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Influence of homocysteine on the physical structure and molec mobility of elastin network in cultured arteries

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A B S T R A C T

The thermal and dielectric properties of the elastin network were investigated in arteries cultured with physiological and pathological concentrations of homocysteine, an aminoacid responsible of histological impairments in human arteries. The glass transition of this amorphous protein was investigated by Differential Scanning Calorimetry (DSC). To explore the molecular dynamics of the elastin network in the nanometer range, we used Thermally Stimulated Currents (TSC), a dielectric technique running at low frequency and measuring the dipolar reorientations in proteins subjected to a static electrical field. Combining TSC and DSC experiments with determination of the activation parameters of relaxation times reveals the molecular mobility of the proteins. The major differences in the relaxation behavior of elastin between arteries cultured with physiological and pathological concentrations of homocysteine are discussed.

Keywords:
Biopolymers
Thermally stimulated and depolarization current
Glass transition

1. Introduction

[1,2].
Arterial lesions associated with hyperhomocysteinemia are characterized by intense extracellular matrix remodeling. One of the main features of this arterial remodeling is the degradation of the arterial elastin network. From histological and macroscopic point of view, this proteolytic process has been clearly characterized for arterial lesions occurring in homocystinuria [3], the most severe form of hyperhomocysteinemia in humans, as well as in dietary-induced mild hyperhomocysteinemia in animals [4,5]. The degradation consists in an enlargement of physiological fenestrae in elastic laminae as a result of a protease dependant degradation of elastin network [6].

The aim of this study was to characterize the influence of homocysteine on the physical structure and the molecular mobility of the arterial elastin network. To this end, we cultured arteries with concentrations of homocysteine matching physiological and pathological levels encountered in humans. The physical structure of the elastin network in cultured arteries was investigated by Differential Scanning Calorimetry (DSC) to analyze thermal transitions such as glass transition and denaturation. To explore the molecular dynamics of the elastin network in the nanometer range, we used Thermally Stimulated Currents (TSC), a dielectric technique running at low frequency and measuring the dipolar reorientations in proteins subjected to a static electrical field. Other techniques like DDS (Dynamic Dielectric Spectroscopy) have been used to study dipolar relaxations in proteins such as elastin and collagen; nevertheless the use of TSC was preferred here to reach low frequency phenomena.

2. Experimental

2.1. Materials

Abdominal aortas were collected from young adult common pigs at a local slaughterhouse and transferred to the laboratory at 4 °C in flasks with phosphate buffered saline (PBS) containing antibiotic–antimycotic (10X-AB–AM: 1000 U/mL penicillin G, 1000 μg/mL streptomycin, 2.5 μg/mL amphotericin). Arteries were prepared under sterile conditions by gently removing the excess fat and interstitial tissue while avoiding stretching the artery or damaging the endothelium. Arterial explants (5-mm-long segments) were randomly put into culture dishes containing 10 mL

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serum free NCTC-135 medium [7] supplemented with 0.2% bovine serum albumin, 2X-AB–AM and homocysteine. Homocysteine was used both at 10 μM corresponding to physiological homocysteinemia (Hcy10) and at 100 μM corresponding to severe hyperhomocysteinemia (Hcy100). Arterial explants were cultured at 37 °C in a humidified incubator at 5% CO_2 for 72 h. At the end of culture, arterial explants were frozen and lyophilized. None procedure was used to remove collagen, and the resulting arteries contain the initial content of extracellular proteins components, i.e. elastin in a large part and a minor quantity of collagen.

2.2. Methods

2.2.1. Differential scanning calorimetry (DSC)

The phase transition thermograms were recorded with a Perkin Elmer DSC7 differential scanning calorimeter. The temperature and energy scales were calibrated using the manufacturer's instructions with Indium and Tin as standards. Freeze-dried arterial explants (5–10 mg in weight) were sealed in aluminum pans. Empty pans were used as references. Investigations were performed between 30 and 250 °C with 20 °C/min heating rates. Determination of transition parameters was performed with Origin software.

2.2.2. Thermally stimulated currents (TSC) – global spectra

This technique was first used to characterize metallic divalent impurities in ionic crystals, and was later applied to polymers because it allows dielectric relaxation processes to be scanned at a lower frequency than conventional dielectric spectroscopy [8]. The relaxations observed by TSC are largely due to the reorientation of polar groups or segments of the macromolecules.

