# Decreased methylglyoxal-mediated protein glycation in the healthy aging mouse model of ectopic expression of UCP1 in skeletal muscle

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**Abstract**

Mice with ectopic expression of uncoupling protein-1 (UCP1) in skeletal muscle exhibit a healthy aging phenotype with increased longevity and resistance to impaired metabolic health. This may be achieved by decreasing protein glycation by the reactive metabolite, methylglyoxal (MG). We investigated protein glycation and oxidative damage in skeletal muscle of mice with UCP1 expression under control of the human skeletal actin promoter (HSA-mUCP1) at aged 12 weeks (young) and 70 weeks (aged). We found both young and aged HSA-mUCP1 mice had decreased advanced glycation endproducts (AGEs) formed from MG, lysine-derived Nε(1-carboxyethyl)lysine (CEL) and arginine-derived hydroimidazolone, MG-H1, whereas protein glycation by glucose forming Nε-fructosyl-lysine (FL) was increased *ca.* 2-fold, compared to wildtype controls. There were related increases in FL-linked AGEs, Nε-carboxymethyl-lysine (CML) and 3-deoxylglucosone-derived hydroimidazolone 3DG-H, and minor changes in protein oxidative and nitration adducts. In aged HSA-mUCP1 mice, urinary MG-derived AGEs/FL ratio was decreased *ca.* 60% whereas there was no change in CML/FL ratio – a marker of oxidative damage. This suggests that, normalized for glycemic status, aged HSA-mUCP1 mice had a lower flux of whole body MG-derived AGE exposure compared to wildtype controls. Proteomics analysis of skeletal muscle revealed a shift to increased heat shock proteins and mechanoprotection and repair in muscle of HSA-mUCP1 mice. Decreased MG-derived AGE protein content in healthy aging of aged HSA-mUCP1 mice is therefore likely related to increased proteolysis of MG-modified proteins and proteostasis surveillance of the skeletal muscle proteome. Decreased formation and increased clearance of MG-derived AGEs may be associated with healthy aging.

1. **Introduction**

There is an increasing need to understand key metabolic determinants of healthy aging with a view to how physical activity, diet and dietary supplements may be optimized to achieve this. Mouse transgenic and gene knockout models provide key experimental evidence on how changes in gene expression – overexpression or deletion – may impact on longevity and sustain healthy aging [1]. One such model has ectopic expression of uncoupling protein-1 (UCP1) in skeletal muscle. UCP1 expression is normally restricted to brown fat mitochondria where it mediates cold-induced non-shivering thermogenesis [2]. Expression of UCP1 in skeletal muscle was achieved under the control of the human skeletal actin promoter (HSA-mUCP1 mice) [3] or rat myosin light-chain 2 promoter (RMYL2-mUCP1 mice) [4]. When expressed in muscle, UCP1 exhibited its usual function of increased uncoupling of mitochondrial respiration activated by fatty acids, with the expression and function of UCP1 in brown fat unaffected by the transgene [5]. In longevity studies, HSA-mUCP1 mice had increased median and maximum lifespan, compared to wildtype (WT) mice on a standard diet [6, 7]. The survival advantage was greater on a high calorific diet: median lifespan of HSA-mUCP1 mice was increased 42% on a high carbohydrate, high fat diet where development of obesity was delayed [7]. HSA-mUCP1 mice had lower lean body mass and increased energy expenditure and insulin sensitivity, compared to WT controls [3, 4]. Insulin resistance correlated negatively and weight-specific energy expenditure correlated positively with longevity [7].

Metabolically, mice with ectopic expression of UCP1 in skeletal muscle had lower fasting serum glucose and insulin levels, compared to WT controls [3, 8]. There was increased basal and insulin-stimulated uptake of glucose by skeletal muscle [9, 10] – with increased GLUT4 protein levels and total hexokinase activity [10], and increased whole body glucose metabolism in transgenic (Tg) mice, compared to WT controls, but similar fasting hepatic glucose production rates [9, 10]. Transcriptomic studies indicated increased expression of enzymes of amino acid metabolism and antioxidant defense [11]. Mitochondrial superoxide formation was markedly decreased (*ca.* 76% lower) in Tg mice, likely due to the uncoupling activity of UCP1 [5]. Characteristics of the HSA-mUCP1 mouse have been recently reviewed [12].

In this study, we analyzed protein glycation, oxidation and nitration adducts in skeletal muscle, plasma and urine of young and aged HSA-mUCP1 mice and WT controls - 12 and 70 weeks old, respectively. The outcome revealed a decrease in advanced glycation endproducts (AGEs) formed by methylglyoxal (MG) - a reactive dicarbonyl glycolytic intermediate derived mainly from the degradation of triosephosphate glycolytic intermediates [13], suggesting an association of the healthy aging response with decrease in MG protein glycation or dicarbonyl stress [13].

1. **Materials and methods**
	1. *Wild type and transgenic mice*

HSA-mUCP1 mice were generated as described previously [3]. Experiments were performed with male Tg and WT controls maintained on a mixed C57BL/6-CBA background. Mice were housed in groups with *ad libitum* access to a standard chow diet and water. At 12 and 70 weeks of age, mice were euthanized in the morning 2 h after food withdrawal.Skeletal muscle tissue samples (20 – 25 mg), plasma (100 µl) from 12 and 70 week-old and urine from 70 week-old wild type and mHSA-UCP1 mice were collected and stored at – 80 oC until analysis, with shipment between the collaborating laboratories in dry ice.

