth Fmoc-Arg(Pbf) was removed. For CycR_4E OAll was removed by incubation with Tetrakis(triphenylphosphine)palladium(0) (10 mg/100 μmol peptide) and dimethylaminoboran (50 mg/100 μmol peptide) in DCM for 20 min, next. Resin was washed with 10% ethanolamine/DCM for 5 min twice followed by washes with DCM, MeOH, DCM and NMP. CycR4E was cyclized with PyAOP (5 eq.) and DIPEA (7.5 eq.) in NMP for 1 h at RT. For CycK(Myr)R_4E and CycK(DOTA)R_4E, Alloc-Lys(Fmoc)-OH was coupled instead of palladium catalyzed removal of OAll. Next myristic acid (10 eq. with 9.8 eq. HBTU and 20 eq. DIPEA in NMP) for CycK(Myr)R_4E or DOTA-tris(tBu)ester (2 eq. with 1.8 eq. COMU and 4 eq. DIPEA in NMP overnight) for CycK(DOTA)R_4E was attached to the side chain of Lys after removal of Fmoc. CycK(Myr)R_4E and CycK(DOTA)R_4E were cyclized with diphenylphosphonic azide (7.5 eq.) and DIPEA (5 eq.) in NMP overnight after palladium catalyzed removal of Alloc and OAll as was described for CycR_4E.

The peptide standards CycK(Myr)R_3MG-H1E, CycK(Myr)R_2MG-H1E, CycK(Myr)RMG-H1R_2E and CycK(Myr)MG-H1R_3E were synthesized equivalent to CycK(Myr)R_4E while one of the four arginines was replaced with MG-H1 in each of the four arginine positions. MG-H1 was introduced as a Fmoc and bis(4-methoxyphenyl)methyl (Dod) protected amino acid (Fmoc-MG-H1(Dod)-OH) which was synthesized as described previously [23]. Coupling of Fmoc-MG-H1(Dod)-OH (2 eq.) was carried out in NMP with DIPEA (4 eq.) and COMU (1.8 eq.) for 2 h at RT and the coupling procedure was repeated once without cleaving off Fmoc.

20 pentapeptides AXRAA were synthesized, where X was replaced with one of each of the canonical amino acids. Synthesis was carried out on CTC resin (1.1 mmol/g, 100 mg resin) which was loaded with 25 μmol of Fmoc-Ala-OH in the presence of DIPEA (2 eq.) in DCM for 1 h. Subsequent amino acids were coupled and the N-terminus was acetylated by incubation with acetic anhydride (10 eq.) and DIPEA (20 eq.) for 30 min in NMP.

The peptides CycK(DOTA)K(Myr)R_4E, K(DOTA)K(Myr)R_4E and CycK(DOTA)K(Myr)MG-H1$_4$E were synthesized on Wang-Resin. MG-H1 was introduced as Fmoc-MG-H1(Dod)-OH. Resin (25 μmol) was loaded with Fmoc-Glu(OAll)-OH (2 eq.) in the presence of triphenylphosphine (3 eq.) and diisopropyl azodicarboxylate (3 eq.) in THF at RT for 2 h. Four Fmoc-Arg(Pbf)-OH or four Fmoc-MG-H1(Dod)-OH followed by Fmoc-Lys(Mtt)-OH and Alloc-Lys(Fmoc)-OH were coupled. DOTA-tris(tBu)ester and myristic acid were coupled to the side chain of Alloc-Lys(Fmoc)-OH and Fmoc-Lys(Mtt)-OH, respectively. The peptides CycK(DOTA)K(Myr)R_4E and CycK(DOTA)K(Myr)MG-H1$_4$E but not K(DOTA)K(Myr)R_4E were cyclized using diphenylphosphonic acid (7.5 eq.) and DIPEA (5 eq.) in NMP overnight after palladium catalyzed removal of Alloc and OAll as described above.

The peptides GERP$_{10}$ and K(DOTA)GERP$_{10}$ (50 μmol) were synthesized on a Rink amide resin on an ABI 433 A peptide synthesizer with 10 eq. of Fmoc-Gly-OH, Fmoc-Glu(tBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Pro-OH. After synthesis of GERP$_{10}$ the resin was split into two parts. One part was cleaved off of the resin and Fmoc-Lys(Alloc)-OH was added to the other part. DOTA-tris(tBu)ester was coupled after palladium catalyzed removal of Alloc as was described above.

Free peptides were obtained by incubation in TFA containing 2.5% TIS and 2.5% H_2O for 2 h at RT. Exceptions were peptides containing DOTA-tris(tBu) and MG-H1(Dod)-OH which were obtained by incubation in 5% ethandithiole in TFA for up to 24 h. Peptides were precipitated and washed in cold diethyl ether and purified by reverse phase HPLC. LC-MS analysis of peptides was carried out with an Exactive Orbitrap instrument (Thermo Scientific, USA) and results are shown in the supplement (Table S1).

2.4. Serum stability

The cyclic peptide CycK(DOTA)K(Myr)R_4E and the linear peptide K(DOTA)K(Myr)R_4E were labeled with ^{177}Lu. In short 20 nmol of peptides was incubated with ^{177}Lu in 0.4 M Na-acetate buffer (pH 5.0) for 10 min at 95 °C. Free ^{177}Lu was removed by C18 solid phase extraction cartridges, the products eluted with EtOH and checked by radio-HPLC and dried under vacuum. The radiolabelled peptides were reconstituted in human serum (H4522; Sigma-Aldrich, Germany) and incubated at 37 °C for the times indicated. Serum was precipitated by addition of two parts acetonitrile centrifuged for 10 min at 13,000*g* and the supernatant analyzed by HPLC (Agilent Technologies, USA) fitted with a radio flow detector using a 10 min gradient of 0–60% acetonitrile in water (0.1% TFA).