Original Paper

Biphasically Modulating the Activity of Carboxypeptidase G2 with Ultrasound

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Key Words

Carboxypeptidase G2 • Dimer • Monomer • Conformation • Ultrasound • Prodrug therapy

Abstract

Background/Aims: Carboxypeptidase G2 (CPG2) has been used for cancer prodrug therapy to realize the targeted release of active drugs, but there yet lacks a means to modulate the CPG2 activity. Here ultrasound was used to modulate the CPG2 activity. **Methods:** The activity of insonated CPG2 was determined, and then underlying biochemical (i.e., monomer, dimer and conformation) and ultrasonic (i.e., heat and cavitation) mechanisms were explored. **Results:** Ultrasound (1.0 MHz) increased or decreased the enzymatic activity; the activity decreased as zero- or first-order kinetics, depending on the intensity. L1 (10 W/cm² for 200 s) improved the activity via increasing the specific activity. L2 or L3 (20 W/cm² for 1200 or 3000 s) decreased the activity via disassembling the dimer, degrading the monomer, inducing glycosylation, transforming conformation and decreasing the specific activity. An increase or a slight decrease of activity attributable to 10 W/cm² was reversible, but the activity decrease due to 20 W/cm² was irreversible. The enzymatic modulation was realized via cavitation. **Conclusion:** Ultrasound can biphasically modulate the CPG2 activity, and can be employed in the CPG2-prodrug therapy to adjust the release and moles of active drugs.

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Introduction

Carboxypeptidase G2 (CPG2) has been applied to the prodrug therapy, with a specific advantage of lacking equivalents in humans; CPG2 is introduced into the tumor, thereby releasing active drugs into cancer tissues to realize a targeted treatment. Preliminary clinical trials have demonstrated that the CPG2-prodrug therapy is effective; however, this modality has been limited by adverse events: the leak of active drugs into circulation results in severe systemic toxicities [1]. Therefore, the yield of active drugs should be under control, i.e., the CPG2 activity should be down-regulated once the drug level within the tumor reaches the goal value. Anti-CPG2 antibodies are injected to neutralize CPG2 in the present regimen [2].

This mode will induce immune reactions limiting following treatments. A safe and simple means that can modulate the CPG2 activity should be developed.

Ultrasound can assist the enzymatic reactions to improve the yield of products. Most trials are performed on monomeric enzymes; thus ultrasound increases the enzymatic activity via inducing conformational transformation [3]. The activity of an oligomeric enzyme depends on both integrity of the peptide chain and correct higher-order structure, suggesting that multiple factors can affect the activity. However, the ultrasonic effects on oligomeric enzymes and underlying mechanisms remain unclear. CPG2 is a homodimer, and subunits assemble into an active dimer through hydrophobic interactions and hydrogen bonds [4]. Such a quaternary structure provides an appropriate model for exploring the responses of a protein to a physical factor: all mechanisms that determine the structure and function of a protein can be evaluated simultaneously [5].

Here we explore the effect of ultrasound on CPG2. The data manifested that ultrasound can biphasically modulate the enzymatic activity.

Materials and Methods

Protein and ultrasonic exposure

Lyophilized recombinant CPG2 (Chongqing Kerun Biomed. Pharm. Co., Chongqing, China) was with a HPLC (high performance liquid chromatography) purity of >97%, and the cryoprotectant was sucrose; the theoretical molecular mass of monomer was 41440 Da. CPG2 was dissolved in deionized water, and the concentration was adjusted to 10 U/ml.

Insonation was performed as described previously, using a device designed by Chongqing Haifu Med. Technol. Co. Ltd. (Chongqing, China) [6]. A concave transducer was located at the bottom of a tank filled with water as the coupling medium; water was prechilled at 4 °C, thereby decreasing temperature rise to stabilize the ultrasound output. This manner can also decrease temperature rise in the insonated solution. 1 ml of CPG2 solution was added into a polystyrene tube, and then placed into the focal volume of the transducer to receive insonation. The intensity was 10 or 20 W/cm², with a frequency of 1.0 MHz in continuous waves. Control samples received sham exposure (i.e., with 0 s of exposure time).

Enzymatic activity

The activity of CPG2 was determined with a kinetic assay, using methotrexate as the substrate [7]. The relative activity was used to quantify an alteration after insonation ([activity after insonation]/[activity in control]).

