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Short communication

Nanosecond electric pulses deprive zinc ions of carboxypeptidase G2

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ABSTRACT

Nanosecond electric pulses (nsEP, 10 kV/cm with a pulse duration of 8, 16 or 24 ns) inhibited the activity of carboxypeptidase G2 (CPG2), a zinc-dependent homodimer; the relative activity was <20% when the total exposure time was >120 s. No alterations were detected in electrophoresis, chromatography, mass spectroscopy and circular dichroism, thus demonstrating intactness of the apoenzyme. Inductively coupled plasma-mass spectrometry indicated that zinc levels were 3.30 μg/mg protein in control CPG2, and decreased to 0.40, 0.12 or 0.38 μg/mg protein after 240 s of 8-, 16- or 24-ns pulses, respectively. In CPG2 exposed to 240 s of 8-, 16- and 24-ns pulses, the reloading of zinc with redialysis recovered the activity to 94.7 ± 3.4%, 84.0 ± 5.2% and 81.7 ± 7.0%, respectively ($p = 0.0853, 0.0741, 0.0668$). These data demonstrated that nsEP inhibited CPG2 via removal of zinc, and that nsEP can be used to modulate CPG2.

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1. Introduction

Nanosecond electric pulses (nsEP) evoke a high voltage in specific cellular structures without producing any heat, thereby triggering responses such as membrane poration and DNA damage [1–3]. nsEP can be used to enhance chemotherapy and ablate tissues non-thermally. However, the electrochemical effect of nsEP on proteins remains unclear. Studies using molecular dynamics simulations suggest that nsEP can alter the free-energy profile of the side-chain orientation of histidine of aquaporin 4, and induce the unfolding of myoglobin [4,5]. However, as yet there is no direct experimental evidence.

Carboxypeptidase G2 (CPG2) is a zinc-dependent homodimer. The molecular mass of the monomer is 41.4–41.8 kDa, and each monomer has two zinc ions [6]. This quaternary structure suggest that those determinants of the function of a protein (i.e., intactness and conformation of each monomer, the assembly of subunits and the prosthetic group) can be assayed simultaneously, providing a better model for exploring the protein's response to nsEP experimentally. Thus, the electrochemical effect of nsEP on CPG2 was investigated in this study.

2. Methods

2.1. CPG2

Recombinant CPG2 was in a lyophilized form (Chongqing Kerun Biomed. Pharm., Chongqing, China). The specific activity was 450 U/mg, and the theoretical molecular mass of monomer was 41,571 Da.

2.2. nsEP exposure

CPG2 was dissolved in deionized water and the concentration was adjusted to 100 U/ml. nsEP exposure was performed as described previously [7]. The pulse duration was 8, 16 or 24 ns. The voltage was 10 kV and the interelectrode distance was 1 cm. Thus, the strength E was 10 kV/cm with a pulse repetition frequency of 10 Hz. The total exposure time was 20, 40, 60, 90, 120, 180 or 240 s. The control received sham nsEP treatment. All experiments were performed in triplicate.

2.3. Determination of enzyme activity

The CPG2 activity was determined with a kinetics assay using methotrexate as the substrate [8]. The reaction mixture was 0.1 M Tris–HCl, pH 7.3, 0.2 mM $ZnCl_2$ and 60 μM methotrexate. The initial rate of substrate consumption was used to deduce the activity, and 1 U represented the hydrolysis of 1 μmol of methotrexate per min at 37 °C. The relative activity [relative activity (%) = (activity in exposed sample / activity in control) × 100%] was calculated to quantify the variation of activity after nsEP exposure, considering inherent errors of a kinetics method [9]. This manner decreased the variance of measured values.

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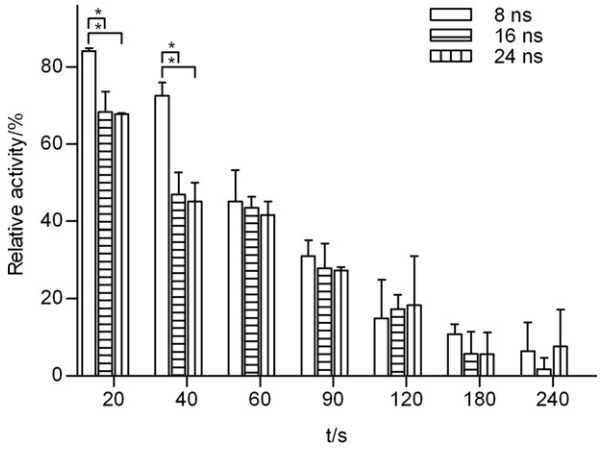

Fig. 1. The relative activity of CPG2 after nsEP exposure; relative activity/% as a function of total exposure time t/s. *$p < 0.05$.

