DOI 10.1002/mnfr.200600110 Mol. Nutr. Food Res. 2007, *51*, 468 – 472

Research Article

Influence of model melanoidins on calcium-dependent transport mechanisms in smooth muscle tissue

Iliyana D. Stefanova[1], Mariana D. Argirova[2] and Athanas D. Krustev[1]

[1] Department of Biophysics, Medical University, Plovdiv, Bulgaria
[2] Department of Chemistry and Biochemistry, Medical University, Plovdiv, Bulgaria

Melanoidins obtained from L-arginine and D-glucose (MW > 3500 Da) were tested for their ability to influence the contractility of gastric smooth muscles. A study within the range 0.1 – 10 mg/mL revealed that at low concentrations, the melanoidins provoked concentration-dependent contraction, whereas a muscle relaxation was registered at high concentrations. The contraction was preceded by changes in the calcium membrane current as measured by single sucrose-gap method and significantly attenuated by the calcium channel blockers D-600 and nifedipine. Measurements with Ca^{2+}-selective electrode showed that the melanoidins decreased the concentration of ionized Ca^{2+} in tissue bath in concentration-dependent manner. Experiments carried out in solutions with lower than normal Ca^{2+} concentration and using melanoidins preliminary saturated with Ca^{2+} confirmed that the calcium chelation by melanoidins was a key contributing cause for the development of relaxant response. The results obtained showed that the melanoidins could influence the contractility of smooth muscles through at least two pathways: at low concentrations they caused depolarization and activation of L-type calcium channels, stimulated the Ca^{2+} influx, and provoked contraction, whereas at high concentrations calcium binding by melanoidins led to significant depletion of extracellular calcium ions and contributed to the relaxation process observed.

Keywords: Arginine / Contractility / Gastric smooth muscle / Maillard reaction / Melanoidins

Received: July 18, 2006; revised: December 5, 2006; accepted: December 14, 2006

1 Introduction

Melanoidins are brown-colored polymeric molecules formed in foods during thermal processing and cooking. They are final products of the Maillard reaction – the interaction between reducing sugars and proteins or other substances containing free amino groups. The Maillard reaction products (MRPs) are ubiquitous component of daily human diet and thus, continuously in contact with the cells of gastrointestinal tract.

The nutritional, physiological, and biological activities of MRPs have received much attention in the last decades. Based on animal models, it was supposed that about 10% of dietary MRPs are biologically insignificant [1]. Some low molecular weight MRPs such as N_{ε}-(carboxymethyl)lysine [2], pyrraline, and pentosidine [3] represent nonreactive products that are readily excreted in the urine, while 3-deoxyglucosone, methylglyoxal, and supposedly many others are reactive intermediates called glycotoxins that may attach onto serum and tissue proteins [4]. Although the high molecular weight melanoidins have been generally considered as inert, some specific biochemical effects of these MRPs have been reported [5]. Among them were the ability of melanoidins to stimulate the growth of intestinal anaerobic bacteria [6] and to influence the activities of Phase I and Phase II xenobiotic enzymes [7 – 10].

Isolated smooth muscle preparations (SMPs) are widely used in medical and biological studies that deal with the mechanism of action of different biologically active substances. The smooth muscle cells have undergone relatively low differentiation and specialization during phylogeny and possess variety of receptors specific to physiologically active compounds. The particular role of the melanoidins in the physiological effects of dietary components on smooth muscles of gastrointestinal tract has not been addressed yet. Gathering such information would extend the knowledge

Correspondence: Dr. Mariana Argirova, Department of Chemistry and Biochemistry, Medical University, 15a Vassil Aprilov Strasse, 4002 Plovdiv, Bulgaria
E-mail: margirova@netbg.com
Fax: +359-32-636-200

Abbreviations: MRP, Maillard reaction product; **SMP,** smooth muscle preparation

about the MRPs, their importance for diet and is a question of present interest.

