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Research Article

Maillard reaction products enriched food extract reduce the expression of myofibroblast phenotype markers

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Advanced glycation end products (AGE) are associated with a wide range of degenerative diseases. The present investigation aimed at analysing the influence of AGE containing nutritional extracts on cardiac fibroblasts (CFs) as the major cell type responsible for cardiac fibrosis. Mice CFs were treated with bread crust extract (BCE) which contained significant amounts of a variety of AGE modifications. BCE treatment with up to 30 mg/mL did not impair cell viability. Furthermore, BCE induced a moderate elevation of reactive oxygen species (ROS) production and activation of redox sensitive pathways like the $p42/44^{MAPK}$, $p38^{MAPK}$ and NF-κB but did not alter Akt kinase phosphorylation. Expression of smooth muscle α-actin and tropomyosin-1, which represent markers for myofibroblast differentiation, was reduced after bread crust treatment. These data suggest a putative antifibrotic effect of melanoidin-rich food.

Keywords: Advanced gylcation endproducts / Bread crust extract / Cardiac fibroblasts / Myofibroblast markers / Oxidative stress

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1 Introduction

Cardiovascular diseases are the leading cause of death in the western world, especially in the elderly [1]. In the last few years, it has been shown that changes in the interstitium like cardiac fibrosis play a major role in the generation of myocardial dysfunctions. Our understanding of the role of the primary participating cell type, the cardiac fibroblast (CF), for this disease and the possible influence of nutritive compounds are meanly, whereby strategies for the treatment of fibrosis are rare.

The cardiac interstitium – the space between the cardiomyocytes – contains primarily fibroblasts and extracellular matrix (ECM) proteins. The major function of this cell type is the homeostatic maintenance of the ECM by secreting collagens (types I and III), extra-cellular matrix degrading enzymes like the metalloproteinases (MMPs) as well as tissue inhibitors of metalloproteinases (TIMPs) and bioactive signalling molecules like growth factors and cytokines [2]. As a result, the ECM of the healthy heart is not inert but subject to continued turnover to provide an optimal environment for cardiac myocytes function [3, 4].

During myocardial diseases CF can be activated by tissue injuries or stress situations which may lead to an exaggerated collagen deposition, known as cardiac fibrosis. These changes in the homeostatic maintenance of the ECM towards augmented amounts of collagens result in increased stiffness followed by a reduced compliance of the heart. This structural remodelling due to external stress is also accompanied by the differentiation of fibroblasts to myofibroblasts which are almost not detectable in the normal cardiac tissue [5]. This cell type is identified by the expression of marker genes like smooth muscle (SM) α-actin (Acta-2), tropomyosin-1 (Tpm-1), nonmuscle myosin-heavy chain-B and connexins. Myofibroblast expression is associated with tissue repair as well as increased col-

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Abbreviations: AGE, advanced glycation end product; **BCE,** bread crust extract; **CF,** cardiac fibroblast; **ECM,** extracellular matrix; **MRP,** Maillard reaction products; **ROS,** reactive oxygen species; **SM,** smooth muscle

lagen production and deposition [6, 7]. Recently, it was shown that myofibroblasts can interfere with electrical properties of cardiomyocytes, thus contributing to arrhythmogenesis [8].

In addition to the quantitative increase in collagen and structural remodelling of the CF, it seems that qualitative changes of the collagen fibres, especially the degree of the crosslinking, are critical for the pathophysiological effect. Some of these crosslinks are generated by the heterogeneous group of advanced glycation end products (AGEs). AGEs are stably modified protein derivatives developed in the presence of reducing carbohydrates and produced by a nonenzymatic glycation reaction, including oxidation and irreversible rearrangements, also established as Maillard reaction [9]. AGEs/Maillard reaction products (MRPs) can arise by two possible ways in the organism. On the one hand they are generated *in situ* under physiological conditions using endogenous substrates; on the other hand they can be absorbed from food.

Endogenously generated AGEs are associated with a number of degenerative diseases like diabetes, Alzheimer's disease, retinopathy, nephropathy and reduced cardiovascular compliance. With age, the accumulation of AGE crosslinks can be detected on proteins with a slow turnover rate like collagens [10] and can cause a reduced degradation, thus contributing to the accumulation and increased cardiovascular stiffness [4, 10].

