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Cyclophosphamide treatment evoked side effect on skeletal muscle actin, monitored by DSC

Péter Farkas[1] · Dávid Szatmári[2] · Franciska Könczöl[3] · Dénes Lőrinczy[2]

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Abstract

Several kind of drugs—used in cancer treatments—such as cyclophosphamide (CP) can also trigger a disease classified as toxic polyneuropathy. Polyneuropathy is a simultaneous malfunction of several peripheral nerves, typical side effect of a cancer therapy. In our previous study, we used CP treated in vitro animal model (Guinea pig) with a comparable dosage and time handling of human protocol to show evidences of this drug-induced effects. We could show a dose-dependent difference between in T_m and ΔH_{cal} of untreated and treated samples assigned to their intact muscle and nerve, blood plasma and red blood cells. In our current study we analyze this side effect on skeletal muscle actin (prepared from *m. psoas* of rabbit) by DSC (differential scanning calorimetry), to follow the possible consequence of drug treatment on the "activator" of muscle contraction. We have demonstrated that run of DSC curves, T_ms together with the ΔH_{cal} exhibit clear CP effect. In case of Ca^{2+} G actin it is manifested in a well separated second high denaturing temperature as a consequence of CP binding into the cleft. This way the nucleotide binding cleft with subdomains 1 and 3 becomes less flexible, indicating clear sensitivity to CP treatment. In F-actin samples, the main peak represents the thermal denaturation of subdomains 1 and 3, and the increased calorimetric enthalpy administrating Ca^{2+} as well as CP refers to a more rigid structure. These alterations can be the molecular background in the malfunction of muscle in case of polyneuropathy after CP treatment.

Keywords Polyneuropathy · Cyclophosphamide · Ca^{2+} · Mg^{2+} · G and skeletal F actin · DSC

Introduction

In the medical practice cyclophosphamide (CP) is applied as an anticancer drug [1]. It has a lot of beneficial effects but as most of the different drugs has some unpleasant short and long term side effects as well [2–7]. Refers to a forensic medicine indication, we have checked its possible malfunction in an animal model using Guinea pig [7]. In case of both sides of peripheral nerve and m. gastrocnemius [8], and in the left ventricle [9] as well as using the red blood cells and blood plasma coming from treated animals, we have observed dose-dependent damages during the thermal denaturation of these samples by DSC [10]. We could separate the effect of the drug in Guinea pig muscle samples for actin and myosin applying the deconvolution procedure in the DSC scans [11, 12] and using similar information collected from psoas muscle fibers [13, 14].

The actin is one of the main components of the cytoskeleton. It plays important roles during muscle contraction as a dynamic compound of actomyosin complex and in the motility of eukaryotic cells [15–21]. The actin can be found in monomeric, but in the muscle mainly in filamentous form. The actin monomer can bind a nucleotide in complex with divalent cations (Ca^{2+} or Mg^{2+}) in the cleft between the two main domains of the protein [22]. During actin polymerization, the ATP is hydrolyzed into ADP and P_i [23–30]. The ADP.P_i state is transient as the inorganic phosphate product is released from actin after polymerization (resulting an ATP and ADP end of the filament). For the characterization of the short-lived ADP.P_i state nucleotide analogues such as ADP.BeF_x or ADP.AlF_4 can be applied, because the P_i release is not reversible during the ATP hydrolysis cycle [31–33]. This way we can get the simulation of "weak" binding state,

✉ Dénes Lőrinczy
denes.lorinczy@aok.pte.hu

[1] Department of Medical Imaging, Medical School, University of Pécs, Szigeti Str. 12, Pécs 7624, Hungary

[2] Department of Biophysics, Medical School, University of Pécs, Szigeti Str. 12, Pécs 7624, Hungary

[3] Department of Forensic Medicine, Medical School, University of Pécs, Szigeti Str. 12, Pécs 7624, Hungary

which has enough long life time for different spectroscopic and thermal investigation.

The effect of the binding of ligands to actin filaments is often cooperative, i.e., the binding of the ligand induces allosteric conformational changes in the actin protomers distant from the bound protomer [34–38]. In the past, we have studied the effect of different toxins—jaspakinoline and phalloidin—on actin prepared from rabbit skeletal muscle [39, 40]. During the thermal denaturation of the treated actin, we have observed a toxin concentration dependent cooperative binding effect of toxins. It was realized that after these toxin treatments at least three actin populations can be identified: those which bound directly a toxin molecule (with highest denaturation temperature, T_m), those which shows the effect of cooperative binding in the vicinity of those actin which has bound toxin (smaller T_m), as well as toxin free actin which has "normal" denaturation temperature.