2 Materials and methods

2.1 Preparation of MRPs

MRPs were obtained from L-arginine and D-glucose as described elsewhere [11]. The product obtained after heating was dissolved in 25 mL of distilled water and packed in a 3 500 Da cut-off dialysis tubing (Spectra/Por® 3; B. V. Breda, The Netherlands). Dialysis was carried on for 72 h against large excess of distilled water and several changes of dialysis fluids. Water-soluble melanoidins were obtained after filtration and freeze-drying of the retained solution. In one experiment, half of the crude MRPs was dialyzed against distilled water, whereas the other half started with dialysis against distilled water for 48 h, and then the product was dialyzed against Krebs solution for 6 h; afterwards, the dialysis continued against distilled water for 18 h. The melanoidins saturated with Ca^{2+} were used in the study of the mechanism of muscle relaxation.

2.2 Registration of bioelectrical and mechanical activity of isolated SMPs

The ability of the MRPs Arg-Glc to stimulate bioelectric and mechanical muscle activity was tested on gastric corpus SMPs obtained from adult male Wistar rats (weight between 280 and 320 g). The internationally approved principles of laboratory animal care (86/609/EEC) were strictly followed. The animals were housed in standard laboratory conditions: temperature, food, and 12-h light/dark cycle, and decapitated under ether anesthesia. Circular gastric corpus SMPs, 12 – 13 mm long and 1.0 – 1.1 mm wide were immediately dissected. They were mounted to a glass holder at one end and to a Swema tensodetector (Stockholm, Sweden) at the other, and immersed in a 20-mL tissue bath. The initial mechanical tension of the SMPs had a value of 10 mN and was set after 60-min equilibration and stabilized spontaneous muscle activity. The bioelectric activity of MRPs was measured by single sucrose-gap method. Krebs, KCl, and sucrose bathing solutions were continuously aerated with a mixture of 5% CO_2 and 95% O_2, and the temperature was maintained at 37°C. The mechanical changes were registered isometrically with a Microtechna amplifier (Prague, Czech Republic) and recorded by a Linseis recorder (Selb, Germany).

SMPs were treated with MRPs by adding aliquots of concentrated melanoidin stock solution (200 mg/mL in Krebs solution). The intactness of the contractile apparatus of SMPs during and at the end of experiments was checked by adding 1×10^{-5} M acetylcholine between each treatment with MRPs. Dose-response data were obtained noncumulatively starting with the lowest dose.

2.3 Solutions and chemicals

The following substances were obtained from Sigma Chemical Company (St. Louis, MO): L-arginine, (±)-methoxyverapamil hydrochloride (D-600), and nifedipine. The Krebs solution had pH 7.40 and the following composition (in mM): 120 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 15.4 $NaHCO_3$, 11.5 glucose. All the ingredients for this solution were of analytical grade and obtained from Merck (Darmstadt, Germany). In the experiments carried out in Krebs solutions with lower than normal Ca^{2+} content, the osmolality was maintained by an increased amount of NaCl. In the experiments carried out in Krebs solution with lower than normal pH, the latter was achieved by changing the ratio of KH_2PO_4 and $NaHCO_3$.

Melanoidin solutions with various concentrations were prepared in Krebs medium in triplicates and used for the determination of ionized Ca^{2+} and pH. The calcium concentration was measured by ion-selective electrode block of clinical analyzer Konelab 60 (Thermo Electron, Waltham, MA) and the pH was determined by Schott handlab 11 pH meter (Schott, Mainz, Germany).

2.4 Statistical analysis

The mean ± standard error (SE) of the effects was calculated for each data point, and the groups were compared using paired or unpaired Student's *t*-test as appropriate by applying INSTAT computer program. Level of significance p was set at 0.05.

3 Results

3.1 Effect of Arg-Glc on the mechanical and bioelectrical activity of SMPs

The melanoidin fraction of the MRPs Arg-Glc demonstrated concentration-dependent effects on the mechanical activity of SMPs. Low concentrations (0.1 and 0.25 mg/mL) evoked muscle contraction. A biphasic effect was registered at concentrations 1 and 2.5 mg/mL; the contraction was preceded by a transient relaxation. Concentrations of 5, 7.5, and 10 mg/mL had a relaxant effect (Fig. 1).