AGE formation was originally thought to tag only senescent proteins, thereby providing a special signal for recognition, degradation and removal of senescent macromolecules [11]. But it was found that AGEs can induce cellular signalling by an interaction with specific receptors like RAGE and AGE-R1–3. The best studied receptor for AGEs implicated in signalling events is RAGE. One consequence of the AGEs–RAGE interaction is the increased reactive oxygen species (ROS) production, the activation of NF-κB and the expression of proinflammatoric cytokines which lead to an increased expression of collagens and stiffening of the myocardium [12].

Besides the endogenous production of AGEs, these compounds can be absorbed by the daily uptake of food. Food contains large quantities of AGEs/MRPs which arise during heating and processing and cause the characteristic flavour and typical brown colour. Different studies that used radioactive labelled AGE molecules showed that between 5 and 30% of high and low molecular mass compounds were absorbed in the intestine and circulated *via* blood stream to all the vital parts of the body like the heart [13, 14]. Somoza *et al.* [15] observed that feeding rats with bread crust caused increased expression of GST in liver and altered the antioxidative capacity of the plasma. These data indicated that compounds from the bread crust were indeed taken up *via* the intestines, and were able to influence physiological parameters.

Therefore, we analysed the influence of BCE as a model for food-AGEs on oxidative stress, signalling events and expression of myofibroblast marker genes in a newly established CF cell line.

2 Materials and methods

2.1 Cell line, culture conditions and treatments

A CF cell line was established from male C57/Bl6 mice (Charles River, Sulzfeld, Germany). Mice were sacrificed by cervical dislocation, the hearts were removed, cut into small pieces, digested by 1 mg/mL collagenase/dispase (Roche Diagnostics, Mannheim, Germany) and seeded on cell culture dishes (TPP, Trasadingen, Switzerland). CFs were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Karlsruhe, Germany), supplemented with 5% calf serum (CS; HyClone, Logan, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) in a 5% CO_2 atmosphere at 37°C. The fibroblasts were immortalised by culturing through the crisis phase and the subsisting cell line was used. For the examination of cell viability, intracellular signalling pathways and gene expression studies, cells were grown to confluence, synchronised by serum reduction to 0.1% CS for 48 h followed by a treatment with water-soluble bread crust extract (BCE) prepared according to Lindenmeier *et al.* [16].

2.2 Dot blot analysis

Dot blot analyses were accomplished using a Minifold Spot-Blot system (Schleicher & Schuell, Dassel, Germany) by transferring various BCE concentrations onto a nitrocellulose membrane. After transfer, the membrane was blocked by 2% BSA in 50 mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl and 0.2% NP40 and incubated with primary antibody in blocking solution overnight at 4°C. After washing three times with 50 mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl, 0.2% NP-40, 0.5% BSA, horseradish peroxidase-conjugated secondary antibody (goat antirabbit IgG or donkey antimouse IgG) from Dianova (Hamburg, Germany) was added for 1 h in room temperature. After three further washes, chemiluminescence detection was performed, with 2.5 mM luminol as the substrate and 400 µM *p*-coumaric acid as an enhancer in 0.1 M Tris-HCl pH 8.5, visualised with an LAS-3000 imaging system (Raytest, Straubenhardt, Germany). Primary antibodies were obtained from U. Friess (University of Tübingen, Germany; anti-*N*ε-carboxymethyllysine (CML)), R. Schinzel (Vasopharm, Wuerzburg, Germany; anti-"AGE"), K. Uchida (University of Nagoya, Japan; antiarginine-pyrimidine (Arg-Pyr)) and R. Nagai (University of Kumamoto, Japan; anti-3-hydoxy-4-hydroxymethyl-1-(5-amino-5carboxypentyl)-pyridinium (GA-Pyr)).

2.3 Propidiumiodid staining for the analysis of necrotic cell death ðisplaystyle

To analyse cell viability after BCE treatment with propidiumiodid (PI) staining, serum starved cells were incubated with indicated BCE concentrations for another 24 h. Thereafter, the harvested CFs were centrifuged for 5 min at $400 \times g$ and stained with PI staining solution (2 μg/mL in PBS) on ice. To analyse the number of living cells relative to the total cell number the FACS Calibur flow cytometer equipped with CellQuest Pro software (Becton Dickinson; Heidelberg, Germany) was used.