Thermally stimulated current measurements were carried out with a dielectric apparatus developed in our laboratory and previously described [9]. Freeze-dried arterial explants (20–40 mg in weight) were compressed (2×10^3 kg/cm^2 during 2 min) resulting in disks 1 mm thick, 8 mm diameter. These samples were placed between two stainless steel plate electrodes. Before experiments, the sample cell was flushed and filled with dry He, to ensure good thermal exchange. To record complex spectra, the sample was polarized with a static electric field (E_p) at a given polarization temperature (T_p) for a time (t_p) long enough for the polarization to reach equilibrium. Then the sample was quenched by a cooling process to $T_0 \ll T_p$, allowing the orientation polarization ($P(T_p)$) to be frozen-in. Finally the electric field was cut off and the sample was short-circuited for a time t_{cc} long enough to remove fast relaxing surface charges and to stabilize the sample temperature. The capacitor was then connected to a very sensitive electrometer (Keithley 642, 10^{-16} A accuracy). The depolarization current $I(T)$ induced by the linear increase in temperature ($T = qt + T_0$) was subsequently recorded against temperature, giving the relaxation spectrum of the sample. The peaks, which are associated with dipolar relaxations, must meet different criteria: their intensity must be proportional to the applied field, and the temperature maximum must be below T_p and stable at with different T_p values. In the present study, the polarization conditions resulting in reproducible dipolar relaxations were as follows: $t_p = 2$ min, $E_p = 400$ V/mm, $t_{cc} = 2$ min and $q = 7$ °C/min.

2.2.3. Thermally stimulated currents (TSC) – fractional polarizations (FP)

In proteins, the TSC peaks corresponding to dipolar relaxations are in general too broad to be associated with only one single relaxation process, i.e. with only one relaxation time. These broad peaks correspond to a distribution of relaxation times connected with the same dipolar mechanism. The FP procedure can be used to decompose a complex TSC peak in elementary processes contributing to a complex dielectric relaxation. In this procedure, each isolated spectrum is well approximated by a single relaxation time, accommodating Bucci–Fieschi's analysis [10]. In the FP procedure, the electrical field E_p is applied at T_p for 2 min, allowing the orientation of dipoles with relaxation time $\tau(T_p)$ lower than 2 min. The temperature is then lowered by ΔT to T_d ($\Delta T = 5$ °C) under field. At T_d, the field is turned off and the sample is then held for 2 min so that dipoles with relaxation time $\tau(T_d)$ lower than 2 min relax. The sample is then quenched to $T_0 \ll T_d$ so that only dipoles whose relaxation time $\tau(T)$ is such that $\tau(T_p) < 2$ min and $\tau(T_d) > 2$ min remain oriented at T_0. The depolarization current is recorded in a fashion similar to that mentioned above for the complex spectrum. The response of this FP experiment is the result of the reorientation of a narrow distribution of relaxation times excited over a $\Delta T = 5$ °C temperature window around T_p. By increasing the value of T_p by steps of 2.5 °C along the temperature axis, the whole TSC spectrum is resolved into a series of fractional depolarization peaks, allowing us to reach experimentally the distribution of the global dipolar relaxation.

In Bucci–Fieschi's framework based on the conventional Debye treatment with a single relaxation time τ the reduction in polarization $P(t)$ after removal of the static field is given by:

$$P(t) = P_0 \exp[-t/\tau], \tag{1}$$

where P_0 is the saturation polarization. The corresponding depolarization current density $J(t)$ at time t is given by:

$$J(t) = -dP(t)/dt = P(t)/\tau. \tag{2}$$

As the reduction in the frozen-in polarization is thermally stimulated, time and temperature are related by a linear relationship, $T = T_0 + qt$, where q is the heating rate. The relaxation time τ is thus temperature dependent and can be written as:

$$\tau(T) = P(T)/J(T) \quad \text{with} \quad P(T) = q^{-1} \int_T^{T_f} J(T')dT' \tag{3}$$

Therefore the temperature dependence of the relaxation time for an elementary peak can be deduced from the ratio between $J(T)$ and the remaining area under the peak from T to T_f, the temperature at which the depolarization current returns to zero.

2.2.4. Statistical analysis

The data are presented as means ± SD. Statistical analysis was performed using Student's t test.