A submaximal concentration of 1.5 mg/mL was used in our further studies on the mechanism of muscle contraction. The intensity of cell membrane depolarization caused by 1.5 mg/mL Arg-Glc was 11.6 ± 2.67 mV (n = 8) as measured by single sucrose-gap method.

3.2 Effects of calcium antagonists on the melanoidin-induced contractile reaction

The effects of L-type Ca^{2+} channel blockers, D-600 (3×10^{-7} mol/L), and nifedipine (1×10^{-6} mol/L) on melanoidin-induced contractions were examined. The initial

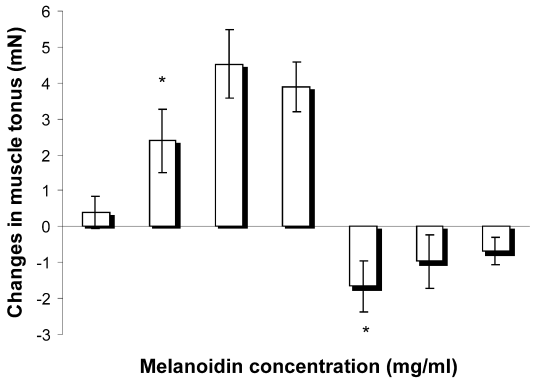

Figure 1. Amplitude of mechanical reactions of SMPs evoked by different concentrations of Arg-Glc. Positive values refer to contractile response; negative values refer to relaxant effect. The initial relaxant phase preceding the contractions at concentrations of 1 and 2.5 mg/mL is not shown.

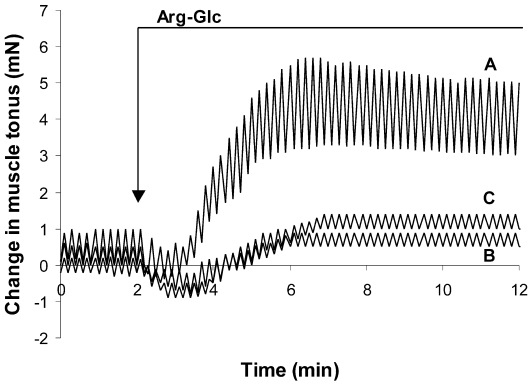

Figure 2. Changes in tonus of SMPs evoked by 1.5 mg/mL Arg-Glc: control record in the presence of melanoidin alone (A), and after pretreatment with 3×10^{-7} mol/L D-600 (B), or 1×10^{-6} mol/L nifedipine (C). Zero level indicates the initial muscle tonus before the addition of Arg-Glc. Positive values of mechanical activity refer to contraction; negative values indicate relaxant response.

contraction of 4.44 ± 1.98 mN evoked by 1.5 mg/mL Arg-Glc was significantly reduced down to 0.52 ± 0.44 mN ($n = 8$, $p = 0.0001$) in preliminary treated with D-600 SMPs. Nifedipine also blocked the contractile phase of the effect caused by Arg-Glc. Control SMPs treated with Arg-Glc had amplitude of contraction 3.44 ± 0.31 mN, which was reduced to 1.36 ± 0.67 mN ($n = 4$, $p = 0.001$) in pretreated with nifedipine SMPs. Records of these mechanical responses are shown in Fig. 2.

3.3 Effect of Arg-Glc on Ca^{2+}-free Krebs solution

Replacement of the standard Krebs solution in tissue bath with Ca^{2+}-free solution caused a muscle relaxation. After 10-min stabilization of the muscle tonus, it reached values that were close to the maximal relaxation. The addition of Arg-Glc (1.5 mg/mL) to the relaxed SMPs ($n = 10$) did not change their tonus.