* 1. *Protein damage marker assessment*

The oxidation, nitration and glycation adduct content of skeletal muscle and plasma protein was quantified in exhaustive enzymatic digests by stable isotopic dilution analysis LC-MS/MS, with correction for autohydrolysis of hydrolytic enzymes as described [14]. Oxidation, nitration and glycation free adducts were determined in the ultrafiltrates of urine samples. Ultrafiltrate of urine (100 μl) was collected by microspin ultrafiltration (3 kDa cut-off) at 4 oC. Skeletal muscle tissue samples (*ca.* 5 mg wet weight) were homogenized in 10 mM sodium phosphate buffer, pH 7.4 and 4 oC, and membranes and fibrils sedimented by centrifugation (20,000g, 30 min, 4 oC). The supernatant was diluted to 500 μl with water and washed by ultradiafiltration over a 10 kDa ultrafilter: 4 cycles of concentration to 50 μl and dilution to 500 μl with water over the microspin ultrafilter at 4 oC. The final washed protein (100 μl) was delipidated and hydrolysed enzymatically as described [14, 15]. Plasma protein was prepared similarly from 100 μl plasma. Total protein in the final concentrate was determined by Bradford assay and an aliquot of protein (100 μg) was digested by exhaustive enzymatic hydrolysis under aseptic, antioxidant conditions using a HTC PAL sample autoprocessor (CTC Analytics, Zwingen, Switzerland). Protein hydrolysate (25 μl, 32 µg equivalent) or ultrafiltrate was mixed with stable isotopic standard analytes and analysed by LC-MS/MS using an Acquity™ UPLC system with a Quattro Premier tandem mass spectrometer (Waters, Manchester, U.K.) [14, 16]. Samples are maintained at 4 oC in the autosampler during batch analysis. The columns were: 2.1 x 50 mm and 2.1 mm x 250 mm, 5 µm particle size HypercarbTM (Thermo Scientific), in series with programmed switching, at 30 oC. Chromatographic retention is necessary to resolve oxidized analytes from their amino acid precursors to avoid interference from partial oxidation of the latter in the electrospray ionization source of the mass spectrometric detector. Analytes were detected by electrospray positive ionization and mass spectrometry multiple reaction monitoring (MRM) mode where analyte detection response is specific for mass/charge ratio of the analyte molecular ion and major fragment ion generated by collision-induced dissociation in the mass spectrometer collision cell. The ionization source and desolvation gas temperatures were 120 oC and 350 oC, respectively, cone gas and desolvation gas flow rates were 99 and 900 l/h and the capillary voltage was 0.60 kV. Argon gas (5.0×10-3 mbar) was in the collision cell. For MRM detection, molecular ion and fragment ion masses and collision energies optimized to ± 0.1 Da and ± 1 eV, respectively, were programmed [14, 16]. Analytes determined were: glycation adducts - Nε-fructosyl-lysine (FL), and AGEs - Nε-carboxymethyl-lysine (CML), Nε-(1-carboxyethyl)lysine (CEL), Nω-carboxymethylarginine (CMA), hydroimidazolones derived from glyoxal, MG and 3-deoxyglucosone (3-DG), G-H1, MG-H1 and 3DG-H, respectively, and pentosidine (PENT); oxidation adducts – protein carbonyls, alpha-aminoadipic acid (AASA) and glutamic semialdehyde (GSA); and the nitration adduct, 3-nitrotyrosine (3-NT). Chemical structures and biochemical and clinical significance of these analytes have been described elsewhere [15-17]. Amino acids quantified were: arg, lys, tyr, and val (valine is determined in protein hydrolysates for the protease autohydrolysis correction) [14]. Analyte adduct residues were normalised to their amino acid residue precursors and given as mmol/mol amino acid modified; and related free adducts in urine are given in nmol/mg creatinine. Creatine was determined in urine by LC-MS/MS as described [18].

*2.3 High mass resolution proteomics analysis of skeletal muscle*

Cytosolic protein extracts of skeletal muscle from 70-week old Tg and WT control mice were analysed by high resolution Orbitrap mass spectrometry of tryptic digests, as described [19]. Cytosolic protein extracts were prepared and washed by ultradiafiltration over 10 kDa ultrafilters, as described for protein damage marker assessment above. The final washed protein concentration was determined by Bradford method. A similar sample of [13C6]lysine-labelled mouse skeletal muscle was processed similarly to produce [13C6]lysine-labelled peptide digest for internal standardization. An aliquot of cytosolic protein extract (89 µg, 50 µl) was treated with dithiothreitol (6 µl, 6 mM) and incubated at 37oC in the dark for 30 min; and then treated with iodoacetamide (5.9 µl, 10.8 mM) and incubated at 37oC in the dark for 30 min. Residual iodoacetamide was then quenched by further addition of dithiothreitol (5.9 µl 6 mM) and incubated at 37oC in the dark for 30 min. An aliquot of Lys-C protease (1 mg/ml, 5 µl) in 500 mM ammonium bicarbonate, pH 8.0, was added and incubated for 1 h at 37oC. Then TPCK-treated trypsin (1 mg/ml, 5 µl) in 1 mM calcium chloride/500 mM ammonium bicarbonate, pH 8.0, was added and samples were incubated at 37oC for 5 h in the dark. Finally, the reaction was stopped by adding 10% TFA (5 µl) in water. The sample was lyophilized to dryness to remove volatile salts and re-suspend in an aliquot (100 µl) 0.1% formic acid in water and analyzed by nanoflow liquid-chromatography-Orbitrap mass spectrometry.