2.4 Intracellular oxidative stress assay

The formation of ROS was determined in response to BCE by using 2′,7′-dichlorofluorescein-diacetate (5 μM H_2-DCF-DA; Molecular Probes, Europe) using confluent, serum starved cells. Nonfluorescent H_2DCF is oxidised to fluorescent 2′,7′-dichlorofluorescein (DCF) by ROS which was determined using a FACSCalibur flow cytometer equipped with CellQuest Pro software (Becton Dickinson). Cells were harvested using Trypsin, resuspended in HBSS buffer (Gibco) containing 10 mmol/L glucose, centrifuged for 5 min at $400 \times g$, resuspended in HBSS/glucose and incubated for 10 min with 5 μmol/L of H_2DCF-DA. Afterwards, cells were washed and incubated with 10 mg/mL BCE or H_2O_2 (25 μM) in HBSS/glucose for 45 min on ice. After another washing step, DCF-fluorescence was assessed in living, PI negative cells. PI (2 μg/mL; Sigma, Deisenhofen, Germany) was added immediately before the analysis.

2.5 Protein extraction and immunoblot analysis

For protein analysis, cells were lysed in lysis buffer (50 mmol/L Tris/HCl, pH 6.7, 2% SDS, 1 mmol/L Na_3VO_4, 0.1 μmol/L ocadaic acid, 1 × complete protease inhibitor mix (Roche Diagnostics) followed by a treatment with 85 U benzonase (Merck, Darmstadt, Germany) for 15 min. After adding 2% β-mercaptoethanol and 0.01% bromophenol blue, lysates were incubated at 60°C for 10 min. Lysates were resolved on SDS-PAGE and transferred onto a nitrocellulose membrane by semidry blotting (BioRad, München, Germany) using 50 mM CAPS, 1 mM 3-mercaptopropionic acid, 10% methanol, pH 10. After transfer, the membrane was blocked by 2% BSA in 50 mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl and 0.2% NP-40 and incubated with primary antibody in blocking solution overnight at 4°C. After washing three times with 50 mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl, 0.2% NP40, 0.5% BSA, horseradish peroxidase-conjugated secondary antibody (goat antirabbit IgG or donkey antimouse IgG) from Dianova were added for 1 h in room temperature. After three further washes, chemiluminescence detection was performed, as described by the Dot blot visualised with a LAS-3000 imaging system (Raytest) and quantified with AIDA 3.5 software (Raytest). Primary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA; phosphospecific antibodies for $p44/42^{MAPK}$, $p38^{MAPK}$ and Akt kinase) and Sigma (Taufkirchen, Germany; actin). Membranes were probed with antiactin antibodies as loading control.

2.6 Reporter gene expression studies

Analysis of the activity of the NF-κB promoter was performed by using the dual luciferase reporter gene system. For transfection experiments, cells were plated at 9×10^4 cells/well in a 96-well noncrosstalk plate (Greiner, Frickenhausen, Germany). pNF-κB-TA-Luc (BD Biosciences, CA, USA) and phRL-CMV (Promega, Heidelberg, Germany) were cotransfected in a 10 : ratio using the TransFectin™ transfection reagent (BioRad Laboratories, Munich, Germany), according to the manufacturer's instructions. Twenty-four hours later, cells were stimulated with 10 mg/mL BCE or 0.2 ng/mL LPS (Sigma) for 18 h. Afterwards, cells were washed with PBS and lysed for 20 min at room temperature using 20 μL of passive lysis buffer (Promega).

The luciferase activities were determined by using a FLUOstar OPTIMA reader (BMG Labtechnologies, Offenburg, Germany). According to Dyer *et al.* [17] 100 μL of reagent A (glycylglycine 25 mM, K_2HPO_4 15 mM, EGTA 4 mM, $MgSO_4$ 15 mM, ATP 2 mM, DTT 1 mM, Luciferin 75 μM pH 8.0) was injected to 20 μL of cell lysate and the firefly luminescence was determined every 0.5 within 10 s. Thereafter 100 μL of reagent B (NaCl 1.1 M, Na_2EDTA 2.2 mM, K_2HPO_4 220 mM, BSA 0.44 mg/mL, NaN_3 1 mM, colenterazine 1.43 μM pH 5.0) was injected and the renilla luciferase luminescence was determined every 0.5 s for 10 s. Finally, the firefly activity was expressed relative to renilla luminescence.