2.4. SDS-PAGE

Reducing and non-reducing (without dithiothreitol) SDS-PAGE were performed to assay the monomer.

2.5. Size exclusion (SEC) and reverse phase-high performance liquid chromatography (RP-HPLC)

HPLC was performed with the Agilent 1100 system, and the eluate was detected at 220 nm.

SEC was performed to determine the molecular mass and purity of the CPG2 dimer. A Zenix-150 column (7.8 × 300 mm, particle size 3 μm; Sepax Tech., Inc.) was used. Samples were eluted at 0.5 ml/min with 50 mM phosphate buffer saline and 0.2 M NaCl, pH 7.0. The molecular mass of each fraction was calibrated with references of 1.35–158 kDa.

The monomer was analyzed with RP-HPLC. A Zorbax 300SB C18 column (4.6 × 250 mm, particle size 5 μm; Agilent) was used. The mobile phase A was 0.075% trifluoroacetic acid, and B was acetonitrile containing 0.075% trifluoroacetic acid. A gradient elusion was applied: B was increased from 25% to 60% in 30 min. The flow rate was 1 ml/min.

2.6. Mass spectroscopy (MS) and circular dichroism (CD)

CPG2 exposed to 240 s of nsEP was analyzed by MS and CD. MS was performed using the system Q Exactive (Thermo Scientific). The spectrum of CD was recorded with the system J-720 (JASCO).

2.7. Determination of zinc

Zinc was assayed in exposed CPG2 (with exposure time of 90 or 240 s). Free zinc ions were removed by dialysis (0.1 M Tris–HCl, pH 7.3, 4 °C, 24 h), and then zinc within the protein was determined with the inductively coupled plasma-mass spectrometry (ICP-MS; ELAN DRC-e, Perkin Elmer).

2.8. Re-incorporation of zinc

Zinc was reconstituted in CPG2 exposed to 240 s of nsEP by direct addition or redialysis. In direct addition, CPG2 was incubated with 0.6 mM $ZnCl_2$ (4 °C, 1 h). In redialysis, CPG2 was dialyzed against 0.1 M Tris–HCl (pH 7.3) containing 0.6 mM $ZnCl_2$ (4 °C, 24 h).

2.9. Statistics

All data were processed with the SAS software (SAS Inst.) and analysis of variance was used. The critical value was set at $p < 0.05$.

3. Results and discussion

The activity of CPG2 decreased with increasing exposure time at each pulse duration, and decreased to <20% when the total exposure time was ≥120 s; values at 240 s were 6.5 ± 7.4% (8-ns), 1.7 ± 2.9% (16-ns) and 7.7 ± 9.5% (24-ns), demonstrating complete deactivation. The 16- and 24-ns pulses caused a lower activity at 20 and 40 s, compared with 8-ns pulses (Fig. 1). These data indicated that nsEP inhibited CPG2 enzyme activity in a pulse duration-dependent manner.

The depolymerization of CPG2 would lead to the loss of activity. The molecular mass of control CPG2 was 80.3 kDa in SEC, confirming that the active form was a dimer. No monomer was detected in nsEP-treated CPG2, and the peak area of dimer was not decreased (Fig. 2). These data showed that nsEP did not disassemble the CPG2 protein.

The integrity of the CPG2 monomer was necessary for the preservation of protein function. No lower-molecular fragments were observed in both reducing and non-reducing SDS-PAGE, demonstrating the