The processed cell lysate samples were submitted to the Mass Spectrometry and Proteomics Facility at Warwick University for a label-free proteomic quantitation analysis. Reversed phase nanoflow liquid chromatography-mass spectrometry for global protein identification was performed on an Orbitrap mass spectrometer (FusionTM TribridTM, ThermoFisher Scientific) equipped with a microspray source operating in positive ion mode. For proteomics analysis the column used was: an Acclaim PepMap µ-pre-column cartridge (trap), 300 µm i.d. x 5 mm, 5 µm particle size, 100 Å pore size, fitted to an Acclaim PepMap RSLC 75 µm i.d. x 50 cm, 2 µm particle size, 100 Å pore size main column (ThermoFisher Scientific). It was installed on an Ultimate 3000 RSLCnano system (Dionex). An aliquot (5 µl) of sample containing a 1: 1 mixture of nature isotopic abundance and [13C6]lysine-labelled digests (*ca.* 1 µg equivalent) was injected. After injection, the peptides were eluted off of the trap onto the analytical column. Mobile phases were: A - 0.1% formic acid in water, and B - 0.1% formic acid in acetonitrile. The flow rate was programmed at 0.3 % to 35% 220 min. Mobile phase B was then increased from 35% to 80% in 5 min before being brought back quickly to 3% in 1 min. The column was equilibrated at 3% of mobile phase B for 15 min before the next sample. Peptides were eluted directly (300 nl min-1) via a Triversa Nanomate nanospray source (Advion Biosciences, NY) into the Orbitrap mass spectrometer. Survey scans of peptide precursors from 350 to 1500 *m/z* were performed at 120K resolution (at 200 *m/z)* with automatic gain control (AGC) 4 × 105. Precursor ions with charge state 2 - 7 were isolated in 1.6 Th intervals in the quadrupole and subjected to high energy collision dissociation fragmentation programmed to 35% and fragments ions detected by rapid scan MS analysis in the ion trap; the AGC was set to 1 x 104 and the max injection time was 200 ms. Dynamic exclusion duration was set to 45 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 2 s cycles. Sequence information from the MS/MS data was managed using MSConvert in ProteoWizard Toolkit (version 3.0.5759) [20] and searched with Mascot engine (Matrix Science, version 2.5.0) against *Mus musculus* protein sequence database (<http://www.uniprot.org/>) assuming enzyme tryptic digestion to determine levels of false-positive peptide identifications; spectra were also searched against the corresponding reverse database, the common Repository of Adventitious Proteins Database

(http://www.thegpm.org/cRAP/index.html). Search parameters for Precursor mass and product ions tolerance were, respectively, ± 5 ppm and ± 0.8 Da, with allowance made for two missed trypsin cleavages, fixed modification of cysteine through carbamidomethylation and methionine oxidation. Only fully tryptic peptide matches were allowed.Scaffold (version Scaffold 4.3.2, Proteome Software Inc.) was used to validate MS/MS based peptide and protein identifications from MS/MS sequencing results. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm and contained at least 2 identified unique peptides; probabilities assigned by the Protein Prophet algorithm [21]. Peptide abundances were normalized to [13C6]lys-labelled internal standards; mean SD for detection of labeled standards was 14.9%, WT 19.9% and HSA-mUCP1 27.2%. For labeled standards, correlations between runs for peptide abundance had r = 0.995.

*2.4 Other measurements*

The concentrations of MG, GSH and GSSG in mouse skeletal muscle were assayed by LC-MS/MS and the activity of glyoxalase 1 (Glo1) and soluble protein thiols in skeletal muscle was assayed spectrophotometrically, as described [22-25].

*2.4 Materials*

L-Lactic dehydrogenase from bovine heart, type III (Cat# L2625), 1,2-diaminobenzene, sublimed (Cat# 694975), pepsin from porcine stomach mucosa (Cat# P6887), prolidase from porcine kidney (Cat# P6675), pronase E - type XIV from *Streptomyces griseus* (Cat# P5147), leucine aminopeptidase - type VI from porcine kidney (Cat# L6007), DL-dithiothreitol (Cat# 43819), iodoacetamide (Cat# I1149), trypsin, N-*p*-tosyl-L-phenylalanine chloromethyl ketone-treated (Cat# 4352157-1KT) and endoproteinase Lys-C - sequencing grade from *Lysobacter enzymogenes* (Cat# 11047825001) were from Merck (Poole, Dorset, U.K.). Radio-immunoprecipitation assay (RIPA) buffer (Cat# 9806) was from Cell Signaling Technology (Leiden, The Netherlands). Glycated, oxidized and nitrated amino acids, natural isotopic abundance and stable isotopic standards, were purchased from Cambridge Isotope Laboratories, Inc, Tewksbury, MA, USA – where available, or otherwise prepared in-house, as previously described [26-28]. [13C6]Lysine-labelled mouse skeletal muscle (>97 atom %, Product no. 252923908) was from Silantes, Munich, Germany.

* 1. *Statistical analysis*

Data are mean ± SD for parametric data and median (upper – lower quartile) for non-parametric data. Significance testing was by paired Student’s t-test and Mann-Whitney U test (for 2 two groups), by one-way ANOVA and Kruskal-Wallis test (for 4 groups) for parametric and non-parametric data, respectively, and correlation analysis by Spearman method. P < 0.05 was considered statistically signiﬁcant. Statistical analyses were performed using SPSS (version 24.0, Armonk, NY, USA).

1. **Results**

*3.1 Physiology of young and aged HSA-mUCP1 and WT control mice*

Both young and aged HSA-mUCP1 mice had an over 30% reduced body weight compared to wildtype controls (Table 1). This is due to a decreased body size concomitant with decreased lean and fat mass as shown previously [9, 29, 30]. Of note, HSA-mUCP1 mice displayed a pronounced muscle atrophy as evident by largely decreased absolute and relative muscle weight in aged mice (Table 1) in line with the previously reported uncoupling induced decrease of grip strength, muscle mass, and muscle fibre size of these mice [31, 32].

*3.2 Protein glycation, oxidation and nitration adducts in skeletal muscle, plasma protein and urine of HSA-mUCP1 and WT control mice*

Major AGEs derived from MG are arginine-derived MG-H1 and lysine-derived CEL. For skeletal muscle, the CEL contents of young and aged HSA-mUCP1 mice and the MG-H1 content of aged HSA-mUCP1 mice were decreased, with respect to WT controls (Figures 1A and 1B). Other changes in protein glycation status in skeletal muscle were: increases in early-stage glucose-derived glycation adduct FL in young and aged mice HSA-mUCP1, +117% and +72%, respectively, compared to WT controls (Figure 1C). There were minor increases in CML residue content of young HSA-mUCP1 mice, compared to WT controls, + 21%, and in in aged WT and HSA-mUCP1 mice, +33%, with respect to young WT controls (Figure 1D). The level of pentose-derived glycation crosslink, pentosidine, increased in skeletal muscle of young HSA-mUCP1 mice, and decreased in aged HSA-mUCP1 and WT mice, with respect to young WT mice. Other changes in glycation adducts and also oxidation and nitration adducts in skeletal muscle of HSA-mUCP1 mice were relatively minor. 3DG-H residue content of skeletal muscle showed similar changes to CML and GSA was below the limit of detection; <0.012 mmol/mol lys. (Supplementary Table 1).