2.7 RNA isolation and cDNA synthesis

Total RNA was prepared using the Qiagen Total RNeasy kit (Hilden, Germany), according to the manufacturer's instructions. RNA concentration was determined by UV extinction at 260 nm. RNA integrity was analysed by electrophoresis on a 1.5% agarose gel followed by ethidium bromide staining. Total RNA (500 ng) in 10 μL RNase-free water was denatured at 72°C for 3 min and cooled to 4°C. Transcription was performed in 25 μL using 200 ng random Primer, 0.5 mmol/L dNTP and 40 U Superscript II (Invitrogen, Karlsruhe, Germany) for 35 min at 42°C and stopped by heating at 75°C for 10 min in a thermocycler (Biometra Trioblock, Goettingen, Germany). This cDNA was finally diluted two-fold with sterile water and was used as a template for PCR.

Table 1. Primers used for RT-PCR

Gene	Primer sequence	Product size (bp)	T_A (°C)	Cycles	GenBank no.
Acta-2	se: CACGAAACCACCTATAACAGCA as: ATCCACAAAACGTTCACAGTTG	354	58	25	NM 009606
Tpm1	se: TGGTGTCACTGCAAAAGAAACT as: TGGATCTCCTGAATCTCCATCT	313	58	28	NM 024427
Col1a1	as: AAGAATGGAGATGATGGGGAAGCT se: TAGGACCAGCAGGACCAGCATCTC	175	61	39	NM 007742
Col3a1	as: TGGTATGAAAGGACACAGAGGC se: TCCAACTTCACCCTTAGCACC	283	61	30	NM 009930
Lamb2	as: GACCCTATGGGTTCTCAAGATG se: AGCCTCCCAGGTTAAATGGT	471	58	30	NM 008483
Fbln5	as: AAACCACACGTGTACCTCACTG se: CTGCCTCTGAAGTTGATGACAG	417	58	31	NM 011812
18S	se: GTTGGTGGAGCCGATTTGTCTGG as: AGGGCAGGGACTTAATCAACGC	346	60	12	X00686

se, sequence; as, antisequence.

2.8 Primerdesign and polymerase chain reaction (PCR)

Primers for RT-PCR were designed using the sequence entries from GenBank. Primers overlapping intron spanning mRNA regions were designed using the Primer3-online-interface provided by the Whitehead Institute for Biomedical Research [18]. Primers were purchased from Invitrogen, (Karlsruhe, Germany).

PCR amplification was performed with the Qiagen PCR Taq Master kit with 10 pmol/µL of specific sense and antisense primers in a final volume of 25 µL. After an initial denaturation step at 95°C for 5 min, PCR cycles were performed in a thermocycler using the following protocol: 30 s at 95°C, 30 s at the primer specific annealing temperature, and 30 s at 72°C. After final extension at 72°C for 5 min, PCR products were separated on a 1.5% agarose gel and visualised by ethidium bromide staining using the LAS-3000 (Raytest) and quantified with AIDA 3.5 software (Raytest). Primer and product specifications are listed in Table 1.

2.9 Statistics

The significance of comparison of mean values was determined by the two sides of unpaired Students's *t*-test. All the data reported are given as mean ± SEM with $p \leq 0.05$ considered as significant difference.

3 Results

3.1 The AGE enriched food extract bread crust does not impair cell viability

First, it was proven that BCE contains numerous AGE/MRP modifications by Dot blot analysis using an Arg-Pyr, CML and GA-Pyr antibodies as well as an antibody against general AGE modifications as shown in Fig. 1.

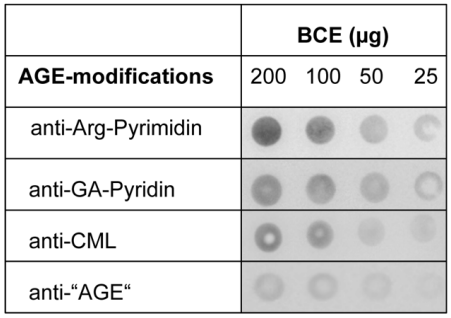

Figure 1. Food extract bread crust contains numerous AGE modifications. The indicated amount of BCE was transferred onto nitrocellulose membrane by vacuum blotting and AGE modification was detected by using different AGE – antibodies as described in Section 2.

