Effect of “in vitro” induced glycation on thermostability of bone tissue

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ABSTRACT

The aim of the study was to test the hypothesis that glycation would influence thermal stability of bone tissue collagen. Bone samples were incubated in buffer or in ribose solution. Then, half of the ribosylated and half of the control samples were completely demineralized in formic acid. Differential scanning calorimetry was performed for temperatures from 40 °C to 220 °C in nitrogen atmosphere on intact (mineralized) and demineralized bone samples, partially dehydrated at room temperature.

Samples were thermally active in temperatures from 110 °C to 210 °C. Few endotherms of a complex nature were found in demineralized and intact bone. Thermodynamics of collagen conformations was affected by glycation, especially in demineralized bone where a significant increase of denaturation temperature (by 3–4 °C) and enthalpy drop (above 20%) were stated after glycation.

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1. Introduction

Bone tissue is a composite material consisting of carbonated hydroxyapatite crystals deposited in an organic matrix of collagen fibres. The highly organized structure of collagen in bone is stabilized by enzymatically mediated covalent cross-links between collagen molecules and fibrils, which provide adequate degree of tissue strength and stability. Even though the primary sequence of a collagen molecule is identical in bones and in other connective tissues, cross-link profile of bone collagen is specific for this tissue [1].

In all collagen containing connective tissues in the presence of sugars in extracellular fluids a nonenzymatic reaction between the sugars and collagen—glycation results in the formation of additional cross-links. Glycation (nonenzymatic glycosylation, NEG) is a spontaneous process and its products—advanced glycation end products (AGEs) accumulate in tissues of living organisms with ageing resulting in depressed solubility of collagen, its lower susceptibility to enzymatic digestion and in increasing stiffness of the tissue [2,3]. In diabetes an increased concentration of glucose in blood results in a considerable acceleration of glycation. Accumulation of destructive AGEs followed by changes in connective tissue collagen is one of the causes of diabetes related problems like renal and cardiovascular disorders or skin lesions [4]. It is unknown to what extent NEG would compromise the integrity of bone tissue. However, the stiffening of collagen in bone, caused by AGEs, could be relevant to age-related increased bone fragility and to a high incidence of bone fracture in diabetes [3,5–7].

There is a growing body of the literature concerning the accumulation of NEG crosslinks in bone in relation to the mechanical performance of the tissue [6–13]. AGEs were shown to alter morphology, microfracture formation and fracture resistance of cancellous bone [7,9,12,13]. In cortical bone an impact of NEG on mechanical properties is not as apparent as in cancellous one. Mechanical behaviour in bending was not influenced by incubation in ribose [13] and in glucose solution [8]. However, glycation was shown to affect significantly mechanical behaviour of bone samples under axial loads and the effects were more pronounced in demineralized bone matrix than in the intact tissue [10,11]. Thus, it seems presumable that a deeper insight into glycation induced modifications of bone collagen properties would be valuable for better understanding of fracture risk in diabetes.

The overall condition of collagen structure can be studied in terms of thermally induced transformations of the material. Thermal techniques, in particular, differential scanning calorimetry (DSC) provides a powerful method for examining conditions in which the stabilization of collagen molecules breaks down [14–18]. DSC studies showed that thermal denaturation of collagen triple helix occurred at much higher temperatures in fibres than in solution and increased dramatically both in dehydrated collagen [15,17,18] and in mineralized collagen in bone tissue and tendon [19–21]. Sensitivity of the thermal behaviour of collagen to
amount of covalent cross-links and to level of collagen hydration was attributed to changes in interaxial separation of the collagen molecules in the fibre [15,18].

DSC was successfully applied to study effects of glycation on collagen in tissues. Effects of cross-links induced by glycation on physicochemical properties of collagen were shown in tendon [22,23], skin [22,24], lens capsules [25] and sclera [26]. So far there is no report on effects of glycation on thermal stability of bone collagen.

The aim of the present study was to test the hypothesis that glycation would influence stability of bone tissue collagen evaluated in terms of DSC analysis.