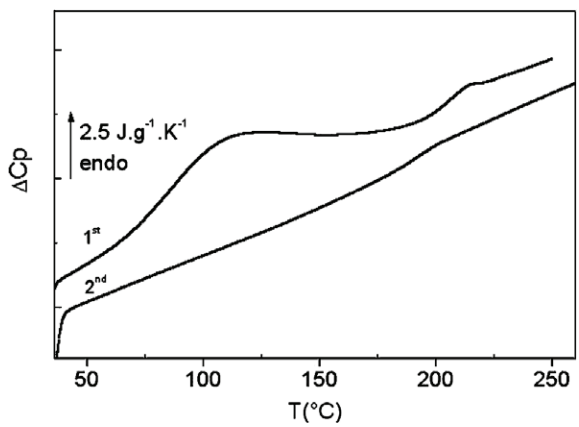

Fig. 1. DSC thermogram of Hcy10 arterial explants (1st and 2nd scan).

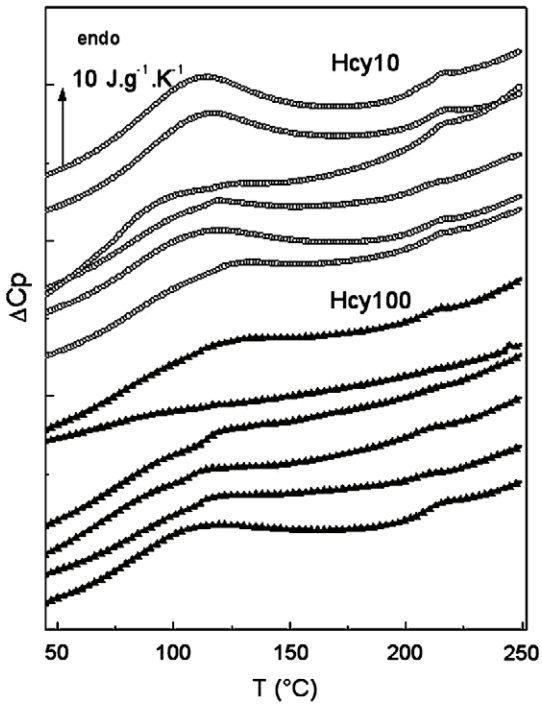

Fig. 2. DSC thermograms of Hcy10 and Hcy100 arterial explants (1st scan).

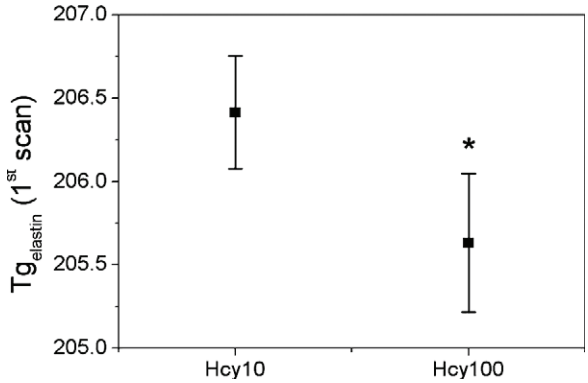

Fig. 3(A). Enthalpy of bound water departure from DSC thermograms (mean ± SD, $n = 6$; $^{*}p < 0.05$).

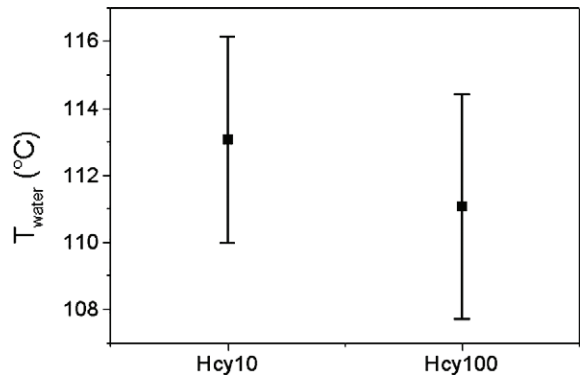

Fig. 3(B). Temperature maximum of bound water departure from DSC thermograms (mean ± SD, $n = 6$).

3. Results and discussion

3.1. Thermal transitions

Fig. 1 presents the DSC thermograms of an Hcy10 arterial explant. On the first scan, a broad endothermic peak is observed at 111 °C; by analogy with previous studies on different proteins and connective tissues in the lyophilized state, this peak, which vanishes on the second scan, is attributed to the evaporation and vaporization of bound water adsorbed by the sample stored at 50–80% relative humidity [11–13]. Therefore, this thermal transition can be considered as an extrinsic transition and is generally evaluated by its temperature maximum (Tmax) and its enthalpy, i.e. the area under the curve (ΔHmax). Here, these two parameters are used to characterize the artery hydration state.