3.4 Effect of KCl depolarization on the melanoidin-induced contractions

Replacement of the standard Krebs solution in tissue bath with Krebs solution, containing 42 mM KCl caused a transient sharp muscle contraction, followed by stabilization of the muscle tonus below the level of the initial tonus. In contrast to the effect demonstrated by 1.5 mg/mL Arg-Glc in normal Krebs solution, the contractile phase was completely abolished in the depolarized tissues; only sustained relaxation with amplitude of 2.01 ± 0.78 mN was registered. The relaxant effect was not significantly different from that developed in normal Krebs solution (1.32 ± 0.66 mN; $n = 4$, $p > 0.05$).

3.5 Changes in the concentration of ionized calcium and pH of the Krebs solution in the presence of Arg-Glc and their effects on the contractility of SMPs

The original Krebs solution contained 2.5 mM Ca^{2+}. This concentration, however, decreased gradually in the presence of 1, 2.5, 4, and 10 mg/mL melanoidins as determined by calcium ion-selective electrode (Fig. 3). Krebs solutions having lower than normal Ca^{2+} concentration and corresponding to those measured in the presence of melanoidins significantly decreased the muscle tonus (Fig. 4).

The model melanoidins Arg-Glc (7.5 mg/mL) obtained after dialysis against distilled water brought about a relaxation with amplitude of 0.98 ± 0.33 mN ($n = 3$). The same concentration of melanoidins preliminary saturated with Ca^{2+} by reverse dialysis against Krebs solution demonstrated significantly diminished relaxant effect with amplitude of 0.43 ± 0.16 mN ($p < 0.05$).

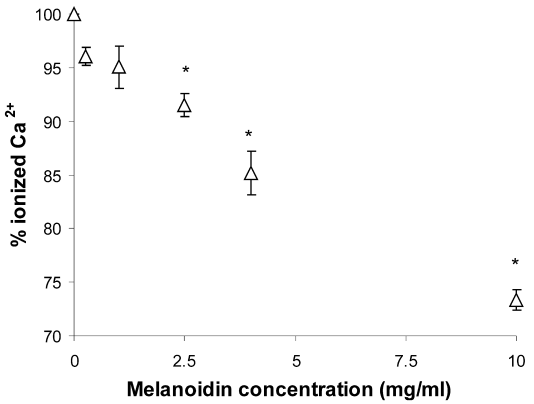

Figure 3. Concentration of ionized Ca^{2+} in the presence of different concentrations of Arg-Glc. Data are presented as a percentage of the initial 2.5 mM Ca^{2+} in the Krebs solution. Asterisks indicate values that are statistically different compared to the initial Ca^{2+} concentration.

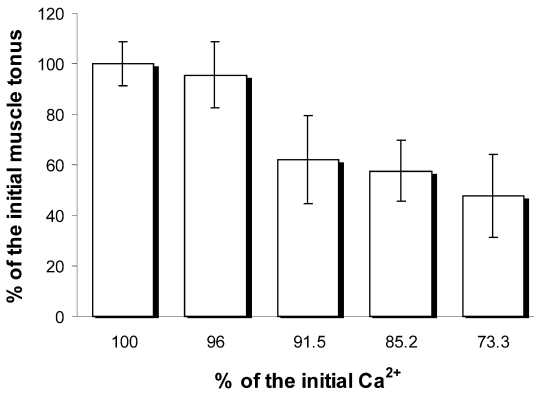

Figure 4. Effects of decreased Ca^{2+} content in tissue bath on the mechanical activity of SMPs.

No significant differences were found between the pH of Krebs solution in the absence and presence of 1, 1.5, 2.5, and 4 mg/mL Arg-Glc. The highest tested concentration of MRP: 10 mg/mL caused a decrease in pH down to 7.21 ($p < 0.05$). Replacement of the normal Krebs solution (pH 7.4) with Krebs solution having pH 7.21 did not induce any mechanical reaction of SMPs.