We also investigated changes in the glycation, oxidation and nitration adduct residue content in plasma protein. Overall there were few changes in plasma protein modifications in HSA-mUCP1 and aged mice. Where changes were found – such as decreased FL residue content in aged WT and HSA-mUCP1 mice, they did not correlate with concomitant changes in skeletal muscle (Supplementary Table 2).

 Changes in whole body flux of protein glycation were explored by measurement of flux of glycated amino acids in urine of aged WT and HSA-mUCP1 mice (Supplementary Table 3). There no changes in urinary flux of these analytes in HSA-mUCP1 mice compared to WT controls. Normalized to the flux of FL free adduct as a marker of glucose exposure, there was a 60% decrease in urinary MG-derived AGEs – (CEL + MG-H1)/FL ratio whereas there was no significant change in urinary CML/FL ratio (Figure 1F and 1G).

 We explored the mechanism of decreased MG-derived AGEs in skeletal muscle protein of aged HSA-mUCP1 mice, compared to WT controls. MG is mainly metabolized by reduced glutathione (GSH) - dependent glyoxalase 1 (Glo1) of the glyoxalase system (Figure 2). MG and Glo1-related variables were measured in skeletal muscle of young and aged WT and HSA-mUCP1 mice. Glo1 activity of skeletal muscle was 172 ± 20 and 218 ± 66 mU per mg protein (n = 8) in young and aged WT mice, respectively, and was not changed significantly in HSA-mUCP1 mice. The concentration of MG in skeletal muscle was 5.60 ± 1.37 and 6.43 ± 1.56 pmol per mg wet weight (n = 8) in young and aged WT mice, respectively, and was not changed significantly in HSA-mUCP1 mice. The activity of Glo1 *in situ* is dependent in the concentration of GSH and MG is also bound reversibly by protein thiols [13], so we also determined the concentrations of GSH, oxidized GSSG and protein thiols in skeletal muscle of WT and HSA-mUCP1 mice. The concentration of GSH in skeletal muscle of young and aged WT mice was 1.54 ± 0.41 and 1.31 ± 0.41 nmol per mg wet weight (n = 8), respectively, and the concentration of GSSG was 0.019 ± 0.008 and 0.029 ± 0.017 nmol per mg wet weight, respectively. The concentrations of GSH and GSSG were not changed significantly in HSA-mUCP1 mice. The concentration of protein thiols in skeletal muscle of young and aged WT mice was 23.8 ± 6.7 and 33.0 ± 6.7 nmol per mg protein (n = 8), respectively, and was not changed significantly in HSA-mUCP1 mice. This indicated there was no change in exposure of skeletal muscle to MG in of HSA-mUCP1 mice in the fasting state of sample collection.

*3.3 Proteomics analysis of skeletal muscle of aged HSA-mUCP1 and WT control mice*

We performed proteomics analysis of cytosolic protein extracts of skeletal muscle samples, as prepared for glycation, oxidation and nitration adduct analysis to assess changes in protein abundance and identify proteins modified by the major MG-derived AGE, MG-H1. We identified and quantified 302 proteins common to the skeletal muscle proteome of aged WT and HSA-mUCP1 mice. MG-H1 modification was detected on 6 proteins (sequence location): myosin-binding protein C (R432), myosin regulatory light chain 11 (R130), creatine kinase B-type (R172), protein kinase C/casein kinase II substrate protein 3 (R137), ß-actin (R210) and γ-actin (R290). Two of these, myosin regulatory light chain 11 and protein kinase C/casein kinase II substrate protein 3, were also detected in aged HSA-mUCP1 mice. Thirty-nine proteins had increased abundance in aged HSA-mUCP1 mice, compared to WT controls, with abundance increases from 17% to 58-fold (Tables 2 and S4); and there were 20 proteins of decreased abundance, with decreases from 15 – 92% (Tables 3 and S5). Protein abundance changes are presented in a volcano plot (Figure 3). Major abundance increases were linked to increased serine synthesis (PSAT1), glycogen storage (STBD1) and muscle contraction (MLRV). The was also a 6-fold increase in heat shock protein beta-1 (HSPB1) – the major chaperone protein of skeletal muscle [33]. Other chaperone proteins were also increased: heat shock 70 kDa protein 4 (HSPA4; + 97%), stress-70 protein, mitochondrial (HSPA9; +65%) and T-complex protein 1 subunit zeta (CCT6A; +54%) – a component of the chaperonin-containing T-complex (TRiC). Caveolae-associated proteins 1 and 2 were also increased (2 and 3-fold).

1. **Discussion**

In this study we found the HSA-mUCP1 transgenic mouse model of healthy aging was associated with decreased MG-derived AGE residues and increased FL residues and in skeletal muscle proteins in young and aged mice. Previous studies of decreased MG-mediated glycation in the nematode *C. elegans* model of aging by overexpression of Glo1 was associated with a *ca.* 30% increase in median and maximum lifespan [34]. Studies of fructosamine 3-phosphokinase deficient mice with increased FL-modification of protein indicated there is no aging phenotype associated with increased early-stage protein glycation [35]. Decrease of MG-derived AGE residues in aged HSA-mUCP1 mice may be associated with healthy aging [34].

The decrease of MG-derived AGEs, CEL and MG-H1, in skeletal muscle aging of HSA-mUCP1 was not linked to decrease in MG concentration or increase of GSH and protein thiol concentrations or activity of Glo1. The effect may rather be due increased proteolysis and turnover of MG-modified proteins. There was a 3-fold increase of HSPB1 and increases in other chaperone proteins in skeletal muscle of aged HSA-mUCP1 mice and this likely increases myocyte proteolysis, decreasing MG-H1 residue content of skeletal muscle protein. HSPB1 is highly expressed in skeletal muscle, is induced by metabolic stress and increased contractile activity [36]. Deficiency of HSPB1 induced ultrastructural abnormalities in the myofibrillar structure [37]. Caveolae-associated proteins 1 and 2 were also increased and are involved in mechanoprotection and repair of muscle fibers [38, 39]. MG-modified proteins are physiological activators of the unfolded protein response, linked to increased proteolysis and inflammation [40]. Decrease of this and proteomic restructuring of skeletal muscle may provide for improved metabolic and muscular health in aging.