A second thermal event, noted as a jump in the specific heat, appears between 190 and 220 °C on the first and second scans: this reversible transition is associated with the glass transition of elastin in the freeze-dried state [14] namely the transition from a vitreous to a rubbery state; by analogy with synthetic polymers, this intrinsic transition is characterized by its temperature (Tg), corresponding to the middle point of the specific heat jump of the step. The value of Tg is an important parameter of the chain dynamics of elastin, and gives specific information on the stiffness of the protein in the artery.

In order to evaluate the influence of a pathological concentration of homocysteine on cultured arteries, the thermograms corresponding to the first scan of Hcy10 and Hcy100 arterial explants ($n = 6$ for each concentration of homocysteine) were performed and superimposed on Fig. 2. All the thermograms present both the broad transition associated with bound water departure and the glass transition of elastin, with different degrees of resolution. To facilitate comparison, the mean values and standard errors of the three parameters (Tmax, ΔHmax and Tg) of the two transitions were extracted from the thermograms and are presented in Figs. 3(A), 3(B) and 4(B).

The most significant difference ($p < 0.05$) between the two groups of samples concerns the enthalpy associated with bound water loss. Bound water falls from 219 J/g for arterial explants cultured with 10 μM homocysteine to 165 J/g for arterial explants cultured with 100 μM homocysteine. This first difference suggests that Hcy10 arterial explants are more hydrated than the Hcy100 arterial explants; thermogravimetric measurements (not shown here) confirmed this assertion: levels of hydration were found to be (20 ± 1%) in Hcy10 arterial explants and (16 ± 1.5%) in Hcy100 arterial explants, respectively.

In contrast to enthalpy values, the temperature maxima of the extrinsic transition are not significantly different for the two kinds of samples, which indicates that water is fixed on similar sites in both cases.

There is a slight difference ($p \approx 0.05$) in the glass transition of elastin for the two sets of samples; Tg varies from 206.4 to 205.6 °C for arterial explants cultured with 10 and 100 μM homocysteine, respectively. By analogy with synthetic polymers [15], this slight but significant decrease in Tg reveals a softening of elastin in arteries cultured in physiopathological conditions.

Another important factor is brought to light with the detailed analysis of the thermal transitions of freeze-dried arteries in Fig. 2: we observe that some thermograms are characterized by an additional jump in the specific heat at around 115 °C; this transition, which appears with more accuracy on the T-derived curves, was identified on each thermogram. Fig. 4(A) gives a detailed account of this additional transition for each set of samples. This transition reappears twice for arterial explants cultured with 100 μM homocysteine. Moreover, the analysis of the *T*-derivated curves highlights a more intense peak associated with this thermal

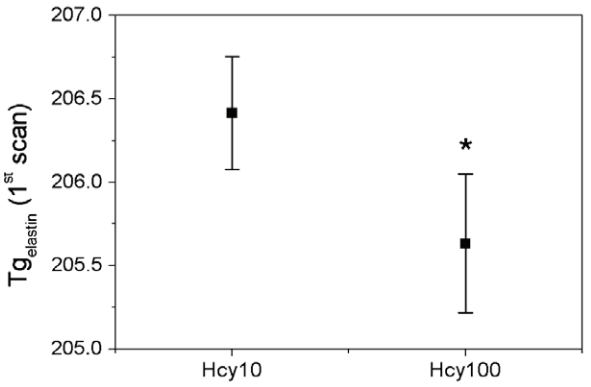

Fig. 4(A). Glass transition temperature of elastin from DSC thermograms (mean ± SD, $n = 6$; $^{*}p < 0.05$).