Based on data in activity, CPG2 received L1 (10 W/cm² for 200 s), L2 (20 W/cm² for 1200 s) and L3 (20 W/cm² for 3000 s) was used to explore mechanisms, with activities of 1.11, 0.37 and 0.05, respectively. K_m and V_{max} were determined. CPG2 was kept at 4 °C and the activity was re-calibrated after 2, 4 and 8 h, thus determining the reversibility of activity. For HPLC, mass spectroscopy (MS), circular dichroism spectroscopy (CD) and fluorescence scan assays, 10 ml of CPG2 solution were concentrated to 1 ml by ultrafiltration (with a cutoff molecular mass of 3 kDa).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

CPG2 received reducing and non-reducing PAGE. The protein was quantified using the software Quantity One (Bio-Rad Lab., Hercules, CA).

Reverse phase (RP)- and size exclusion (SEC)-HPLC

HPLC was performed using a UltiMate3000 system (Thermo Sci., Germering, Germany). The elute was detected at 220 nm, and the ratio of peak area of intact CPG2 dimer/monomer was calculated to quantify the decrease ([area after insonation]/[area in control]).

SEC-HPLC was performed to detect the dimer, using a Zenix-150 column (7.8×300 mm, particle size 3 µm; Sepax Technol., Newark, DE). The mobile phase was 0.2 M NaCl, 50 mM PBS, pH 7.0, with a flow rate of 0.5 ml/min. Molecular mass of each fraction was calibrated with a gel filtration standard (1.35–158 kDa; Bio-Rad).

RP-HPLC was performed to assay the monomer. A Zorbax 300SB-C18 column (4.6×250 mm, particle size 5 µm; Agilent Technol., Santa Clara, CA) was employed. The mobile phase A was 0.1% trifluoroacetic acid, and B was acetonitrile containing 0.1% trifluoroacetic acid. B was increased from 10% to 60% in 30 min, and the flow rate was 0.8 ml/min.

Molecular mass

Molecular mass of monomer was determined with MS (Triple-TOF 5600+; AB Sciex, Framingham, MA). L2- and L3-insonated CPG2 also received PNGase F treatment to cleave glycans before MS.

CD and synchronous fluorescence

The secondary structure was analyzed using CD (Chirascan; Appl. Photophys., Surrey, UK).

Synchronous fluorescence scan (Varioskan Flash; Thermo Sci., Vantaa, Finland) was performed to measure the intrinsic fluorescence, thereby reflecting conformational transformation. The interval between λ_{ex} and λ_{em} was 60 nm [8]. Phe, Tyr and Trp references were used to identify the characteristic λ_{ex} , with values of 214, 232 and 282 nm, respectively; data in the mixture (11:13:1; the molar ratio in CPG2) manifested that λ_{ex} was 232 nm for Tyr and 278 nm for Trp, and that Phe displayed no peak.

Temperature, free radicals and glucose

Temperature in the CPG2 solution during insonation was recorded using a 100 µm-diameter thermocouple (Guangzhou Sungun Meas. Ctrl. Technol. Co., Ltd., Guangzhou, China).

Free radicals attributable to cavitation were determined using the terephthalate assay, and the control was used for zero adjustment [9]. Glucose was determined with the oxidase assay (Nanjing Jiancheng Bioeng. Inst., China).

Statistics

Data were processed with the statistics software SAS (SAS Inst., Cary, NC). Analysis of variance was used, and multiple comparisons were corrected with the Student-Newman-Keuls method or Dunnett's test (enzymatic activity). The critical value was set $p < 0.05$.

Results and Discussion

Ultrasound improved the activity via increasing the specific activity

Under 10 W/cm², the activity was improved when the insonation time was ≤400 s, with a peak value of 1.11 at L1 ($p = 0.0004$) (Fig 1A). The activity relied on integrity of the dimer. Interestingly, SEC-HPLC demonstrated that the CPG2 dimer (82.5 kDa) was decreased to 0.76 ($p < 0.05$) (Fig 2G, H). Molecular mass of monomer was 41438 Da in control and was 41440 Da in L1-treated CPG2, indicating that the ultrasonically increased activity was not due to protein modifications. These data manifested that the improvement of activity was realized via increasing the specific activity (1.46-fold after L1 exposure). A protein may display heterogeneity (i.e., conformational isoforms) [10]. Certain forms can be activated by insonation but certain forms would be destroyed. Further, the distribution of ultrasonic filed was not uniform – molecules were activated within definite areas but destructed within other definite areas [11]. Therefore, the activity observed reflected a cumulative effect.