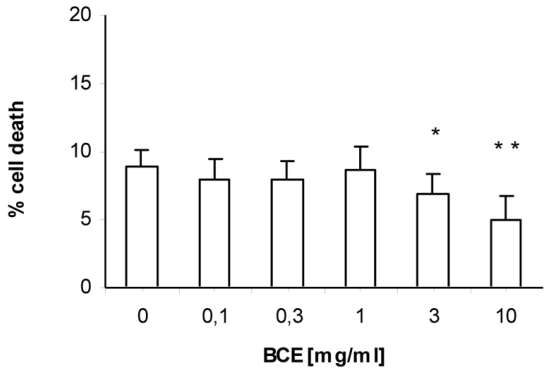

Figure 2. Effect of BCE on cell viability. Confluent serum starved cells were incubated with indicated BCE concentrations for 24 h and cell death was determined by flow cytometry using PI-staining. As shown, BCE did not induce cell death but instead enhanced viability at concentration higher than 3 mg/mL. Values are given as mean ± SEM ($n = 3$) of three independent experiments with $^{**}p < 0.001$ compared with corresponding control by Student's *t*-test.

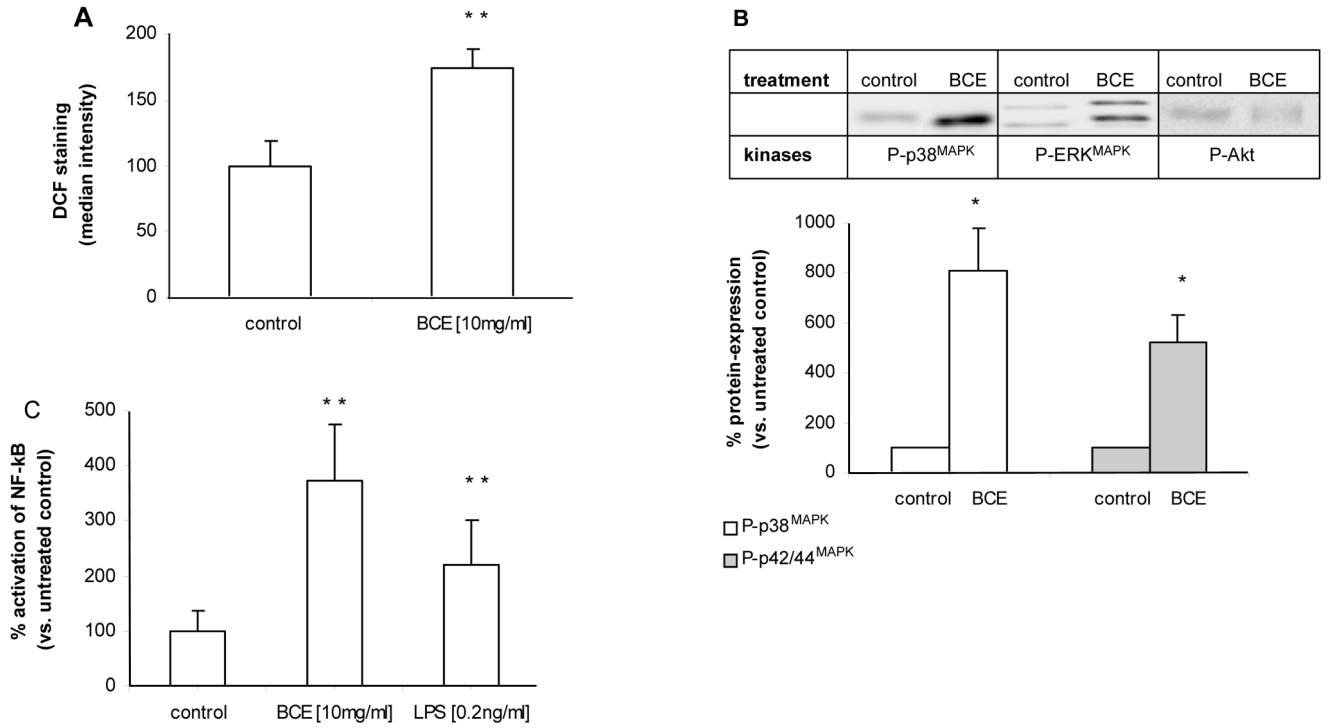

Figure 3. Influence of BCE on the production of ROS and cell signalling. (A) Formation of ROS triggered by BCE in comparison to untreated control cells. ROS were determined by monitoring the increase in fluorescence of 2′,7′-dichlorofluorescein (DCF) relative to control cells after 10 min exposure to 10 mg/mL BCE. Results are given as mean ± SEM ($n = 4$) of three independent experiments with $^{**}p < 0.01$ *vs.* untreated control analysed by Student's *t*-test. (B) Confluent CFs were stimulated with 10 mg/mL BCE for 60 min. Subsequently, cells were lysed and the activation of kinases were analysed by Western blot analysis using phospho-specific antibodies for $p38^{MAPK}$, $p42/44^{MAPK}$ and Akt kinase. Similar results were obtained in three independent experiments. (C) CFs were seeded into 96-well noncrosstalk plate at 20 000 cells/cm^2 and cultured for 24 h in medium containing 5% calf serum. After 24 h of cell transfection with pNF-κB-TA-Luc and phRL-CMV, cells were stimulated with 10 mg/mL BCE for 6 h and the NF-κB activity was determined using the dual luciferase reporter gene assay according to Section 2. Values are given as mean ± SEM ($n = 7$) of three independent experiments with $^{**}p < 0.01$ *vs.* untreated control analysed by Student's *t*-test.