Whole body exposure to MG-derived AGEs, assessed by urinary excretion of CEL + MG-H1 free adducts, normalized to urinary excretion of FL free adduct to correct for glycemic control, was decreased 60% in aged HSA-mUCP1 mice in whereas CML/FL ratio – a marker of oxidative stress [41] – was unchanged. This indicates that for a given glycemic exposure, aged HSA-mUCP1 mice produce markedly lower MG-derived AGEs than WT controls and thereby experience lower dicarbonyl stress. In mouse muscle, the period of lowest and highest glycolytic intermediates occurs after periods of physical activity and sedentary behaviour, respectively [42]. Decreased fasting plasma glucose in HSA-mUCP1 mice may produce lower glucose disposal in the fasting phase and decreased unscheduled glycolysis and formation of MG [43]. This may lead to the decreased total body MG exposure and decreased formation of MG-derived AGEs in aged HSA-mUCP1 mice.

 Increased FL content of skeletal muscle protein of young and aged HSA-mUCP1 mice is indicative of increased cellular concentration of glucose in skeletal muscle of HSA-mUCP1 mice; *cf*. increase of FL residue content of cytoplasmic proteins of endothelial cells incubated in high glucose concentration [44]. This is consistent with previous reports of increased glucose uptake and GLUT4 protein activity in skeletal muscle of HSA-mUCP1 mice [9, 10]. There was expected related increases of FL-derived AGEs, CML and 3DG-H [45, 46], and increase of CML in skeletal muscle of aged wildtype mice, consistent with increased oxidative stress. Levels of pentosidine reflect activity through the pentose pathway (PPP) activity [47] and the increase in young HSA-mUCP1 mice may indicate increased PPP activity - for which evidence was found previously [11]. PPP activity in skeletal muscle declines in old age [48] and corresponding decrease in pentosidine was found herein. Decreased FL, 3DG-H and AASA residue content of plasma protein in aged WT mice may relate to increased capillary permeability and albumin transcapillary escape rate in aged mice where plasma protein has increased dwell time in the lower glycating and oxidizing environment of interstitial fluid [49]

 In summary, decreased MG-derived AGE protein content in healthy aging of aged HSA-mUCP1 mice is likely related to increased proteolysis of MG-modified proteins and proteostasis surveillance of the skeletal muscle proteome. Decrease of MG-mediated protein glycation in aging, such as may be achieved with dietary supplement inducers of Glo1 [23], may be associated with healthy aging.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**Author contributions**

JM analysed skeletal muscle, plasma and urine samples, PW and NR analysed the proteomics data, SKe performed animal experiment and organ collection, MO, NR, PJT and SKl designed the study and checked all experimental data; and PJT and SKl drafted the manuscript. All authors checked, amended and approved the manuscript.

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**Table 1.**  Animal morphometrics

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|

|  |  |  |  |
| --- | --- | --- | --- |
|  | Young | Aged | Significance P |
|  | Wild-type | HSA-UCP1 | Wild-type | HSA-UCP1 |  |
| Body weight (g) | 28.48 ± 0.34 | 19.48 ± 0.29 | 37.60 ± 1.81 | 23.3 ± 0.70 | <0.0001 |
| Quadriceps (g) | nd | nd | 0.403 ± 0.03 | 0.167 ± 0.001 | <0.0001 |
| Quadriceps (% BW) | nd | nd | 0.55 ± 0.02 | 0.37 ± 0.01 | <0.0001 |

 |  |  |  |  |  |
|  |  |  |  |  |
| Data are mean ± SEM, n=7-8. Significances are calculated via Students t-test for differences between WT and HSA-mUCP1 mice. BW: body weight, nd: not determined. |  |  |  |  |  |
|  |  |  |  |  |
|  |  |  |  |  |
|  |  |  |  |  |

**Table 2. Ten proteins of highest increased abundance change in cytosolic extracts of skeletal muscle of aged HSA-mUCP1 mice, with respect to aged wildtype controls.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| No | Accession no | Description | Abundance ratio(HSA-mUCP1/WT) | P-value | Pathway involvement/function |
|  | Q3U6K9 | Phosphoserine aminotransferase (PSAT1) | 57.57 | 0.027 | Serine biosynthesis |
|  | Q8C7E7 | Starch-binding domain-containing protein 1 (STBD1) | 11.91 | 0.011 | Glycogen storage |
|  | P51667 | Myosin regulatory light chain 2 (MLRV) | 8.65 | 0.024 | Muscle contraction |
|  | Q9CZD3 | Glycine--tRNA ligase | 8.39 | 0.044 | Protein translation |
|  | P14602 | Heat shock protein beta-1 (HSPB1) | 6.44 | 0.014 | Major skeletal muscle chaperone |
|  | P47738 | Aldehyde dehydrogenase, mitochondrial | 4.79 | 0.011 | Metabolizes lipid peroxidation products |
|  | E9PZF0 | Nucleoside diphosphate kinase | 4.28 | 0.002 |  |
|  | P14211 | Calreticulin | 4.22 | 0.001 | Calcium-binding chaperone |
|  | Q8VHX6 | Filamin-C | 3.69 | 0.005 | Sarcomere assembly and organization |
|  | P26041 | Moesin | 3.43 | 0.002 | Connects the actin cytoskeleton to the plasma membrane |