It was also shown by previous DSC data [8, 11, 12] that muscle filament system was changed by the CP treatment. Using all the above-mentioned data, we have planned the recent investigation to look for a possible effect of CP on filamentous actin and actin monomers supposing that the chemo-therapeutic agent binding can cause similar effects in their structure as toxin binding.

Materials and methods

Actin preparation from rabbit skeletal muscle

G and F-actin in Ca^{2+} and Mg^{2+} form was prepared from acetone powder of rabbit skeletal muscle as described earlier by Spudich and Watt [41] and stored in MOPS-buffer (2 mM MOPS, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.1 mM β-mercaptoethanol, pH 7.4). Actin concentration was determined from the absorption spectra (Jasco V-550 spectrophotometer) (as the average concentration by $\varepsilon = 1.11$ mL mg^{-1} cm^{-1} at 280 nm and $\varepsilon = 0.63$ mL mg^{-1} cm^{-1} at 290 nm). We applied 2 mM EGTA then 2 mM $MgCl_2$ treatment for exchange calcium to magnesium on 2 mg mL^{-1} actin monomers. It was required to use actin in samples as concentrated as possible, but based on our experience the concentration of actin should be fixed at maximum 2 mg mL^{-1} because this concentration of monomers and filaments show stable thermodynamical equilibrum. High concentration of monomers can start spontaneous polymerization and high number of filaments can be in superstructural remodeling by their interfilamental interactions. Actin polymerization process was initialized by 100 mM KCl addition.

Cyclophosphamide treatment

For in vitro measurements, applied dosage of cyclophosphamide (CP) is comparable to the human dosage (150 mg kg^{-1} b.m.) [7–10]. The average actin content of skeletal muscle is roughly 10% [41] thus the average mass of Guinea pig gastrocnemius muscle (from our previous study [8]) divided by 10 then by the mass of CP passed in the muscle (150 mg kg^{-1} $*$ $\frac{massof\,\text{gastrocnemius}}{massofthebody}$) resulted that the actin to CP ratio has to be $\frac{2000}{3}$ (it means 2 mg actin to 3 µg CP) as a single dose. However, as we used actin from rabbit skeletal muscle, we can assume that the distribution of CP in rabbit skeletal muscle should be the same as in Guinea pig skeletal muscle. We carried out experiments—to achieve a more pronounced effect—with 5 times dose of CP to treat actin followed by incubation at room temperature for 1 h (in case of model experiment the animal underwent to a real, long lasting chemotherapeutic protocol).

DSC measurements

The samples were freshly prepared before all measurements. The analysis was made by a SETARAM Micro-DSCII calorimeter between 0 and 100 °C with heating rate of 0.3 K min^{-1}. Conventional Hastelloy batch vessels ($V_{max} = 1$ mL) were used for the experiment to investigate denaturation with 950 µL sample volume (sample + buffer) in average. Samples' masses were between 920 and 970 mgs. MOPS buffer was used as a reference. The reference and sample vessels were equilibrated with a precision of ± 0.1 mg; this way we did not need to do any correction between vessels' heat capacity. With the help of a two-point SETARAM peak integration setting, calorimetric enthalpy was calculated from the area under the heat absorption curve, and then, the results [denaturation or melting temperature (T_m) and calorimetric enthalpy (ΔH_{cal}) data of samples] were compared.

Results

Figure 1 shows our summarized denaturation results on different form of actin before and after CP treatment. The scans are average of three measurements. Statistical analysis was not performed because of the small number of observation. The main goal of measurements was to see the tendency of CP treatment on different form of actin to find explanation for our former study.