To study the influence of AGE/MMP enriched food on cell viability, CF were treated with BCE for 24 h. As shown by PI-staining, the viability of serum starved CF was not negatively affected by BCE treatment over this period of time compared to control cells (Fig. 2). Instead, 10 and 30 mg/mL BCE treatment resulted in a slightly enhanced viability. In parallel, there was no impairment of cell proliferation over a period of 7 days (data not shown).

3.2 BCE induces ROS production and activation of $p38^{MAPK}$, $p42/44^{MAPK}$ and NF-κB in CFs

To elucidate the effect of BCE in more detail, the activation of intracellular signalling pathways was analysed. It is well known that AGE modified proteins can induce cellular radical formation. Indeed, the treatment of CF with 10 mg/mL BCE for up to 10 min resulted in a 1.7-fold significant, but moderate increased ROS production (Fig. 3A). Furthermore, we analysed the activation of the stress related $p38^{MAPK}$, the growth appropriated $p42/44^{MAPK}$ and survival associated Akt kinase after 60 min by Western blotting. Phosphorylation of $p38^{MAPK}$ was eight-fold and the $p42/44^{MAPK}$ was five-fold increased, respectively, compared to untreated control cells whereas the phosphorylation of the Akt kinase was not altered (Fig. 3B). As the transcription factor NF-κB is well known to be associated with intracellular ROS formation and the stimulation with model Maillard products, the activity of this factor was analysed using a reporter gene assay. A five-fold significant activation of NF-κB after 18 h of BCE-treatment was observed in comparison to three-fold activation by a 0.2 ng/mL LPS treatment for 18 h (Fig. 3C).

3.3 BCE reduced the expression of myofibroblast markers and ECM-proteins

As the transdifferentiation of fibroblasts into myofibroblasts is implicated in the development of cardiac fibrosis, we analysed whether BCE was able to interfere in this process. The expression of two typical myofibroblast marker