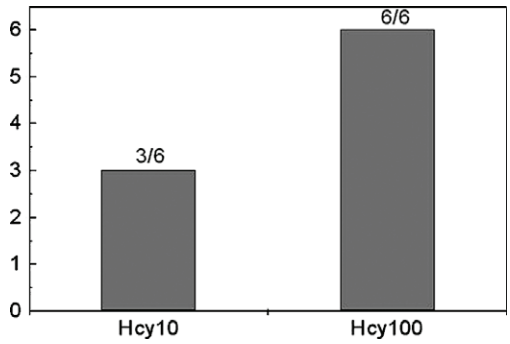

Fig. 4(B). Occurrence of an additional glass transition at 115 °C (from DSC thermograms).

event for the Hcy100 arterial explants when compared with the Hcy10 arterial explants ones (0.07 arbitrary units and 0.04 arbitrary units, respectively). The thermal characteristic of this transition suggests a pseudo-second-order transition, namely a glass transition: the occurrence of a new glass transition in arterial explants is thus attributed to a new amorphous component, more precisely an amorphous protein, whose origin needs to be determined. By comparison with extensive data on the jump in the specific heat in synthetic polymers [16], the value of the jump of ΔCp (0.2–0.5 J K^{-1} g^{-1}) indicates that a large quantity of this amorphous component is present in the arteries. Neither proteins present in small quantities in arteries, nor possible elastin fragments resulting from elastin hydrolysis in cultured arterial explants can explain this transition. Previous studies by differential scanning calorimetry on kappa-elastin and enzymatically digested elastins showed that the glass transition of elastin was strongly dependent on the molecular architecture of this protein [17]. As a matter of fact, the temperature of this glass transition significantly decreases with a high degree of chemical or enzymatic digestion. Therefore we suggest this thermal event, which is emphasized with pathological concentrations of homocysteine, is associated with a new phase of elastin, corresponding to a degraded network. It is noteworthy that if we apply this hypothesis, two phases of elastin corresponding to intact network and impaired network coexist in arterial explants cultured with 100 μM homocysteine.

3.2. Dielectric relaxations

Dielectric analysis of cultured arterial explants is an additional tool to determine with greater accuracy the chain dynamics of the major proteins in these samples. Fig. 5 presents the global

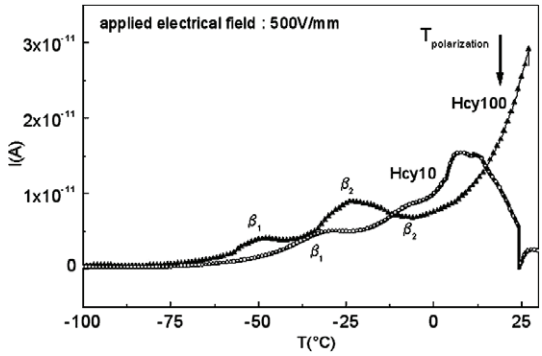

Fig. 5. Global TSC spectra of Hcy10 and Hcy100 arterial explants in the low temperature range.

TSC spectra of Hcy10 and Hcy100 arterial explants obtained after a polarization temperature of 20 °C with a static electrical field of 500 V/mm, and illustrates the dipolar mobility of the major proteins of arteries in the low temperature range. We present a representative spectrum for each set of samples in order to simplify the figure. The TSC spectrum of Hcy10 arterial explants is characterized by two dipolar relaxations that appear as shoulders (labeled β_1 and β_2) at −31 ± 2 °C and −8 ± 3 °C, respectively. By analogy with TSC studies on a large series of biological and synthetic polymers [18–21] the intensity and temperature position of the β_1 and β_2 modes indicate a link with localized movements of the polypeptidic chains. Previous dielectric studies on various connective tissues in the freeze-dried state have indeed linked these two relaxations to the reorientation of polar groups of some nanometers along elastin and collagen backbone [22,23].

The TSC spectrum of Hcy100 arterial explants also presents these two relaxation modes, which appear as peaks and not shoulders at −49 ± 4 °C and −23 ± 3 °C, respectively. The intensity of the two modes is relatively similar in Hcy10 and Hcy100 arterial explants, but a major difference is observed in the temperature position of the relaxations: the TSC spectrum of Hcy100 arterial explants is largely shifted toward low temperature, which indicates plastification. The shift in relaxations toward low temperature is generally interpreted as an increase in molecular mobility of the dipolar groups involved. In TSC experiments, dipolar reorientations are thermally activated: thus, the greater is the energy requirement for the movement, the greater the temperature. In accordance with the thermal results, the difference in global dielectric behavior between the two groups of arterial explants

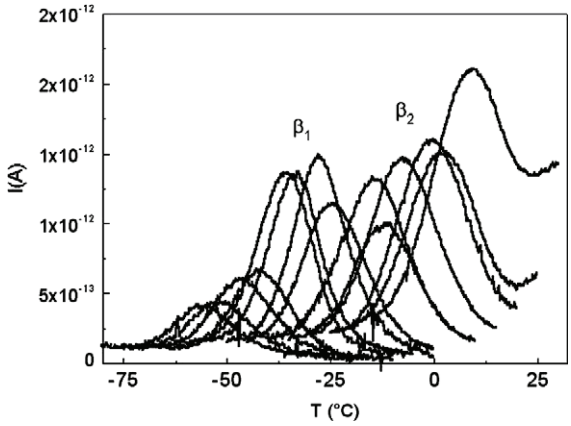

Fig. 6. Experimental decomposition of the low temperature spectrum of Hcy10 arterial explants (FP procedure).

reinforces the degrading effect of 100 μM homocysteine on the arterial elastin network.