2. Materials and methods

Cortical bone samples were wet machined from a shaft of bovine femur from an 18 months old cattle. Bone cubes (4.5–4.7 mm) were grouped in pairs of specimens obtained from adjacent anatomical position. One sample from each pair was subjected to in vitro glycation by incubation for 21 days in 100 mg/mL of ribose in Hanks buffer/1.3 mM CaCl₂ at 37 °C supplemented with 5 mg/10 mL of gentamicin as a prevention against bacterial growth. It was stated previously [11] that such a procedure results in a significant accumulation of advanced glycation end-products in bovine bone specimens. The other sample from each pair was incubated for the same time period in the same buffer without ribose.

Six pairs of samples (one ribosylated and one control sample in each pair) were then decalcified in 45% formic acid with 1 mM sodium citrate [11]. Completeness of demineralization was checked by ashing of additional test samples (ribosylated and control) of the same size in a muffle furnace at 620 °C for 10 h. After 20 days in the acid solution test samples were completely ashed without any residuum. Five pairs of incubated samples were left with intact mineral phase.

0.5–0.7 mm thick slices from each decalcified and intact bone sample were prepared as samples for calorimetric measurements. Samples were dehydrated at room temperature for 24 h in excicator and weighted with accuracy of 10⁻⁴ g just before measurements. The mass of dried samples was 4–7 mg. Mass fraction of water in samples was determined using test slices of similar mass weighted after drying in excicator and then in 105 °C.

Stability of collagen in intact and decalcified samples was investigated using differential scanning calorimetry (Q2000, TA Instruments, US). The calorimeter was calibrated with indium as a standard. Samples were sealed in hermetic aluminium pans and scanned in temperatures from 40 °C to 220 °C in nitrogen atmosphere with an empty capsule as a reference. Six pairs of decalcified and five pairs of intact bone slices (one slice from one bone cube) were heated at scanning rate of 3 °C/min. Two additional pairs of decalcified slices were scanned only to 165 °C for visual examination at that temperature. For each endothermal process obtained on the thermogram, the initial (onset) temperature (T_{on}) of the peak temperature at maximum heat absorption (T_{max}) and the area of the peak, corresponding to transition enthalpy (ΔH) were determined using a software integrated with the calorimeter.

As the shape of endotherms was complex, decomposition of the traces was done using Gramms Al, v.9.00 R2 software. The decomposition procedure enabled to find the peak temperature and the relative area of each component of the endotherm.

All results given in the text are given as mean (±S.D.). Statistical analysis of the differences between glycated and control samples was performed in terms of Mann–Whitney U test (Statistica 6.1).

3. Results

Occurrence of AGEs in samples incubated in ribose was presumed on the basis of the colour change from white to pale brown while samples incubated in buffer remained white during the period of incubation. In other experiments using the same or similar procedure of glycation existence of AGEs was confirmed by fluorescence measurements [9,11,13].

Hydration level was 12.6% (±0.9%) in demineralized and 9.8% (±0.7%) in intact bone samples. In each of samples at least two endothermal processes of different complexity and cooperativity were stated in temperatures above 100 °C without any sign of thermal activity in lower temperatures. Samples of decalcified bone matrix were thermally active in temperatures from 110 °C to 210 °C (Fig. 1a) while the intact bone samples were active in temperatures between 110 °C and 190 °C (Fig. 1b). In each of demineralized samples three endotherms at 110–135 °C, 150–160 °C and 190–210 °C were found. Two first endotherms were complex in shape and followed by a considerable increase of heat flow rate. Intact bone samples were thermally active at 130–145 °C, 145–165 °C and 165–180 °C, however in glycated samples the second area was much less distinct and probably overlapped with the last endotherm. Moreover, in each of intact samples a first subtle sign of thermal activity was noticed between 118 °C and 122 °C. In the main body of samples the first endotherm had a double peak separated by less than 1 °C, followed by an additional broad tail. The last endotherm, above 190 °C in decalcified and above 165 °C in intact bone, was considerably larger than all former thermal processes, both in demineralized and intact samples, glycated and control. That endotherm can represent an irreversible melting of collagen involving decomposition of fibres into smaller units. The enthalpy of the process was about 100 J/g in demineralized and 120 J/g in intact bone which is much higher than enthalpies of collagen denaturation reported to be not higher than 85 J/g in fully hydrated state and at most about 40 J/g in dehydrated state [14–18]. Moreover, samples were examined visually just after completion of that last endotherm. When a sample pan was opened the decalcified samples were visibly melted in a form of pale-yellow (control) or pale brown (glycated) viscous liquid. Intact bone samples did not show any evidence of changes. Decalcified samples scanned to the completion of the smaller endotherms, i.e. to 165 °C were markedly deformed but without any sign of melting.