4 Discussion

The model melanoidins Arg-Glc had an influence on the physiological state of smooth muscle tissues and caused changes in their mechanical activity. The responses were concentration-dependent and varied from contraction, through a biphasic effect of relaxation/contraction up to a relaxant effect caused by high melanoidin concentrations.

One of the most ubiquitous signaling substances that modulates the excitability of smooth muscles is the calcium ion [12]. It was plausible to suppose an influence of the melanoidins on cellular mechanisms related to Ca^{2+} homeostasis in smooth muscle cells. The experiments with Ca^{2+}-free Krebs solution in tissue bath showed that nominal lack of extracellular Ca^{2+} eliminated the development of smooth muscle contraction. This finding led to the conclusion that the melanoidins evoked the contractile reaction through influence upon Ca^{2+} influx. The membrane depolarization, registered by single sucrose-gap method, and preceding the contraction, was an indication for the activation of potential-dependent ion channels in the presence of melanoidin. Furthermore, in the presence of inhibitors of L-type Ca^{2+} channels, D-600 or nifedipine, the melanoidin-induced contraction was significantly blocked. Thus it can be concluded that these channels were the major gate for Ca^{2+} influx from the extracellular surrounding that were affected by the presence of melanoidins, but most likely not the only one. The fact that these Ca^{2+} antagonists did not completely abolish the contraction suggested the possible involvement of other Ca^{2+}-determined mechanisms. When most of the potential-dependent ion channels were preliminary opened by membrane depolarization with 42 mM KCl, only the relaxant phase of the melanoidin-induced mechanical reaction was observed. Therefore, the melanoidins were not able to influence over already depolarized membranes.

Decrease in cytosolic level of Ca^{2+} is a typical event causing muscle relaxation. Gomyo and Horikoshi [13] reported that the melanoidins behave as anionic hydrophilic polymers, which can form stable complexes with metal ions. Numerous investigations have reported chelating properties of different model melanoidins [14–16], including complexation of calcium. The measurement of concentration of ionized calcium in bathing solution in the presence of melanoidins showed that concentrations of 5.0, 7.5, and 10.0 mg/mL Arg-Glc significantly decreased calcium levels; only 73.3 ± 0.9% of the initial 2.5 mM Ca^{2+} remained in the presence of the highest Arg-Glc concentration tested. The experiments with Krebs solutions containing lower than normal Ca^{2+} concentration, corresponding to those in the presence of MRP, showed significantly decreased contractile activity and development of relaxation.

Evidence that the depletion of free calcium ions as a result of chelation by melanoidins is related to the relaxation observed was also found in experiments with melanoidins preliminary saturated with Ca^{2+} by reverse dialysis. They elicited significantly reduced relaxation compared to the normal, nonsaturated melanoidins. These findings supported the assumption that the melanoidin-induced relaxation was related to the depletion of extracellular Ca^{2+} and most likely coupled to the intracellular calcium levels. On the other hand, the relaxation was not completely abolished and this finding shows that the calcium chelation is important but not the only factor causing muscle relaxation in the presence of high concentrations of melanoidins. The slight decrease in pH of the Krebs solution in the presence of high melanoidin concentrations does not contribute to the relaxation since the bathing solutions with lower pH corresponding to those measured in the presence of different melanoidin concentrations did not cause any mechanical reaction of SMPs.

In conclusion, the present results reveal that the model melanoidins Arg-Glc possess a complex, concentration-dependent profile of influence on cellular mechanisms involved in muscle contraction/relaxation. Leading role in the melanoidin-induced contractions had the depolarization of smooth muscle tissues, which increased the Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels. One of the major factors causing smooth muscle relaxation at high melanoidin concentration was the decrease in extracellular Ca^{2+} in tissue bath as a result of calcium chelation by the melanoidins.

5 References

[1] Sgarbieri, V. C., Amaya, J., Tanaka, M., Chichester, C. O., Response of rats to amino acid supplementation of brown egg albumin, *J. Nutr.* 1973, *103*, 1731 – 1738.