In order to define more clearly the influence of pathological concentrations of homocysteine on the local chain dynamics of proteins, the technique of fractional polarizations was performed on each set of samples in the low temperature range, according to the protocol described in the Section 2.

Fig. 6 shows the set of elementary spectra obtained for Hcy10 arterial explants when the polarization window is displaced from −65 °C to 0 °C. The sum of these peaks reproduces the global TSC spectrum: the FP procedure allows us to perform an experimental decomposition of the global relaxation in elementary relaxations, corresponding to single relaxation times as described in Eq. (3). The same FP protocol was applied to Hcy100 arterial explants; the experimental decomposition is presented in Fig. 7. In this case too, the sum of the elementary peaks fits the global spectrum, and the β_1 and β_2 modes can be detected.

Bucci–Fieschi's framework based on the conventional Debye treatment was used to determine the temperature dependence of each relaxation time associated with an elementary peak following Eq. (3), and thus to access the experimental distribution of relaxation times in the low temperature range for the two kinds of arteries. When the variation of $\tau(T)$ was plotted vs. temperature, we noted that all the extracted relaxation times are well fitted by an Arrhenius law:

$$\tau_i(T) = \tau_{0i} \exp\left(E_{ai}/\mathrm{RT}\right), \tag{4}$$

where τ_i is the isolated relaxation time from the ith peak, T the temperature, τ_{0i} an exponential factor, E_{ai} the activation energy and R the ideal gas constant.

This result is in good agreement with previous studies on the local chain dynamics of polymers, which generally present an Arrhenius dependence for the relaxation time associated with sub-Tg movements. This temperature dependence can be interpreted in terms of Eyring's activated state rate theory where $\tau(T)$ follows the law:

$$\tau_i(T) = \frac{h}{kT} \exp\left(\frac{-\Delta S_i}{R}\right) \exp(\Delta H_i/\mathrm{RT}), \tag{5}$$

where ΔS_i and ΔH_i are the activation entropy and the activation enthalpy of an elementary dipolar reorientation, respectively, k Bolzmann's constant and h Plank's constant.

The local chain dynamics of the Hcy10 and Hcy100 arterial explants are thus well represented by Fig. 8, where we plot the variation of preexponential factor τ_0 versus the activation energy E_a for each elementary peak in a semi-logarithmic scale. In both Hcy10 and Hcy100 arterial explants, a linear relationship is observed for the parameters associated with the $\beta 1$ mode and can be expressed by the following equation:

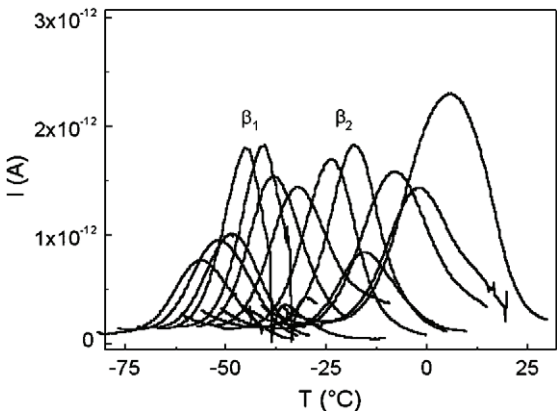

Fig. 7. Experimental decomposition of the low temperature spectrum of Hcy100 arterial explants (FP procedure).

