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Original article

Oxidants produced by methylglyoxal-modified collagen trigger ER stress and apoptosis in skin fibroblasts

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ABSTRACT

Methylglyoxal (MG), a highly reactive dicarbonyl, interacts with proteins to form advanced glycation end products (AGEs). AGEs include a variety of compounds which were shown to have damaging potential and to accumulate in the course of different conditions such as diabetes mellitus and aging. After confirming collagen as a main target for MG modifications *in vivo* within the extracellular matrix, we show here that MG-collagen disrupts fibroblast redox homeostasis and induces endoplasmic reticulum (ER) stress and apoptosis. In particular, MG-collagen-induced apoptosis is associated with the activation of the PERK-eIF2α pathway and caspase-12. MG-collagen contributes to altered redox homeostasis by directly generating hydrogen peroxide and oxygen-derived free radicals. The induction of ER stress in human fibroblasts was confirmed using collagen extracts isolated from old mice in which MG-derived AGEs were enriched. In conclusion, MG-derived AGEs represent one factor contributing to diminished fibroblast function during aging.

1. Introduction

In recent decades life expectancy at birth has increased dramatically and as a consequence the number of elderly people is rising [1]. However, with increasing age many physiological alterations occur which lead not only to the development of an aged phenotype but also to enhanced risk for numerous diseases. Therefore, it is essential to understand causal events in the aging process. On a molecular level various hallmarks are reported to explain the aging process [2]. These include mitochondrial dysfunction, loss of proteostasis, cellular senescence, deregulated nutrient-sensing, altered intercellular communication and stem cell exhaustion. Moreover, telomere shortening, genomic instability and epigenetic alterations may contribute to age-related changes. Besides cellular alterations, age-related changes occur in the extracellular matrix (ECM), the non-cellular component of tissues and organs [3]. In general, the ECM provides structural support for cells and is important for a series of biomechanical and biochemical processes. For example, the components of the ECM are necessary for cell division, cell adhesion and cell motility. The most abundant fibrous protein in a multicellular organism is collagen. The collagen family consists of many different collagen types of which collagen type I is the most frequent. During aging several factors affect the ECM and an increase of cross-linked collagen was reported [4,5]. One major step involved in enhanced collagen cross-linking is the formation of cross-linking products due to non-enzymatic glycation. In addition to products with cross-linking properties, the group of the so-called advanced glycation end products (AGEs) includes further products shown to have damaging potential and to accumulate during aging [6,7]. One very reactive

Abbreviations: AGE, Advanced glycation end product; ATF4, Activating transcription factor 4; BID, BH3 interacting-domain death agonist; CHOP, CCAAT-enhancer-binding protein homologous protein; COL1A1, Collagen type I α1-chain; DAPI, 4',6-diamidino-2-phenylindole; ECM, Extracellular matrix; eIF2α, Eukaryotic initiation factor 2 α; ER, Endoplasmic reticulum; ESR, Electron spin resonance; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GF-AFC, Glycylphenylalanyl-aminofluorocoumarin; GSH, Reduced glutathione; GSSG, Oxidized glutathione; H_2O_2, Hydrogen peroxide; IRE1α, Inositol-requiring enzyme 1 α; MG, Methylglyoxal; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; MTT, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetyl-L-cysteine; ·OH, Hydroxyl radical; PARP, Poly(ADP-ribose)-Polymerase; PERK, Protein kinase R-like kinase; PRX, Peroxiredoxin; ROS, Reactive oxygen species; RPL13A, Ribosomal protein L13a; UPR, Unfolded protein response

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compound leading directly to the formation of AGEs is methylglyoxal (MG). MG is formed as a by-product in a series of physiological pathways. The most important endogenous source of MG is glycolysis in which MG is generated mainly by the fragmentation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate [8]. Moreover, MG is an intermediate in the Maillard reaction, initiated by the non-enzymatic reaction of reducing sugars with amino groups of *e.g.* proteins and also occurring in the human body. MG primarily reacts with arginine or lysine residues to form *via* Schiff bases irreversible products such as methylglyoxal-lysine dimer, methylglyoxal-derived hydroimidazolones such as MG-H1 (N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine), argpyrimidine or N^{ϵ}-(carboxylethyl)lysine [9–14]. On the one hand, these products may affect the structure and function of proteins. On the other hand, glycated proteins were shown to influence physiological pathways. This may play an important role in the aging process and in age-related complications. Nevertheless, the knowledge about the role of MG-derived AGEs and their associated mechanisms is still limited. Therefore, our aim was to investigate the molecular mechanisms by which an aged, MG-AGE-enriched ECM would affect cellular homeostasis.

2. Material and methods

2.1. Animals

Heart tissue was obtained from C57BL/6J (Charles River Laboratories) mice which were kept at the Institute of Nutrition in Jena, Germany. Mice were housed under controlled environmental conditions (temperature of 22–24 °C and 12 h light/dark schedule) and had access to food and water *ad libitum*. Tails for collagen isolation were obtained from C57BL/6J (The Jackson Laboratory) mice which were kept at the animal facility of the German Institute of Human Nutrition Potsdam-Rehbruecke (Nuthetal, Germany). Mice were housed under controlled environmental conditions (temperature of 20 ± 2 °C and 12 h light/dark schedule) and had access to food and water *ad libitum*. Tissues were collected immediately after sacrification from young (≤ 10 weeks), adult (10 months) and old mice (≥ 15 months), snap frozen in liquid nitrogen and stored at −80 °C. Animal experiments were performed according to the German animal protection law (TierSchG). Animal experiments were approved by local authorities. Mice were housed and handled in compliance with good animal practice as defined by the national animal welfare body GV-SOLAS (www.gv-solas.de/index.html) and FELASA (www.felasa.eu/guidelines.php).