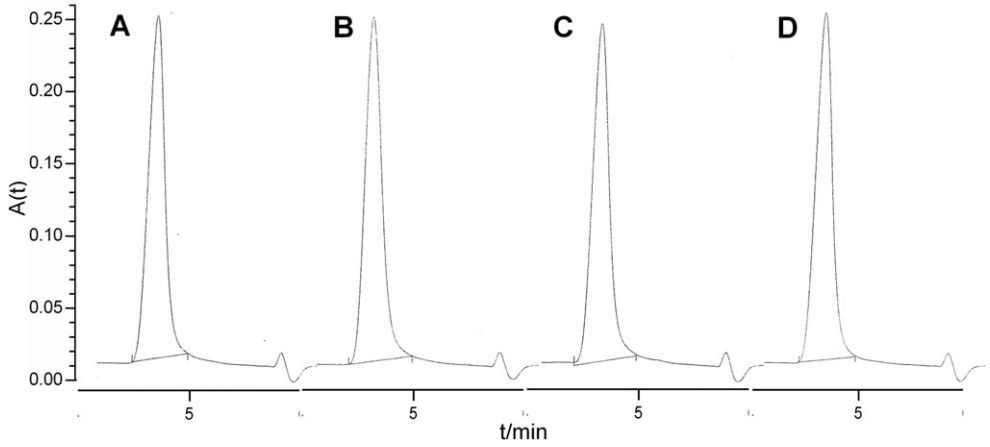

Fig. 2. SEC chromatograms; absorbance A(t) as a function of retention time t/min. Control (A), and CPG2 exposed to 8- (B), 16- (C) and 24-ns (D) pulses. The total exposure time was 240 s. No monomer was detected.

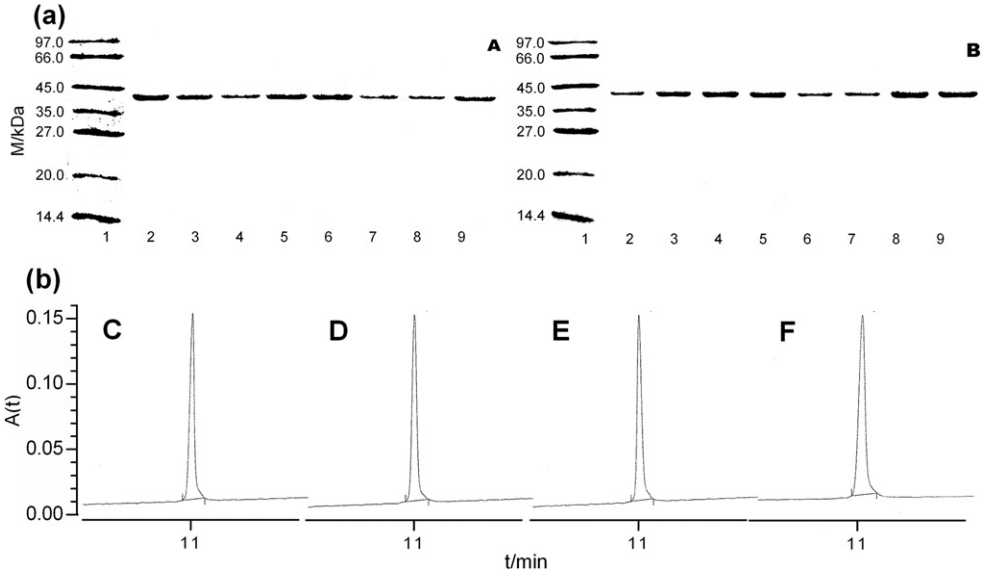

Fig. 3. (a) SDS-PAGE lane profiles of CPG2 treated with non-reducing (A) and reducing (B) conditions, after exposure to 24-ns pulses; lanes (1 to 9): molecular mass references M/kDa, and CPG2 exposed to 0, 20, 40, 60, 90, 120, 180 and 240 s of nsEP, respectively. (b) RP-HPLC chromatograms; absorbance A(t) as a function of retention time t/min. Control (C) and CPG2 treated by 8- (D), 16- (E) and 24-ns (F) pulses (with a total exposure time of 240 s); no new peak occurred.

intactness of monomer (Fig. 3A–B). Considering the lower sensitivity of SDS-PAGE to small peptides, we then performed MS analysis. The molecular mass of control CPG2 was 41,571 Da, and 41,571, 41,570 and 41,571 Da in 8-, 16- and 24-ns pulse-treated CPG2, respectively. The SDS-PAGE and MS data indicated that nsEP did not break the CPG2 monomer.

The CPG2 monomer was also assayed by RP-HPLC. No new peak occurred in nsEP-treated CPG2 (Fig. 3C–F). The RP-HPLC behavior depended on the structure and conformation of a protein. Further, there was no difference in the CD spectrum between the samples (Fig. 4). Overall, these data suggested that the CPG2 subunit was not affected by nsEP.