Ultrasound decreased the activity via multiple pathways

The intensity of 10 W/cm² decreased the activity when the insonation time was ≥700 s ($p < 0.0001$). The CPG2 activity decreased linearly, with a rate of 2.66×10^{-4} /s ($t \in [200, 2000]$). However, longer exposure time caused only a limited reduction, indicating a plateau (Fig 1A). The activity cannot be decreased to <0.50, and therefore the intensity was increased to 20 W/cm². Under 20 W/cm², the activity was decreased when the insonation time was ≥600 s ($p < 0.0001$); the decrease of activity followed zero-order kinetics when the insonation time was ≤1000 s (with a rate of 3.36×10^{-4} /s), and displayed first-order kinetics when the

Fig. 1. Relative activity of CPG2 after insonation. Activity under 10 W/cm² (A): the activity was increased when the insonation time was ≤400 s, and then decreased linearly; there was a plateau when the insonation time was ≥2000 s. Activity under 20 W/cm² (B): the decrease followed zero-order kinetics when the insonation time was ≤1000 s, and displayed first-order kinetics when the insonation time was ≥1200 s. Data were mean ± standard deviation for 3 independent experiments. a: vs. Ctrl, p<0.05.

insonation time was ≥1200 s (with a rate of $1.15 \times 10^{-3}/s$) (Fig 1B). These data confirmed that the intensity was the determinant for enzyme inhibition.

SEC-HPLC indicated that the peak area of intact CPG2 dimer was decreased after L2/L3 exposure, with a relative amount of 0.09 in L3-treated protein (i.e., the specific activities were 0.81- and 0.52-fold after L2 and L3 insonation, respectively) ($p < 0.0001$); smaller fragments (e.g., 42.0, 3.5 and 2.6 kDa) were detected (Fig 2G, H). The 42.0 kD-fraction was monomer and others were degraded fragments. These suggested that ultrasound decreased the activity via disassembling and/or decomposing the apoenzyme.

The formation of a correct homodimer depended on the integrity of monomer, and therefore the monomer was analyzed by PAGE, RP-HPLC and MS. Reducing and non-reducing PAGE, and RP-HPLC demonstrated a decrease of intact monomer after insonation, and L2/L3 led to a lower level compared with L1 ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$); there appeared a new peak in RP-HPLC (Fig 2A–F). Considering a lower sensitivity to small peptides, CPG2 exposed to 10 W/cm² for 100 s (with an activity of 1.06) and 20 W/cm² for 1000 s (with an activity of 0.68) also received PAGE; a gradual decrease verified the reliability of results. These were consistent with data in SEC-HPLC. A reduction of monomers decreased moles of the dimer, thereby leading to a lower activity. MS demonstrated higher-mass fragments (41461–44082 Da) in L2-treated samples, and both higher- and lower-mass fragments (39486–44995 Da) in L3-treated CPG2; there was a decrease in mass (34756–41458 Da) after L2/L3-insonated sample was digested with PNGase. Therefore, lower-mass fractions should be degraded subunits (i.e., the new peak in RP-HPLC), and those higher-mass fragments were mainly due to glycosylated monomers because sucrose was the cryoprotectant. Glycosylation was with the hydrolytic products (i.e., glucose and fructose) since sucrose was a non-reducing sugar; indeed, glucose was detected after L2/L3 insonation (with levels of 0, 0, 2.1 ± 0.2 , and 10.6 ± 5.7 µg/ml in control, L1-, L2-, and L3-treated samples, respectively). Glycosylation impacted on the function of a protein [12,13]. Ultrasound can accelerate the hydrolysis of sucrose, and enhance the protein-sugar reactions to form conjugates via free –OH, –COOH or –NH₂ in the protein [14,15]. These indicated that the 42.0 kDa-fraction in SEC-HPLC in L2/L3-treated CPG2 contained intact, truncated and glycosylated monomers, and that the 82.5 kDa-fraction reflected dimers formed from native and/or modified subunits. Ultrasound inhibited CPG2 via disassembling the dimer, degrading the monomer, inducing glycosylation, and decreasing the specific activity. Noticeably, sucrose was the preferred stabilizer in drug formulations. Ultrasound was used to enhance the action of drugs but without synergism in some cases [16]. The present data suggested a mechanism: drug molecules were glycosylated