Average values of thermal parameters for demineralized samples are given in Table 1. The first endotherm was significantly influenced by glycation in terms of all parameters. In glycated samples the onset temperature (T_{on}) and the peak temperature (T_{max}) were in average 4.4 °C and 3.4 °C higher than in controls. Moreover, the process occurred with lower enthalpy than in the controls. The temperatures and enthalpy of the second endotherm were not influenced by glycation. The temperatures of last, melting endotherm were very similar in glycated and control samples but enthalpy of the process was significantly lowered by glycation.

Average results for samples with intact mineral are given in Table 2. Temperatures of the first and the third endotherms were not influenced by glycation. The temperature of the second endotherm appears to rise in glycated samples. However, that endotherm was not described quantitatively because was flat and broad, and probably overlapped with the melting endotherm (Fig. 1b). Enthalpy of the first endotherm was very low (did not exceed 2.61 J/g in any sample) and not significantly different in glycated and control samples. The enthalpy of the melting endotherm was significantly lowered by glycation. The small activity around 120 °C in intact bone samples (not shown in Table 2), resembling glass transition, was not affected by ribosylation. The mid-point temperature of this process was 119.5±2.5 °C in the control and 120.4±2.1 °C in the glycated samples.
Fig. 1. Example of DSC thermograms of (a) demineralized and (b) intact bone samples; dotted line—a control sample, solid line—a glycated sample. The onset temperature ($T_{on}$) corresponding to the start of transition, max temperature ($T_{max}$) representing the temperature at maximum heat absorption, and the area between the trace and the baseline corresponding to transition enthalpy ($\Delta H$) were determined for each endothermal process on the thermograms. Inset area is indicated in the main thermogram; endothermal processes are directed down.

Decomposition procedure was applied to the first and the second endotherm in decalcified samples. An example of curve fitting results applied to the first endotherm is given in Fig. 2. The first endotherm revealed three main processes with peak temperatures equal to 113.0 ± 1.6 °C, 116.6 ± 1.2 °C, and 124.8 ± 2.7 °C in controls and 117.0 ± 2.6 °C, 119.1 ± 2.5 °C, and 132.1 ± 4.3 °C in ribosylated. The differences between peaks in ribosylated and controls were significant with p-values = 0.006, 0.038 and 0.003, respectively. The areas of the subsequent components of the endotherm were 31%, 29% and 40% of the total area in controls and 38%, 25% and 37% in ribosylated.

Table 1
Thermal characteristics of demineralized bone samples.

<table>
<thead>
<tr>
<th></th>
<th>$T_{on1}$ (°C)</th>
<th>$T_{m1}$ (°C)</th>
<th>$\Delta H1$ (J/g)</th>
<th>$T_{on2}$ (°C)</th>
<th>$T_{m2}$ (°C)</th>
<th>$\Delta H2$ (J/g)</th>
<th>$T_{on3}$ (°C)</th>
<th>$T_{m3}$ (°C)</th>
<th>$\Delta H3$ (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>109.6 (2.7)</td>
<td>114.1 (2.8)</td>
<td>31.2 (2.1)</td>
<td>152.3 (3.1)</td>
<td>153.0 (3.0)</td>
<td>8.5 (1.3)</td>
<td>195.1 (4.8)</td>
<td>195.6 (4.9)</td>
<td>109.4 (4.0)</td>
</tr>
<tr>
<td>R</td>
<td>114.0 (2.7)</td>
<td>117.5 (2.4)</td>
<td>23.4 (2.8)</td>
<td>153.2 (3.4)</td>
<td>155.5 (2.1)</td>
<td>9.2 (2.1)</td>
<td>190.8 (9.8)</td>
<td>191.7 (10.5)</td>
<td>89.0 (15.8)</td>
</tr>
<tr>
<td>n=6</td>
<td>0.010</td>
<td>0.025</td>
<td>0.001</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>0.009</td>
</tr>
</tbody>
</table>