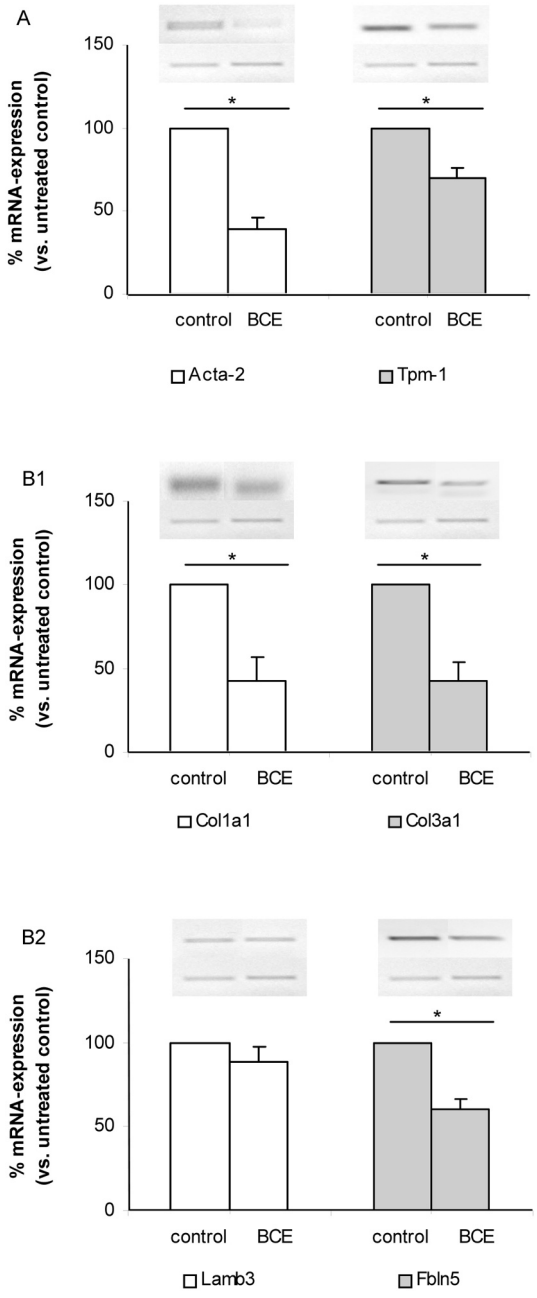

Figure 4. Effects of BCE on gene expression of myofibroblast markers and ECM proteins. Confluent serum starved CFs were incubated with 10 mg/mL BCE for 64 h. Following isolation of total RNA, gene expression was analysed by semiquantitative RT-PCR according to Section 2. Bars represent means ± SEM obtained from three independent experiments. *$p < 0.05$ compared with control by Student's *t*-test. (A) Quantification of RT-PCR results for Acta-2 and Tpm-1. Inset: PCR products for Acta-2 (upper left) and Tpm-1 (upper right) and 18 S (lower panel), respectively. (B) Quantification of RT-PCR results for collagen-1 and -3, fibulin-5 and laminin β3. (B1) Inset: PCR products for Col1a1 (upper left) and Col3a1 (upper right) and 18 S (lower panel), respectively. (B2) Inset: PCR products for Lamb3 (upper left) and Fbln5 (upper right) and 18 S (lower panel), respectively.

genes SM α-actin (Acta-2) and tropomyosin-1 (Tpm-1) were determined 64 h after stimulation with 10 mg/mL BCE. This time point was chosen to analyse the long-lasting effects induced by BCE.

As shown in Fig. 4A, the expression of both myofibroblast markers was reduced by BCE treatment. The Acta-2 mRNA was significantly down regulated by 60% and the mRNA content of Tpm-1 by 30%, compared to control cells. Furthermore, we analysed the expression of some ECM proteins because myofibroblast transdifferentiation is associated with an increased expression of these proteins. After treating CF for 64 h with BCE, a reduced expression of the ECM proteins collagen-1 (Col1a1), -3 (Col3a1) and fibulin-5 (Fbln5) could be found, whereas the mRNA level of laminin beta 2 (Lamb2) was not altered.

4 Discussion

MRPs and AGEs are the principal aromatic and flavourful components of heated food [19]. They arise during normal processing by Maillard reaction when protein- and carbohydrate containing food is exposed to high temperatures. Although we consume relatively high amounts on a daily basis, little is known about their impact on human health, whereas *in situ* generated AGEs are clearly associated with a wide range of degenerative diseases like Alzheimer, diabetes and cardiovascular diseases.

To analyse the influence of food-derived AGEs/MRPs with regard to cardiac diseases, a cardiac mice fibroblast cell line was treated with a model food extract (BCE) rich in AGE structures as confirmed by Dot Blot analysis against several AGE modifications (Fig. 1). Using specific anti-AGE antibodies, we could demonstrate the presence of Arg-Pyr, CML, GA-Pyr and further not well-characterised AGE structures.

Most of these AGE modifications can be produced by a reaction between dialdehydes and aminoacids. Arg-Pyr is a major reaction product of methylglyoxal and Arg [20], whereas CML can be formed either by oxidation of an Amadori product, or the reaction of glyoxal with lysine. GA-Pyr was first described as an AGE structure that is formed from lysine and the α-hydroxy-aldehyde glycolaldehyde [21]. The less well characterised anti-AGE antiserum recognised AGE structures that were formed by thermal glycation [22] of BSA with glucose (Urszula Kanska, Wroclaw, Poland, unpublished results). The presence of these compounds might indicate that either dialdehydes are present in bread dough or that they are formed during the baking process by the oxidation and rearrangement of carbohydrates.