Fig. 2. Amounts of CPG2 monomer and dimer after insonation. Monomer analyzed with 10% reducing (A, B) and non-reducing PAGE (C, D): insonation decreased monomer; a gradual decrease demonstrated the reliability of results. Mr: mass references (kDa); lane 1–6: Ctrl, CPG2 exposed to 10 W/cm² for 100 or 200 s, 20 W/cm² for 1000, 1200 or 3000 s. Monomer detected with RP-HPLC (E, F): monomer was decreased after sonication, and 20 W/cm² led to a less level compared with 10 W/cm²; there appeared a new peak (↓; i.e., degraded fragment) after 20 W/cm² insonation. Dimer assayed by SEC-HPLC (G, H): insonation decreased the level of dimer (82.5 kDa); there was a slight decrease after 10 W/cm² for 200 s, suggesting that ultrasound improved the activity via increasing the specific activity; lower-mass fragments were detected after exposure to 20 W/cm² for 1200 or 3000 s, indicating disassembly and degradation of the apoenzyme; 1.1 and 0.4 kDa were extrapolated values for beyond the detection limitation. Data were mean ± standard deviation for 3 independent experiments. a: vs. Ctrl, *p* < 0.05; b: vs. 10 W/cm² for 200 s, *p* < 0.05; c: vs. 20 W/cm² for 1200 s, *p* < 0.05.

under insonation decreasing the therapeutic potency; this needed particular concerns in ultrasonically assisted chemotherapy.

Conformational transformation occurred in insonated CPG2

The secondary structure was analyzed with CD. Helices were 52.6%, 39.8%, 29.0% and 21.6%, β -sheets were 7.1%, 11.8%, 21.2% and 37.0%, and random coils were 23.9%, 31.1%, 37.0% and 40.7%, in control, L1-, L2- and L3-treated CPG2, respectively (Fig 3A). These suggested that a slight decrease of helices combined with a slight increase of β -sheets favored catalysis. The catalytic domain of a subunit consisted of 8 helices and 12 β -strands, and the dimerization domain contained 2 helices and 4 β -strands [4]. Therefore, insonation modulated the activity mainly via affecting structure of the catalytic domain. A remarkable increase of random coils in L2/L3-treated CPG2 showed that insonation relaxed the peptide chain.

There were 13 Tyr (4 in helix, 5 in β -strand and 4 in random coil) and 1 Trp (in β -strand) in a subunit, and therefore alteration of the higher-order structure was assessed with synchronous fluorescence. A higher fluorescent intensity at 232 and 278 nm demonstrated

Fig. 3. Higher-order structure analyzed with CD (A) and synchronous fluorescence scan (B). CD demonstrated a decrease of helices and an increase of β -sheets after insonation. Higher fluorescence intensities at 232 and 278 nm revealed externalization of Tyr and Trp after insonation (10 W/cm² for 200 s); however, a decrease of fluorescence intensities was noted after exposure to 20 W/cm² for 1200 or 3000 s, demonstrating internalization of Tyr and Trp; there had a 2-nm blue-shift in insonated CPG2 (20 W/cm²), showing a decrease of polarity.

Fig. 4. Temperature (A) and the level of free radicals (B). Temperature was <30 °C, even under an intensity of 20 W/cm² with insonation time of 3000 s. Free radicals detected with the terephthalate assay: a higher intensity led to a higher yield. Data were mean $\pm$ standard deviation for 3 independent experiments. b: vs. 10 W/cm² for 200 s, $p < 0.05$; c: vs. 20 W/cm² for 1200 s, $p < 0.05$.

Table 1. K_m and V_{max} of insonated CPG2

Insonation	K_m (μ M)	V_{max} (μ M/s)	K_m/V_{max}
Ctrl	9.361	0.028	334
L1	7.748	0.024	323
L2	12.610	0.021	600
L3	0	0.003	0

* V_{max} and K_m demonstrated complete inhibition after L3 exposure

the externalization of Tyr and Trp after L1 exposure, and a lower fluorescent intensity revealed internalization of Tyr and Trp after L2/L3 insonation (Fig 3B). Trp had a 2-nm blue-shift in L2/L3-treated sample, indicating a decrease of polarity [17]. These data manifested that the conformational transformation played a part in the enzymatic modulation (i.e., alteration of the specific activity). Conformational transformation can result from directly ultrasonic effects on protein molecules, or protein modifications after insonation; which played the leading role should be explored. There arose a peak at 226 nm in insonated CPG2, but underlying mechanisms remained unclear.

K_m decreased in L1-exposed CPG2, suggesting that ultrasound increased the activity via improving the affinity to substrates. This was due to conformational transformation. L2-insonated CPG2 had a less V_{max} and a higher K_m . A decrease of V_{max} resulted from the decrease of amount of active CPG2, and an increase of K_m was attributable to conformational alteration. K_m was decreased to 0 in L3-exposed enzyme, demonstrating complete inhibition, which complied with the data in activity (Table 1).