[2] Bergmann, R., Helling, R., Heichert, C., Scheunemann, M., *et al.*, Radio fluorination and positron emission tomography (PET) as a new approach to study the *in vivo* distribution and elimination of the advanced glycation endproducts N epsilon-carboxymethyllysine (CML) and N epsilon-carboxyethyllysine (CEL), *Nahrung/Food* 2001, *45*, 182 – 188.

[3] Forster, A., Kuhne, Y., Henle, T., Studies on absorption and elimination of dietary Maillard reaction products, *Ann. NY Acad. Sci.* 2005, *1043*, 474 – 481.

[4] Koschinsky, T., He, C. J., Mitsuhashi, T., Bucala, R., *et al.*, Orally absorbed reactive glycation products (glycotoxins): an environmental risk factor in diabetic nephropathy, *Proc. Natl. Acad. Sci. USA* 1997, *94*, 6474 – 6479.

[5] Somoza, V., Five years of research on health risks and benefits of Maillard reaction products, *Mol. Nutr. Food Res.* 2005, *49*, 663 – 672.

[6] Ames, J. M., Wynne, A., Hofmann, A., Plos, S., Gibson, G. R., The effect of a model melanoidin mixture on faecal bacterial populations *in vitro*, *Br. J. Nutr.* 1999, *82*, 489 – 495.

[7] Kitts, D. D., Wu, C. H., Powrie, W. D., Effect of glucose-lysine Maillard reaction product fractions on tissue xenobiotic enzyme systems, *J. Agric. Food Chem.* 1993, *41*, 2359 – 2363.

[8] Hofmann, T., Ames, J., Krome, K., Faist, V., Determination of the molecular weight distribution of non-enzymatic browning products formed by roasting of glucose and glycine and studies on their effects on NADPH-cytochrome c-reductase and glutathione-S-transferase in Caco-2 cells, *Nahrung/Food* 2001, *45*, 189 – 194.

[9] Faist, V., Lindenmeier, M., Geisler, C., Erbersdobler, H. F., Hofman, T., Influence of molecular weight fractions isolated from roasted malt on the enzyme activities of NADPH-cytochrome c-reductase and glutathione-S-transferase in Caco-2 cells, *J. Agric. Food Chem.* 2002, *50*, 602 – 606.

[10] Borrelli, R. C., Mennella, C., Barba, F., Russo, M., *et al.*, Characterization of coloured compounds obtained by enzymatic extraction of bakery products, *Food Chem. Toxicol.* 2003, *41*, 1367 – 1374.

[11] Obretenov, C., Demyttenaere, J., Tehrani, K. A., Adams, A., *et al.*, Flavor release in the presence of melanoidins prepared from L-(+)-ascorbic acid and amino acids, *J. Agric. Food Chem.* 2002, *50*, 4244 – 4250.

[12] Akbarali, H. I., Signal-transduction pathways that regulate smooth muscle function. II. Receptor-ion channel coupling mechanisms in gastrointestinal smooth muscle, *Am. J. Physiol.* 2005, *288*, G598 – G602.

[13] Gomyo, T., Horiikoshi, M., On the interaction of melanoidin with metallic ions, *Agric. Biol. Chem.* 1976, *40*, 1 – 12.

[14] O'Brien, J., Morrissey, P. A., Metal ion complexation by products of the Maillard reaction, *Food Chem.* 1997, *58*, 17 – 27.

[15] Morales, F. J., Fernándes-Fraguas, K., Jiménes-Pérez, S., Iron-binding ability of melanoidins from food and model systems, *Food Chem.* 2005, *90*, 821 – 827.

[16] Delgado-Andrade, C., Seiquer, I., Navarro, M. P., Comparative effects of glucose-lysine versus glucose-methionine Maillard reaction products consumption: *in vitro* and *in vivo* calcium availability, *Mol. Nutr. Food Res.* 2005, *49*, 679 – 684.