2.2. Tissue decellularization

Decellularization was performed with heart tissue from young, adult and old mice. For similar tissue amounts, organs of 3 young animals were pooled while one organ was used from adult and old mice. Murine heart tissue was homogenized in KCP buffer (150 mM KH_2PO_4, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4; pH 7.4) with a Potter-Elvehjem homogenizer (motor-driven) on ice. Tissue homogenates were centrifuged at 45,000*g* for 1 h at 4 °C and supernatants (cytosols) were kept for method validation. To decellularize tissue, pellets were incubated in 1% SDS/PBS with protease inhibitor cocktail (Roche) at room temperature in an overhead shaker and the solution was changed 2–3 times until the remaining tissue was white and transparent. For validation of the decellularization process, decellularized heart tissue was resuspended in 4 × sample buffer (0.25 M Tris (pH 6.8), 8% SDS, 40% glycerol, 0.03% orange G) containing 100 mM DTT (according to [15]). Additionally, decellularized heart tissues were sonificated. 1 × sample buffer containing 100 mM DTT was added to intermediate fractions of the decellularizations process (homogenates, cytosols). All lysates were boiled for 5 min at 95 °C. Protein amounts were determined by dotting 2 µl of the sample on a nitrocellulose membrane (0.45 µm pore size, GE Healthcare Life Sciences) followed by total protein staining (REVERT protein stain, LI-COR Biosciences, 926–11010). Protein amounts were adjusted. The decellularization process was characterized by SDS-PAGE followed by Coomassie staining or immunostaining for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and for collagen type I α1-chain (COL1A1) as a cytosolic and extracellular marker, respectively.

2.3. Collagen extraction

For collagen extraction, tail tendons from young and old mice were treated as it was done before by Damodarasamy et al. [16] with some modifications. Tendons were washed in PBS and subsequently rinsed in acetone and 70% iso-propanol for 5 min at RT. Tendons were incubated overnight in 0.05 N acetic acid at 4 °C. After homogenization, extracts were incubated for another 3 days at 4 °C. To remove undissolved material, collagen extracts were centrifuged (20,000*g*, 30 min, 4 °C). Collagen extracts were adjusted to the hydroxyprolin content (Sigma-Aldrich, MAK008) and collagen was polymerized by adding 1 part of NaOH to 9 parts of collagen extracts.

2.4. Analysis of AGE formation

AGE formation was determined with the dot blot method. In addition, AGE-specific autofluorescence was determined by fluorescence measurements at 370 nm excitation and 440 nm emission with a plate reader (Infinite® M200 Pro, Tecan).

2.5. Preparation of MG-modified collagen gels (MG-collagen)

Collagen gels were prepared by adding 1 part of NaOH to 9 parts of acid soluble rat tail collagen type I (Thermo Fisher Scientific, A1048301) to a final concentration of 1 mg/ml. After polymerization at 37 °C and 5% CO_2 for 2 h, gels were modified with different concentrations of MG for 2 h using the same conditions. Before cell seeding gels were washed with PBS intensively. As a control (native/unmodified collagen), collagen gels were incubated with PBS and treated as described for MG-collagen.

2.6. Cell culture and inhibitions studies

Primary human skin fibroblasts were obtained from foreskin tissue of a 1-year old donor (kind gift of Prof. Scharffetter-Kochanek from the University of Ulm, Germany). Fibroblasts were cultured in DMEM with 10% fetal bovine serum and 1% L-alanyl-L-glutamine under standard cell culture conditions (37 °C, 5% CO_2, 95% humidity). Cell culture reagents were obtained from Merck Millipore/Biochrom AG.

For inhibition studies, cells were harvested and incubated with specific inhibitors 30 min prior to their seeding on collagen gels. For caspase inhibition, fibroblasts were incubated with 20 µM pan caspase inhibitor Z-VAD-FMK, 20 µM caspase-8 inhibitor Z-IETD-FMK, 20 µM caspase-9 inhibitor Z-LEHD-FMK, 20 µM caspase-10 inhibitor Z-AEVD-FMK and 20 µM caspase-12 Z-ATAD-FMK (all purchased from R&D Systems; FMK001, FMKSP01, FMK013). PERK (Protein kinase R-like kinase) was inhibited using 25 µM of GSK2606414 (Merck Millipore, 516535).

2.7. Cell viability

Cell viability was determined with the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [17]. Moreover, it was assessed by glycylphenylalanyl-aminofluorocoumarin (GF-AFC) assay. GF-AFC-substrate and assay buffer were obtained from Promega (G608A and G610A) and used as described in the manufacturer's instructions. Fluorescence was measured at 400 nm excitation and 505 nm emission with a plate reader (Infinite® M200 Pro, Tecan).