First, it could be established that BCE had no toxic effects on CF (Fig. 2) but triggered cell signalling through ROS production, activation of MAP kinases and NF-κB. Although we did not analyse the sequence of signalling

events by specific inhibitors, recent studies have demonstrated that ROS plays a role as a second messenger to regulate cellular processes like the transduction of mitogenic signals. A moderate rise in the intracellular ROS concentration, like it was found in our experiments, can lead to an activation of mitogen-activated protein kinases (MAPK) p42/p44^{MAPK} and p38^{MAPK} as well as activation of the redox sensitive transcription factor NF-κB [23, 24]. Indeed, an eight-fold increase of p38^{MAPK} phosphorylation and a five-fold increased activation of the p42/44^{MAPK} was observed after BCE-treatment of CF (Fig. 3B) as well as a five-fold elevation of NF-κB activity compared to control cells (Fig. 3C). On the other hand, the cell survival-related Akt-kinase remained unaffected.

Yeh *et al.* [25] demonstrated that CML-modified albumin induced an increased ROS production, p38^{MAPK} and NF-kB activation through RAGE signalling asin human THP-1 monocytes. It was also shown previously, that BCE-treatment activated MAP-kinases in the CACO-2 enterocyte cell model, in an, at least to some degree, RAGE-dependent manner [26, 27]. Moreover, AGEs and RAGE are linked to pathways involved in cellular oxidative stress perception by multiple mechanisms [28] and it is likely that the response of CF to BCE also relied on such a mechanism as these cells do express RAGE at low levels (data not shown). In chronic disorders, a prolonged activation of RAGE is often associated with the propagation of the disease due to the continuous presence of endogenous AGE –ligands, and are therefore unfavourable. However, due to the complex chemical composition of BCE, an interaction with additional receptors is also possible.

It is known that in response to myocardial diseases and pressure overload of paracrine and autocrine, signals are released in the cardiac interstitium that stimulate fibroblasts to participate in wound healing but later to differentiate into myofibroblasts. Thus, fibroblast transformation is associated with an increased production of ECM proteins like collagen I/III and a decreased matrix turnover, resulting in cardiac fibrosis and increased chamber stiffness of the heart [29]. Thus, agents that block the differentiation or promote the redifferentiation into normal fibroblasts have the potential to improve the organ function following injury and subsequent healing processes by suppressing the development of fibrosis. The CF cell line used in our studies expressed SM α-actin in small, but significant amounts which made it possible to investigate whether nutritive compounds might affect the transition towards the fibroblast phenotype. In our experiments BCE treatment reduced the expression of two typical myofibroblast markers SM α-actin and tropomyosin-1 as well as the expression of ECM proteins like collagen I/III and myofibulin-5. Recent studies by Greenberg *et al.* [30] suggest that the successive activation of the focal adhesion kinase (FAK), FGF-signalling *via* the fibroblast growth factor receptor substrate 2 (FRS2) and p42/44^{MAPK} pathway prevent SM α-actin expression and the differentiation into myofibroblasts. BCE also activates the p42/44^{MAPK} pathway and this might contribute to the reduced SM α-actin expression in BCE treated cells. However, further studies on growth factors like TGF-β and FGF and the possible interference of BCE in these cells are required to understand this process in detail. Furthermore, myofibroblasts produce increased amounts of ECM proteins. Again, treating of CF with BCE decreased the expression of ECM proteins and this also argues for the maintenance of the fibroblast phenotype.

As stated above, a prolonged RAGE activation is considered to be implicated in chronic diseases. Nevertheless, our data point towards a protective, antifibrotic effect of BCE. This might be due to the short exposure time or the different chemical structure and composition of the AGE moieties that are present in BCE in contrast to the endogenously formed entities. It is imperative to further analyse which component of the heterogeneous class of food AGE/MRPs is responsible for this protective effect. Lindenmeier and coworkers [16] have already reported that BCE contains pronyl-lysine which was able to increase phase II GST activity in an enterocyte model system. Further investigations are necessary to analyse the influence of this substance and other chemically defined AGE compounds from BCE on our cell culture model.

In summary, our data suggest an antifibrotic effect of BCE on CFs but further studies are necessary to identify the bioactive ingredients of BCE and to optimise the food processing technologies to obtain optimal amounts of health beneficial AGEs/MRPs.

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