We assayed the levels of zinc, as it was necessary for catalysis [6]. There was no difference in the activity of CPG2 before and after dialysis (29.1 ± 1.4% vs. 26.0 ± 3.3% in CPG2 exposed to 90 s of 24-ns pulses, $p = 0.3159$), suggesting that zinc was the prosthetic group of CPG2. Zinc levels were 3.30 μg/mg protein in control CPG2 (corresponding to 4.2 zinc ions per mol CPG2). By contrast, after 90 and 240 s of nsEP exposure, zinc levels decreased to 1.74 and 0.40, 1.60 and 0.12, and 1.42 and 0.38 μg/mg protein, at 8-, 16- and 24-ns pulses, respectively. These findings suggested that nsEP inhibited CPG2 via removal of zinc:

$$\mathrm{CPG2}\left(\mathrm{Zn}^{2+}\right) \overset{\mathrm{nsEP}}{\rightarrow} \mathrm{CPG2} + \mathrm{Zn}^{2+}. \quad (1)$$

Next, zinc was reloaded via direct addition or redialysis. The activities of CPG2 exposed to 8-, 16- and 24-ns pulses recovered to 81.1 ± 3.9%, 70.0 ± 4.4% and 67.9 ± 3.6% ($p = 0.1258, 0.0011, 0.0002$) after direct addition, and to 94.7 ± 3.4%, 84.0 ± 5.2% and 81.7 ± 7.0% ($p = 0.0853, 0.0741, 0.0668$) after redialysis, respectively. These findings confirmed that zinc loss was the mechanism of CPG2 deactivation following nsEP. Dialysis was a dynamic equilibrium process, thus favoring the rebinding of zinc ions.

The present findings suggested that nsEP inhibited CPG2 via deprivation of zinc ions, with no effect on apoenzyme integrity. Zinc may be removed from CPG2 by the electric forces. Within CPG2, each zinc ion was coordinated by one histidine, one glutamate and one aspartate [6]. The nsEP field can alter the free-energy profile of histidine [4], which relaxed the covalent bonds, thus releasing a zinc ion. As pulse duration was a determinant of the electric energy [10], longer pulses would lead to lower levels of zinc in CPG2, resulting in a lower activity. The binding of zinc within a protein with tertiary structure differed from that within CPG2; whether nsEP caused the zinc eviction in those proteins therefore should be explored. Similarly, nsEP may deactivate a protein with quaternary structure but without prosthetic groups, via other mechanisms such as the disassembly and conformation changes.

CPG2 can release an active drug from a nontoxic prodrug (i.e., prodrug therapy). Because of enzymolysis, prodrugs became rapidly activated, leading to a high level of active drugs within the cancer; the

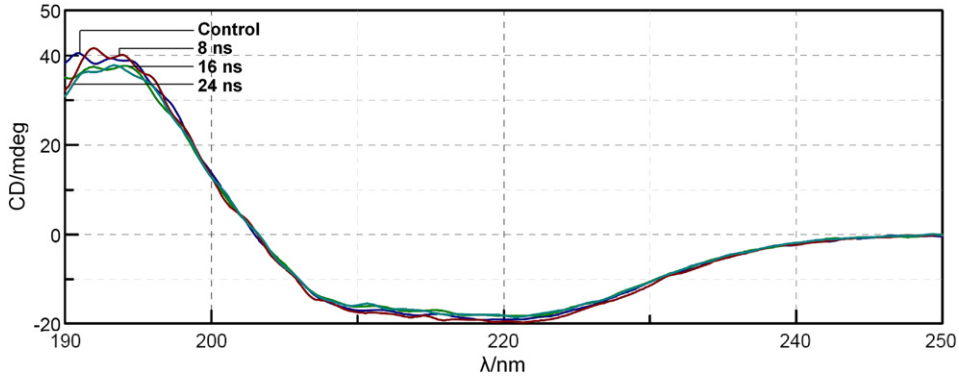

Fig. 4. CD spectrums of CPG2 exposed to 240 s of nsEP; ellipticity CD/mdeg as a function of wavelength λ/nm. There was no difference between the samples.

leak of active drugs back into the circulation can cause systemic toxicity [11]. The present data suggested that nsEP modulate CPG2 activity, which may be useful to assist prodrug therapy. Further, nsEP was previously reported to produce anticancer effects [1,12]. Thus, the combination of these two modalities may improve therapeutic efficacy and decrease toxicities.

4. Conclusion

Our data demonstrated that nsEP inhibited CPG2 via dissociating the prosthetic groups. nsEP can be used to modulate CPG2, thereby assisting the prodrug therapy. The dissociation kinetics of zinc and the interaction between apoenzyme and zinc under nsEP, as well as the electric and biochemical mechanisms of zinc loss, will be explored in future studies.

Acknowledgments

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