$q$—onset temperature; $T_{m}$—peak temperature; $\Delta H$—enthalpy change per gram of a sample (for three subsequent endotherms). C= control samples; R= samples after three weeks of glycation in ribose solution; mean values and (S.D.) are given; $n$—number of samples in the group. $p$-Value from Mann-Whitney $U$ test.
glycated, and the differences between glycated and controls were not significant. The second endotherm was decomposed into two peaks at 152.9 ± 2.8 °C and 157.2 ± 5.6 °C in controls and 154.9 ± 2.0 °C and 156.6 ± 1.5 °C in glycated samples. The areas of both peaks were 70% vs 30% in controls and 55% vs 45% in glycated samples. None of the parameters for the second endotherm components was significantly different in glycated and control samples.

4. Discussion

The results of the study demonstrated that partially dehydrated collagen in bone matrix was thermally very stable both in demineralized and in intact bone samples. Moreover, the thermograms revealed that a complex pattern of endothermal processes in bone collagen was influenced by glycation in ribose. Thermal activity of samples, both decalcified and mineralized, up to 160 °C can be attributed to the denaturation of collagen, i.e. to rearrangement of the collagen triple helix into a random chain configuration. In previous studies denaturation temperature in demineralized bone was reported to be from 69 °C in fully hydrated avian bones [27] to 134 °C in bovine bone collagen with 12% water [21]. In another experiment on sheep bone at different hydrations, denaturation temperature rose from about 65 °C to 120 °C for hydrations decreasing from 79.5% to 18.9% [15]. Denaturation temperature of collagen in situ in mature, fully mineralized bovine bone was reported to be 158 °C [20] and from 152 °C to 163 °C depending on hydration of bone [21]. Endothermal peak at temperature as high as 215 °C described as irreversible melting was found in fully dehydrated bone collagen [28]. Enthalpies of denaturation were found to be from 50 J/g to 60 J/g in hydrated decalcified bone [20], about 30–40 J/g at hydrations below 20% [15] and about 25 J/g in fully dehydrated samples [21]. In the present study, denaturation in decalcified samples occurred in two distinct and complex endothermal processes with average enthalpy equal to 31.2 J/g in the first and 8.5 J/g in the second one.

In intact bone samples a small activity found in temperatures corresponding to the temperatures of the first endotherm in demineralized bone, can be a sign of thermal denaturation of a new synthesized, nonmineralized collagen present in a certain amount even in a mature bone tissue. A more distinct, high and narrow endotherm was found at temperatures around 135 °C. The enthalpy of this process was very low, about 2 J/g. Even considering that only 30–35 wt% of mineralized bone constitutes collagen, the value is still much lower than 14–25 J/g [21], 40 J/g [27] or 50 J/g [20] reported for mineralized bone collagen at different hydrations. The value about 30 J/g of collagen can be obtained only together with the energy of the next broad process with maximum close to 160 °C. Such a stepwise pattern of thermograms is consistent with the hypothesis of Miles and coworkers, that collagen in bone can exist in few different populations resulting from domains of different thermal lability caused by intra- and intermolecular cross-links [16,17]. They hypothesized that earlier peaks corresponded to the thermal denaturation of a collagenous fraction with a lower level of heat-labile crosslinks. So, some of the endotherm characteristics could be attributed to heterogeneity of fibrils making up the fibres composing the scaffold of the tissue. A complex nature of endotherms was reported previously for collagen in bone [20,27], as well as in rat skin [29] and lens capsules [25]. It is presumable, that the broad endotherm (145–165 °C) in mineralized samples corresponds to the thermal activity of more cross-linked collagen fraction, but the presence of mineral pushing apart collagen fibres lowers the cooperativity of the denaturation processes.