**Table 3. Ten proteins of highest decreased abundance change in cytosolic extracts of skeletal muscle of aged HSA-mUCP1 mice, with respect to aged wildtype controls.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| No | Accession no | Description | Abundance ratio (HSA-mUCP1/WT) | P-value | Pathway involvement/function |
|  | P62806 | Histone H4 | 0.08 | 0.038 | Nucleosome |
|  | Q64523 | Histone H2A type 2-C | 0.16 | 0.040 | Nucleosome |
|  | P70695 | Fructose-1,6-bisphosphatase isozyme 2 | 0.24 | 0.001 | Regulation of glycolysis |
|  | Q8VCR8 | Myosin light chain kinase 2 | 0.41 | 0.012 | Muscle contraction |
|  | Q3V1D3 | AMP deaminase 1 | 0.42 | 0.003 |  |
|  | P16858 | Glyceraldehyde-3-phosphate dehydrogenase | 0.43 | 0.046 | Glycolysis |
|  | Q9CZ30 | Obg-like ATPase 1 | 0.57 | 0.026 | ATP hydrolysis |
|  | P45376 | Aldose reductase | 0.58 | 0.040 |  |
|  | P12787 | Cytochrome c oxidase subunit 5A, mitochondrial | 0.62 | 0.004 | Cell respiration |
|  | Q9ET78 | Junctophilin-2 | 0.70 | 0.035 |  |

**Figure legends**

**Figure 1. Changes in protein glycation status in young and aged HSA-mUCP1 transgenic mice, with respect to wild-type controls.** Glycation adduct residue content of skeletal muscle protein: A. CEL, B. MG-H1, C. FL, D. CML and E. PENT. Urinary excretion of glycation free adducts as a percentage of FL free adduct flux: F. CEL+MG-H1 and G. CML. Data are mean ± SEM (n = 8). Significance: \*, \*\* and \*\*\*, P<0.05, P<0.01 and P<0.001 with respect to young WT control and o, oo and ooo, P<0.05, P<0.01 and P<0.001 with respect toagedWT control; *Student’s t-test*. For ANOVA analysis, see supplementary Table 1.

**Figure 2. The glyoxalase system.** The glyoxalase system is in the cytosol of all cells and catalyses the GSH-dependent conversion of methylglyoxal to D-lactate via intermediate, S-D-lactoylglutathione [13].

**Figure 3. A volcano plot of change** **in abundance in cytosolic extracts of skeletal muscle of aged HSA-mUCP1 mice**. Each circle represents a protein identified. The horizontal line represents the P = 0.05 significant threshold (-log10 = 1.3). The vertical lines indicate 2-fold decrease and increase in protein abundances. Proteins of decreased and increased abundance shown in red and blue filled circle, respectively. Total number of proteins shown: 302.







**Supplementary Tables**

**Table S1**. **Protein glycation, oxidation and nitration adduct residues in skeletal muscle protein and young and aged WT and UCP1 transgenic mice.**

|  |  |  |  |
| --- | --- | --- | --- |
| Protein damage marker  | Young | Aged | Significance(P-value; ANOVA) |
| Class |  Marker | WT | HSA-mUCP1 | WT | HSA-mUCP1 |
| Glycation | FL (mmol/mol lys) | 0.117 ± 0.056 | 0.254 ± 0.042\*\*\* | 0.118 ± 0.025 | 0.201 ± 0.074\*,OO | <0.001 |
| CML (mmol/mol lys) | 0.090 ± 0.008 | 0.109 ± 0.021\* | 0.120 ± 0.018\* | 0.119 ± 0.017\*\* | 0.003 |
| CEL (mmol/mol lys) | 0.0263 ± 0.0037 | 0.0196 ± 0.0046\*\* | 0.0240 ± 0.0032 | 0.0159 ± 0.0076\*\*,OO | 0.002 |
| G-H1 (mmol/mol arg) | 0.0095 ± 0.0031 | 0.0130 ± 0.0067 | 0.0095 ± 0.0031 | 0.0114 ± 0.0027 |  |
| MG-H1 (mmol/mol arg) | 0.461 ± 0.135 | 0.476 ± 0.149 | 0.370 ± 0.052 | 0.268 ± 0.025\*\*,OOO,++ | 0.003 |
| 3DG-H (mmol/mol arg) | 0.084 ± 0.031 | 0.118 ± 0.033\* | 0.114 ± 0.008\* | 0.116 ± 0.013\* | 0.023 |
| CMA (mmol/mol arg) | 0.0243 ± 0.0038 | 0.0319 ± 0.0078\* | 0.0216 ± 0.0046 | 0.0259 ± 0.0054 | 0.008 |
| PENT (mmol/mol lys) | 0.0136 ± 0.0025 | 0.0160 ± 0.0019\* | 0.0104 ± 0.0023\* | 0.0112 ± 0.0023+++ | <0.001 |
| Oxidation | AASA (mmol/mol lys) | 0.0177 ± 0.0067 | 0.0221 ± 0.0108 | 0.0206 ± 0.0073 | 0.0189 ± 0.0058 |  |
| Nitration  | 3-NT (mmol/mol tyr) | 0.0044 ± 0.0022 | 0.0053 ± 0.0027 | 0.0032 ± 0.0017 | 0.026 ± 0.0015+ |  |

Data are mean ± SD (n = 8). Significance: 4 group comparison – ANOVA (righthand column) and 2-group comparisons – Student’s t-test. Key: \*, \*\* and \*\*\*, P<0.05, P<0.01 and P<0.001 with respect to young WT; oo and ooo, P<0.01 and P<0.001 with respect to **aged** WT; and +, ++ and +++, P<0.05, P<0.01 and P<0.001 with respect to young HSA-mUCP1 mice.