In case of thermal denaturation of biological samples, it is generally accepted that the difference of nearly 1 °C in T_m is a remarkable deviation. Because the usual instrument accuracy in the determination of calorimetric enthalpy in

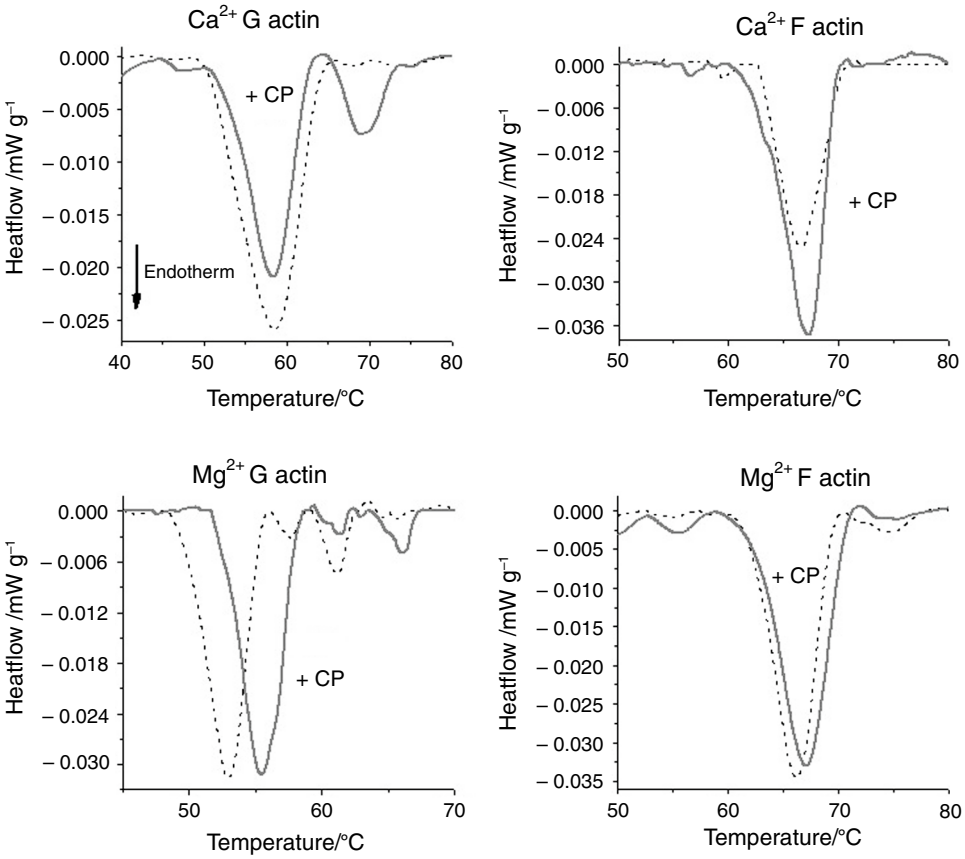

Fig. 1 Thermal denaturation curves of different G and F-actin in the presence (solid gray line) and in the absence of CP (dotted line) (endotherm effect is deflected downward)

Table 1 Remarkable thermal parameters of the denaturation of different native and CP treated actin solutions

	Ca^{2+}		Mg^{2+}	
	Thermal parameters			
	T_m/°C	ΔH_{cal_total}/Jg^{-1}	T_m/°C	ΔH_{cal_total}/Jg^{-1}
G-actin				
Native	57.89	0.041	52.3	0.037
			61.2	
CP treated	57.94	0.042	55.46	0.038
	69.42		66.03	
F-actin				
Native	66.52	0.044	65.9	0.039
CP treated	66.82	0.05	66.8	0.041

Data are averages of three different measurements. The calorimetric enthalpy refers for the whole denaturation range and normalized on the sample mass

case of commercial DSC has ~ 5% error, the deviations in this range are also negligible. In line with these information, we observed the following effects for the different actin samples (see Fig. 1 and Table 1):

Protocols of actin isolation and preparation from different laboratories show negligibly small differences which can have influence on the thermodynamical responses of actin. Actin concentration-dependent intermolecular forces and charges can modify the parameters of actin denaturation what we tried to avoid thus fixed it at 2 mg mL^{-1}. As a result of them the main denaturation peaks for untreated Ca^{2+}–Mg^{2+} G and F-actin are in a good agreement with former literature data [42–49]. After CP treatment in both G-actin plus endotherm peak appeared in the higher temperature range. It can prove—beside the structural data [32, 39, 40, 49, 50]—that the nucleotide binding clefts between the two big domains of G actin underwent a structural change (became more rigid) as a consequence of CP binding. The calorimetric enthalpy in case of untreated Mg^{2+} G-actin was 10% smaller than in case of Ca^{2+} G actin. To surprise, the CP treatment has only mild effect on ΔH_{cal} in the case of Ca^{2+} G as well as Mg^{2+} G actin.