Thermal parameters of decalcified bone samples obtained in our study were considerably influenced by glycation in ribose solution. The temperature of the denaturation endotherm and peak temperatures of the endotherm components were significantly higher after glycation while the enthalpy of the process was lower than in the control. The higher temperature required to denature the collagen after in vitro incubation in glucose can be a consequence of sugars-mediated intermolecular cross-linking between the helical parts of collagen molecules [16]. Physico-chemical analyses of glycated fibrous collagen in tendon and skin revealed that glycation resulted in cross-links of different types [30]. DSC thermograms of scleral collagen stabilized by glycerol aldehyde revealed formation of several fractions of collagen, possessing different level of cross-links [26]. Melling et al. [24] showed an increase in denaturation temperature in diabetic human skin compared to controls. The changes were consistent with increased collagen stability due to glucose-mediated cross-linking. Elevations of collagen denaturation temperature by several degrees were observed also by Bailey et al. [25] in the in vitro glycosylation of lens capsules after incubation in the presence of glucose. In vitro incubation of collagen in glucose resulted in an increase of the collagen denaturation temperature in rat tail tendon [23]. Flandin et al. [29] found similar results in rat skin.

Glycation induced changes in collagen properties were shown previously to influence mechanical behaviour of decalcified bone.

![Fig. 2. An example of curve fitting applied to the first endotherm in one of the demineralized, control samples revealing three separate components of the endotherm.](image-url)
matrix under axial compression [10,11]. Bone matrix from glycated samples was stiffer and revealed reduced ductility and post-yield deformation [11]. In our previous experiment on bone incubated in ribose [10] deformability of collagenous bone matrix was also significantly influenced by glycation. Moreover, anisotropy of mechanical behaviour of bone matrix was lowered markedly in ribosylated samples.

From the mechanical point of view, fully mineralized bone tissue is a brittle material deriving its resistance against fracture by absorbing energy in a form of microcracks that provide an appropriate ductility of the material and delay the propagation of fracture. Studies revealed that mechanisms of bone failure depend on the effectiveness of microcracks related processes of energy dissipation within the tissue [31,32].

Even though the load bearing capacity in bone arises essentially from mineral phase [33,34], an increasing number of studies underline a crucial role of collagen quality and microstructure for energy absorbed in bone during deformation and for fracture toughness [35–38]. Any change of collagen deformability can reduce ductility of bone material and impair toughening behaviour and as a result increase a propensity of bone to undergo a brittle failure.

In the present study effects of ribose incubation on thermostability of collagen in mineralized bone were noticeable, however less apparent than in decalcified one. On the other hand, results of mechanical tests of glycated cortical bones are ambiguous [8,11,13]. NEG products did not significantly affect the overall stiffness of the mineralized bone under axial loads but properties associated with yield and final fracture were affected [11]. In experiments with three-point-bending tests none of mechanical characteristics of bone was influenced by incubation in ribose [13] and in glucose solution [8]. However, three-point-bending test can be inadequate to demonstrate effects of glycation products on bone, since it was previously stated that bending of cortical bone is influenced mainly by its mineral phase [34]. Moreover, mineral deposited in and between collagen fibres constrains and separates collagen molecules and it is presumable, that the effect of additional cross-links is suppressed to some extent. However, any modification of collagen-mineral interaction may influence complex processes of bone turnover as well as impair the mechanical properties of bone tissue.

On the basis of thermograms obtained in the present study it can be stated that thermodynamics of collagen conformations in bone tissue is affected considerably by glycation. Although collagen may have less effect on bone’s strength and stiffness than does mineral, it may have a profound effect on bone fragility. In bone resistance to fracture arises from deformability of collagen and its ability to absorb energy of deformation, so any alterations in these mechanisms will affect bone toughness. Results of the study demonstrated that non-enzymatic glycation plays a significant role in modifying organic matrix properties in cortical bone, which can contribute to non-physiological stiffening of the bone tissue and its increasing fragility in advanced age and in diabetes.

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