**Table S2**. Protein glycation, oxidation and nitration adduct residues in plasma protein and young and aged WT and UCP1 transgenic mice.

|  |  |  |  |
| --- | --- | --- | --- |
| Protein damage marker  | Young | Aged | Significance(P-value; ANOVA) |
| Class |  Marker | WT | HSA-mUCP1 | WT | HSA-mUCP1 |
| Glycation | FL (mmol/mol lys) | 2.01 ± 0.27 | 1.91 ± 0.17 | 1.51 ± 0.32\*\* | 1.41 ± 0.52\*,+ | 0.006 |
| CML (mmol/mol lys) | 0.120 ± 0.015 | 0.110 ± 0.015 | 0.122 ± 0.020 | 0.128 ± 0.013,+ |  |
| CEL (mmol/mol lys) | 0.0159 (0.0110 – 0.0458) | 0.0163 (0.0088 – 0.0362) | 0.0093 (0.0057 – 0.0151) | 0.0171 (0.0135 – 0.0230) |  |
| G-H1 (mmol/mol arg) | 0.0154 ± 0.0042 | 0.0091 ± 0.0054\* | 0.0118 ± 0.0062 | 0.0178 ± 0.0079+ |  |
| MG-H1 (mmol/mol arg) | 0.143 ± 0.036 | 0.145 ± 0.051 | 0.132 ± 0.028 | 0.135 ± 0.025 |  |
| 3DG-H (mmol/mol arg) | 0.097 ± 0.021 | 0.098 ± 0.035 | 0.074 ± 0.015\* | 0.082 ± 0.014 |  |
| CMA (mmol/mol arg) | 0.0521 ± 0.0181 | 0.0432 ± 0.0098 | 0.0453 ± 0.0120 | 0.0521 ± 0.0141 |  |
| PENT (mmol/mol lys) | 0.0056 ± 0.0024 | 0.0046 ± 0.0027 | 0.0011 ± 0.0005\*\* | 0.0005 ± 0.0004\*\*,++ | <0.001 |
| Oxidation | AASA (mmol/mol lys) | 0.0125 ± 0.0086 | 0.0134 ± 0.0026 | 0.0052 ± 0.0015\* | 0.0068 ± 0.0.0023+++ | 0.003 |
| Nitration | 3-NT (mmol/mol tyr) | 0.0242 ± 0.0097 | 0.0126 ± 0.0023\* | 0.0192 ± 0.0077 | 0.0182 ± 0.0049+ |  |

Data are mean ± SD (n = 8). Significance: 4 group comparison – ANOVA (righthand column) and 2-group comparisons – Student’s t-test. Key: \* and \*\*, P<0.05 and P<0.01 with respect to young WT; and +, ++ and +++, P<0.05, P<0.01 and P<0.001 with respect to young HSA-mUCP1 mice.

**Table S3. Urinary protein glycation free adduct excretion in aged WT and UCP1 transgenic mice.**

|  |  |  |
| --- | --- | --- |
| Protein damage marker class | Marker | Age |
| WT | HSA-mUCP1 |
| Glycation | FL (nmol/mg creatinine) | 5.14 (2.17 – 11.27) | 4.44 (2.82 – 8.48) |
| CML (nmol/mg creatinine) | 0.92 (0.36 – 1.93) | 0.58 (0.50 – 0.73) |
| CML/FL (%) | 14.9 (12.6 – 22.9) | 12.8 (10.8 – 17.6) |
| CEL (nmol/mg creatinine) | 0.131 (0.061 – 0.405) | 0.103 (0.083 – 0.185) |
| G-H1 (nmol/mg creatinine) | 0.0073 (0.0034- 0.0182) | 0.0076 (0.0051 – 0.0120) |
| MG-H1 (nmol/mg creatinine) | 0.0438 (0.0209 – 0.0774) | 0.0252 (0.0190 – 0.0312) |
| (CEL+MG-H1)/FL (%) | 4.3 (3.4 – 4.6) | 1.7 (1.5 – 2.9)\* |
| 3DG-H (nmol/mg creatinine) | 0.199 (0.109 – 0.564) | 0.163 (0.142 – 0.247) |
| CMA (nmol/mg creatinine) | 0.025 (0.015 – 0.114) | 0.025 (0.020 - 0.031) |
| PENT (nmol/mg creatinine) | 0.00061 (0.00023 – 0.00094) | 0.00070 (0.00046 – 0.00104) |

Data are mean ± SD (n = 10). Significance: \*, P<0.05; Mann-Whitney U test.

**Table S4. Proteins increased in abundance in cytosolic extracts of skeletal muscle of aged HSA-mUCP1 mice, with respect to aged wildtype controls.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| No | Accession no | Description | Abundance ratio(HSA-mUCP1 /WT) | P-value | Pathway involvement/function |
|  | Q3U6K9 | Phosphoserine aminotransferase | 57.57 | 0.027 |  |
|  | Q8C7E7 | Starch-binding domain-containing protein 1 | 11.91 | 0.011 | Glycogen storage |
|  | P51667 | Myosin regulatory light chain 2 | 8.65 | 0.024 | Muscle contraction |
|  | Q9CZD3 | Glycine--tRNA ligase | 8.39 | 0.044 | Protein translation |
|  | P14602 | Heat shock protein beta-1 (HSPB1) | 6.44 | 0.014 | Major skeletal muscle chaperone |
|  | P47738 | Aldehyde dehydrogenase, mitochondrial | 4.79 | 0.011 | Metabolizes lipid peroxidation products |
|  | E9PZF0 | Nucleoside diphosphate kinase | 4.28 | 0.002 |  |
|  | P14211 | Calreticulin | 4.22 | 0.001 | Calcium-binding chaperone |
|  | Q8VHX6 | Filamin-C | 3.69 | 0.005 | Sarcomere assembly and organization |
|  | P26041 | Moesin | 3.43 | 0.002 | Connects the actin cytoskeleton to the plasma membrane |
|  | P27546 | Microtubule-associated protein 4 | 3.31 | 0.038 | Microtubule assembly |
|  | P60843 | Eukaryotic initiation factor 4A-I | 3.28 | 0.042 | Protein translation |
|  | P16125 | L-lactate dehydrogenase B | 3.03 | 0.020 | Glycolysis |
|  | Q63918 | Caveolae-associated protein 2 | 2.92 | 0.012 | Caveolar biogenesis and morphology |
|  | Q00623 | Apolipoprotein A-I | 2.88 | 0.011 | Lipid and cholesterol transport in HDL |
|  | P51885 | Lumican | 2.46 | 0.029 | Caveolae formation and organization |
|  | G3UXL2 | Ribose-phosphate diphosphokinase | 2.27 | 0.033 |  |