In case of F-actins the main T_m was above 65 °C which is higher than in case of G-actin as the consequence of the polymerization-based structural stabilization. After the CP treatment in case of Ca^{2+} F-actin the main denaturation peak was not changed remarkably, while the lower one decreased by 3 °C and a shoulder was appeared at 63 °C. In the contrast with Mg^{2+} F-actin a lower peak was appeared at 55 °C, the main one increased by 1 °C and the peak at 74 °C (together with the 55 °C) practically does not give

remarkable enthalpy contribution. The calorimetric enthalpy of Ca^{2+} F-actin was increased compared to G-actin, and the CP treatment increased it by ~ 20%. (Mg^{2+} F-actin exhibited ~ 5% higher enthalpy change than in G form, and CP treatment increased it ~ 5% too, unfortunately both are in the range of error of enthalpy determination.)

Discussion

The reason—of some differences between our recent denaturation temperatures compared to the literature data—can be the difference in the concentration of samples, in the heating rate as well as in the different measuring principle of our system (most of the devices use capillary sample holders why our is a big [V = 1 mL] stainless steel cylinder. The instrument is a heat-flux calorimeter.). Run of DSC curves (see Fig. 1), and the denaturation temperatures (see Table 1) together with the calorimetric enthalpy exhibit the effect of CP treatment. In case of Ca^{2+} G actin, it is manifested in a well separated second high denaturing temperature (see Fig. 1) as a consequence of less flexible side of nucleotide binding cleft with subdomains 1 and 3 [51], thus the actin monomers are sensitive to CP treatment, similarly as in case of P_i analogues or toxins [39–49]. The Mg^{2+}-G-actin has smaller denaturation temperature because Ca^{2+} is a structure stabilizing agent. ΔH_{cal} well demonstrates this effect too (see Table 1). The increased T_m for CP treated Mg^{2+} G-actin proves also a more rigid state in the cleft compared with the native one.

In F-actin samples the higher denaturation temperature in both cases is the consequence of polymerization. The main peak represents the thermal denaturation of subdomains 1 and 3, while a pre-transition at ~ 60 °C and ~ 55 oC in case of Ca^{2+} and Mg^{2+} F-actin, respectively, could be the contribution of a more flexible structure of nucleotide binding cleft (see Fig. 1). The T_m of CP treatment in Ca^{2+} F actin shows a mild stabilization of structure, while in the presence of Mg^{2+} indicates a more remarkable strengthening (~ 1 °C). The main T_m practically is not affected by CP treatment in case of Ca^{2+} F-actin, but the remarkable increase in ΔH_{cal} is a strong sign of the more rigid structure. F-actin with Mg^{2+} nucleotide showed an increased T_m and a mild ΔH_{cal} increase of the main denaturation as the sign of more rigid structure.

Conclusions

The name *actin* refers to its activator ability [52–54]. It means that the actual molecular dynamic capability of actin crucial way affects the actin–myosin interaction, so force generation during contraction. Referring to our forensic medical case of the polyneuropathic condition after CP treatment [7] as well as to the animal model experiments [8–12] which motivated our present study, we can state that CP treatment can make such a structural change in muscle protein which can be manifested in burning and panging pain sensation in the limbs, muscle spasm, progressive distal weakness because of more rigid structure of actin [7]. These symptoms are typical signs of polyneuropathy. This way we can confirm the finding that toxic effects, such as industrial poisons [39, 40] and other *pharmaceutical products* play an important role in the occurrence of polyneuropathy [55].

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Authors' contributions Dr. Péter Farkas involved in sample collection and handling with cyclophosphamide and data analysis. Dr. Dávid Szatmári participated in sample preparation and handling, data analysis, manuscript writing. Dr. Franciska Könczöl involved in rising the problem, sample collection and handling with cyclophosphamide. Prof. Dr. Dénes Lőrinczy, corresponding author and principle investigator, involved in DSC experiments, data analysis and manuscript writing.

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Availability of data and materials There are no additional available data to upload.

Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval and consent to participate All procedures followed were approved and in accordance with the ethical standards of the responsible committee on animal experimentation (institutional and national) and with the revised Helsinki Declaration of 1975.

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