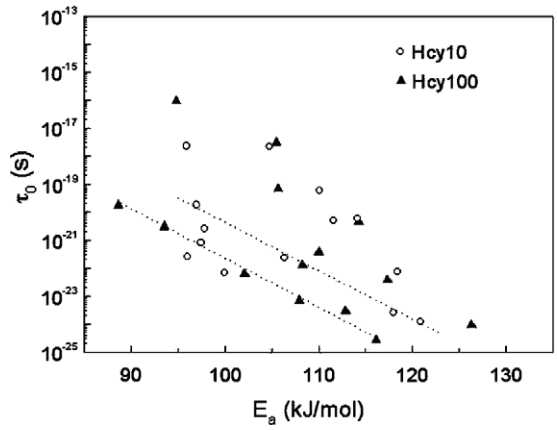

Fig. 8. Compensation diagram of Hcy10 and Hcy100 arterial explants in the low temperature range.

$$\log(\tau_{0i}) = a + bE_{ai}, \tag{6}$$

where a is the origin ordinate and b the slope of the straight line, respectively. These parameters with their associated errors are reported in Table 1 for the two sets of points.

This relationship can be also written as follows:

$$\tau_{0i} = \tau_c \exp\left(-\frac{E_{ai}}{\mathrm{RT_c}}\right), \tag{7}$$

where $\tau_c = 10^a$ (in seconds) and $T_c = -1/(bR\ln 10)$ (in degrees K).

At temperature T_c, all the related relaxation times would have the same value τ_c, which is considered in the literature as a compensation phenomenon [24,25]. We present in Table 2 the values of T_c and τ_c for Hcy10 and Hcy100 arterial explants, giving a good indication of the size of the implied movements. By analogy with previous studies on wide range of polymers, the values of τ_c, in the order of 10^{-5} s in both cases, can be associated with local movements of some nanometers along the polypeptide chain. Values for T_c are significantly different between the Hcy10 and Hcy100 arterial explants; in fact decrease in T_c for 100 μM homocysteine reflects the behavior of the global β1 mode, which was shifted toward low temperature; the plastification of the local chain dynamics at this pathological concentration of homocysteine is therefore confirmed. Another interesting point is related to the energy distribution range of the elementary processes involved in the β1 mode (Table 2). This energy range is shifted toward low values in Hcy100 arterial explants: in agreement with the Williams–Hoffmann–Passaglia theory [26], the reorienting dipolar sequences are smaller in Hcy100 arterial explants than in Hcy10 arterial explants. This information is in good agreement with the previous hypothesis from DSC results regarding a possible fragmentation of the elastin network. In fact, the formation of hanging chains subsequent to a degradation process could explain such a shift in energy distribution in the β1 mode, and could also be responsible for plastification. It is important to note that a similar shift in energy distribution was observed on the main mode of the elastin network from bovine arteries degraded by elastases.

A final important point of comparison between the local chain dynamics of the two cultured arterial explants concerns the value of the preexponential factor, τ_0, which is related to the activation entropy by the following formulae (combination of Eqs. (4) and (5)) [27]:

Table 1
Parameters of the linear fit log τ_0 = A + BEa (from Fig. 8).

	A	ΔA	B	ΔB	R	N	SD	P
Hcy10	−4.67	1.08	$-1.597 \cdot 10^{-4}$	$9.37 \cdot 10^{-6}$	0.99828	4	0.10136	0.00226
Hcy100	−4.13	0.36	$-1.763 \cdot 10^{-4}$	$3.56 \cdot 10^{-6}$	0.99939	5	0.07835	<0.0001

Table 2
Compensation parameters of the β1 mode.

	T_c (°C)	ΔT_c (°C)	τ_c (s)	Energy range (kJ mol^{-1})
Hcy10	54	19	$2 \cdot 10^{-5}$	From 97 to 121
Hcy100	23	6	$7 \cdot 10^{-5}$	From 88.5 to 116

$$\tau_{0i} = \frac{h}{kT} \exp\left(\frac{-\Delta S_i}{R}\right). \quad (8)$$

Fig. 8 shows that the τ_0 values for the β1 mode of Hcy100 arterial explants are lower than the τ_0 values for Hcy10 arterial explants; when considering Eq. (8), this decrease in τ_0 is necessarily linked in to an increase in the activation entropy. Activation entropy is related to the number of conformations accessible for the dipolar group and its environment in the activated state leading to reorientation. An increase in this number is thus an indication of local disorder around the dipolar sequence when it reorientates.

4. Conclusion

In this study, we show that high levels of homocysteine severely impair the physical properties of the arterial elastin network. Suggesting degradation of the elastin network, these results give new insights into the well known degradation of the elastic structures in the arterial wall associated with hperhomocysteinemia [4]. These molecular alterations could account, at least in part, for the impairment of arterial rheology in hyperhomocysteinemic patients [28,29].

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