Fig. 5. Dynamic pattern of activity (A) and the cavitation level (B). Dynamic pattern of activity: the activity of CPG2 exposed to 10 W/cm² (200/1000 s) did not differ from that in control after 2 h, suggesting that an increase or a slight decrease of activity was reversible; the activity after exposure to 10 W/cm² for 3000 s, or 20 W/cm² (1000/1200/3000 s) cannot return to the level in control CPG2, demonstrating irreversible decrease. a: vs. Ctrl @ the same time point, $p < 0.05$. Cavitation level: using 10 W/cm² for 200 s as the reference, exerted ultrasonic energies of 10 W/cm² for 200/1000/3000 s, and 20 W/cm² for 1000/1200/3000 s, were 1×, 5×, 15×, 10×, 12× and 30×, respectively; these manifested that intensity was a crucial determinant of the cavitation level under equal ultrasonic energy. *, $p < 0.05$. Data were mean $\pm$ standard deviation for 3 independent experiments.

Reversibility depended on the degree of activity alteration and the intensity

The activity of insonatated CPG2 (10 W/cm² for 200 or 1000 s, with an activity of 1.11 or 0.85) did not differ from that in control after 2 h ($p = 0.3071$), suggesting that an increase or a slight decrease of activity was reversible. CPG2 received 10 W/cm² for 3000 s (with an activity of 0.58), or 20 W/cm² for 1000–3000 s (with an activity of 0.68–0.05) yet had a lower activity after 2 h ($p = 0.0009$, $p < 0.0001$), demonstrating irreversible decrease (Fig 5A). Thus, the reversibility depended on the degree of activity alteration, and the intensity was a crucial determinant. A lower intensity should be employed to realize reversible modulation, and a higher intensity can be used to inactivate enzymes perpetually.

In reversible cases, the activity alteration was mainly due to conformational transformation. The decrease of activity was mostly mediated by other mechanisms in irreversible inhibition. The dynamic pattern of activity suggested that conformational transformation after L2/L3 insonation may result from protein modifications.

Ultrasonically enzymatic modulation was mediated by cavitation

Ultrasound impacted on molecules via heat and cavitation. Temperature was $< 30^{\circ}\text{C}$ in the CPG2 solution, indicating that heat did not play an important role in the CPG2 modulation (Fig 4A). Free radicals were detected after insonation, and a higher intensity and longer insonation time led to a higher yield, confirming the occurrence of cavitation ($p < 0.0001$) (Fig 4B). Therefore, ultrasonically enzymatic modulation was mediated by cavitation. Cavitation caused shear forces and active radicals. Shear forces can attack the carbon-oxygen, hydrogen, acetamido and unsaturated bonds, and active radicals can react with free $-\text{OH}$, $-\text{SH}$, $-\text{COOH}$ and $-\text{NH}_2$ radicals [14,15,18,19]. Cavitation induced the in situ formation of formic acid, hydrolyzing adjacent molecules [14]. These effects ultimately resulted in protein degradation and/or modification. L2/L3 caused a much higher yield of free radicals compared with L1 (> 40 -fold). This indicated that a low level of cavitation can activate CPG2 but a high level led to inhibition. The intensity was a crucial determinant of the cavitation level when exerting equal ultrasonic energy (Fig 5B). This was the reason why most effects observed here depended on the intensity. Therefore, the intensity should be precisely set to adjust the cavitation level.

Potential applications

In the prodrug therapy, an insufficiency of active drugs decreases the therapeutic efficacy, but an overdose leads to severe toxicities. Therefore, the CPG2 activity should be modulated precisely. The present data manifested that ultrasound can biphasically modulate CPG2 using a lower intensity. Ultrasound can penetrate into the deeper volume without harming overlying tissues, suggesting a noninvasive strategy to modulate the CPG2 activity; consequently, the release rate and moles of active drugs can be adjusted promptly according to the therapeutic responses. Further, insonation can enhance the action of drugs, and permeabilize the vessels and cells to modulate the intratumoral/intracellular pharmacokinetics [20–22]. These can improve the therapeutic effects. A higher intensity can be employed for detoxification, i.e., inactivating CPG2 completely to terminate the production of active drugs. These suggest that ultrasound can be an adjuvant for the CPG2-prodrug therapy.

Conclusion

Ultrasound can biphasically modulate the CPG2 activity. Ultrasound up-regulated the activity via increasing the specific activity, and down-regulated the activity via disassembling the dimer, degrading the monomer, inducing glycosylation, transforming the conformation and decreasing the specific activity. An increase or a slight decrease of activity was reversible. These data suggest that ultrasound can be an adjuvant for the CPG2-prodrug therapy.

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Disclosure Statement

The authors declare no conflict of interest.

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