**Table S4. Proteins increased in abundance in cytosolic extracts of skeletal muscle of aged HSA-mUCP1 mice, with respect to aged wildtype controls** (cont’d).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| No | Accession no | Description | Abundance ratio(HSA-mUCP1 /WT) | P-value | Pathway involvement/function |
|  | P99027 | 60S acidic ribosomal protein P2 (Eukaryotic initiation factor 4A-I) | 2.22 | 0.020 | Peptide elongation in protein synthesis |
|  | P04117 | Fatty acid-binding protein, adipocyte | 2.18 | 0.042 | Lipid transport protein |
|  | O54724 | Caveolae-associated protein 1 | 2.12 | 0.045 | Caveolae formation and organization |
|  | P09542 | Myosin light chain 3 | 2.03 | 0.031 | Muscle contraction |
|  | P45591 | Cofilin-2 | 2.00 | 0.001 | Controls reversible actin polymerization |
|  | Q61316 | Heat shock 70 kDa protein 4 (HSPA4) | 1.97 | 0.033 | Chaperone protein |
|  | Q91X72 | Hemopexin | 1.91 | 0.048 | Heme transport |
|  | G3UY93 | Valine--tRNA ligase | 1.78 | 0.037 | Protein translation |
|  | Q99NF7 | Protein-serine/threonine phosphatase | 1.70 | 0.023 |  |
|  | K3W4S6 | Glycogenin-1 | 1.68 | 0.004 | Glycogen synthesis |
|  | P38647 | Stress-70 protein, mitochondrial (HSPA9) | 1.65 | 0.045 | Chaperone protein |
|  | Q08642 | Protein-arginine deiminase type-2 | 1.59 | 0.026 |  |
|  | P97457 | Myosin regulatory light chain 2 | 1.56 | 0.012 | Muscle contraction |
|  | Q6PF96 | Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial | 1.55 | 0.036 |  |
|  | P80317 | T-complex protein 1 subunit zeta | 1.54 | 0.031 | Component of the chaperonin-containing T-complex (TRiC) |

**Table S4. Proteins increased in abundance in cytosolic extracts of skeletal muscle of aged HSA-mUCP1 mice, with respect to aged wildtype controls** (cont’d).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| No | Accession no | Description | Abundance ratio(HSA-mUCP1 /WT) | P-value | Pathway involvement/function |
|  | Q9JLV1 | BAG family molecular chaperone regulator 3 | 1.49 | 0.031 | Co-chaperone for HSP70 and HSC70 chaperone proteins |
|  | Q9CQ65 | S-methyl-5'-thioadenosine phosphorylase | 1.42 | 0.004 |  |
|  | P54822 | Adenylosuccinate lyase | 1.38 | 0.018 |  |
|  | P20801 | Troponin C | 1.35 | 0.047 | Muscle contraction |
|  | P05977 | Myosin light chain 1/3 | 1.32 | 0.018 | Formation and/or maintenance of myofibers |
|  | P56480 | ATP synthase subunit beta, mitochondrial | 1.29 | 0.032 | Cell respiration |
|  | Q91ZJ5 | UTP--glucose-1-phosphate uridylyltransferase | 1.17 | 0.041 | Glycogen synthesis |

**Table S5. Proteins decreased in abundance in cytosolic extracts of skeletal muscle of aged HSA-mUCP1 mice**, **with respect to aged wildtype controls**.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| No | Accession no | Description | Abundance ratio (HSA-mUCP1/WT) | P-value | Pathway involvement/function |
|  | P62806 | Histone H4 | 0.08 | 0.038 | Nucleosome |
|  | Q64523 | Histone H2A type 2-C | 0.16 | 0.040 | Nucleosome |
|  | P70695 | Fructose-1,6-bisphosphatase isozyme 2 | 0.24 | 0.001 | Regulation of glycolysis |
|  | Q8VCR8 | Myosin light chain kinase 2 | 0.41 | 0.012 | Muscle contraction |
|  | Q3V1D3 | AMP deaminase 1 | 0.42 | 0.003 |  |
|  | P16858 | Glyceraldehyde-3-phosphate dehydrogenase | 0.43 | 0.046 | Glycolysis |
|  | Q9CZ30 | Obg-like ATPase 1 | 0.57 | 0.026 | ATP hydrolysis |
|  | P45376 | Aldose reductase | 0.58 | 0.040 |  |
|  | P12787 | Cytochrome c oxidase subunit 5A, mitochondrial | 0.62 | 0.004 | Cell respiration |
|  | Q9ET78 | Junctophilin-2 | 0.70 | 0.035 |  |
|  | E9Q1W3 | Nebulin | 0.70 | 0.003 | Component of the skeletal muscle thin filament |
|  | Q9WUB3 | Glycogen phosphorylase | 0.70 | 0.017 | Glycogenolysis |
|  | E9Q8P5 | PDZ and LIM domain protein 5 | 0.71 | 0.037 |  |
|  | Q564E2 | L-lactate dehydrogenase-A | 0.72 | 0.013 | Glycolysis |
|  | P50247 | Adenosylhomocysteinase | 0.73 | 0.031 |  |
|  | Q9DCZ1 | GMP reductase 1 | 0.77 | 0.039 |  |
|  |  |  |  |  |  |

**Table S5. Proteins decreased in abundance in cytosolic extracts of skeletal muscle of aged HSA-mUCP1 mice**, **with respect to aged wildtype controls** (cont’d).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| No | Accession no | Description | Abundance ratio (HSA-mUCP1/WT) | P-value | Pathway involvement/function |
|  | P10649 | Glutathione S-transferase M1  | 0.78 | 0.032 | Metabolizes lipid peroxidation products |
|  | P47754 | F-actin-capping protein subunit alpha-2 | 0.78 | 0.013 |  |
|  | P58771 | Tropomyosin alpha-1 chain | 0.84 | 0.027 | Regulation of muscle contraction |
|  | P16015 | Carbonic anhydrase 3 | 0.85 | 0.027 |  |