Soluble adenyl cyclase mediates mitochondrial pathway of apoptosis and ATP metabolism in oyster *Crassostrea gigas* exposed to elevated CO₂

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Soluble adenylyl cyclase mediates mitochondrial pathway of apoptosis and ATP metabolism in oyster *Crassostrea gigas* exposed to elevated CO₂

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Abstract

Ocean acidification (OA) has deleterious impacts on immune response and energy homeostasis status of Mollusca. In the present study, the apoptosis ratio of hemocytes and the adenosine triphosphate (ATP) allocation in gill tissues were determined after Pacific oysters *Crassostrea gigas* were exposed to elevated CO₂ environment (pH=7.50) for 16 days.. The apoptosis ratio in CO₂ exposure group (35.2 %) was

significantly higher ($p < 0.05$) than that in the control group, and the increased apoptosis ratio induced by elevated CO_2 could be significantly inhibited ($p < 0.05$) by KH7, a specific inhibitor of a bicarbonate sensor soluble adenylyl cyclase (sAC). After CO_2 exposure, sAC in oyster (CgsAC) was found to be clustered with mitochondria in the cytoplasm, and the pro-caspase-3 was cleaved into two small fragments. Moreover, the activities of caspase-3 and caspase-9 also increased post CO_2 exposure and these increases could be inhibited by KH7. However, the activities of caspase-8 did not change significantly compared with that in the control group. After CO_2 exposure, the ATP content in the gill increased significantly ($p < 0.05$) and such increase could also be inhibited by KH7. The ATP content in purified gill mitochondria decreased significantly ($p < 0.05$) after CO_2 exposure, which was also inhibited by KH7. These results implied that the elevated CO_2 could activate the mitochondria-CgsAC pathway of apoptosis and ATP metabolism in oyster, and this pathway played essential roles in maintaining the homeostasis and the balance of energy metabolism in response to OA.

Keywords: *Crassostrea gigas*; Elevated CO_2 ; Apoptosis; ATP; Soluble adenylyl cyclase; Mitochondria

1 Introduction

Pacific oyster (*Crassostrea gigas*) is one of the most widely cultured species in the second largest animal phylum Mollusca [1]. They are successful colonizers of estuaries and intertidal zones where environmental balance is easily interfered by series of environmental stresses, such as temperature, salinity, pathogens and carbonic chemistry disturbance [2-4]. As important marine calcifiers and ecosystem engineers in the marine environment, oysters are regarded as model organisms in the investigation of marine environmental stresses such as dry exposure, temperature, heavy metal, and low oxygen [5-7]. Recently, the rising of atmospheric carbon dioxide is predicted to cause the decrease of ocean pH by about 0.3-0.4 units at the end of this century, which is termed ocean acidification (OA) [8]. It changes the carbonate chemistry of seawater (i.e. speciation of inorganic carbon) and reduces calcium carbonate saturation state [9]. OA could pose severe impacts on shell-forming marine organisms from plankton to benthic mollusks, echinoderms, and corals [10]. There are various reports about the negative effects of OA on oysters, such as on fertilization, larval development, growth, immune response and energy metabolism [5, 11-14].

Apoptosis, a type of programmed cell death process, plays crucial roles during development and aging in both vertebrates and invertebrates. It is involved in immune responses against biotic stresses like various pathogens and adaptive responses to environmental stresses [15, 16]. The apoptosis pathway can be divided into extrinsic

or death receptor pathway and intrinsic or mitochondrial pathway [17]. Environmental contaminations such as heavy metals could trigger the intrinsic apoptosis pathway. For example, Cd induced mitochondrial apoptosis pathway in the hepatopancreas of freshwater crab *Sinopotamon yangtsekiense* [18] and in the gill cells of *Mytilus edulis* [19]. It has been reported that OA could increase the apoptosis ratio of hemocytes in oyster [11]. However, the underlying mechanism of the regulation has not yet been well understood.

Energy metabolism is critical to maintain physiological homeostasis in response to environmental changes, such as oxygen deficiency, temperature stress, salinity, pollution and OA [20, 21]. The decreasing of pH value may result in deleterious impacts on energy homeostasis which are closely related to the survival of mollusks [11, 20, 22-24]. For example, exposure to OA may cause the shifts of the energy budget in cells, tissues or the whole organism due to shifted acid-base regulation [25]. The balance of ATP synthesis and consumption plays essential roles in adaptation to environmental stresses [21]. Oysters are filter feeders and their gills are constantly exposed to the outside environment for respiration and filtration, and frequently in contact with the chemically altered water [26]. Environmental stresses like hypercapnia, hypoxia, air exposure or copper treatment could affect the energy metabolic activities in gill [27, 28]. CO₂ exposure may cause metabolic alteration to maintain sufficient energy for the necessary physiological processes such as ion transportation and acid-base regulation [20, 22, 23, 29]. Thus, the ATP allocation in gill is worthy of investigating and it might provide a better understanding of

biological responses to OA.

Soluble adenylyl cyclase (sAC) is a conserved bicarbonate sensor and also an important intracellular source of signaling molecule cyclic AMP (cAMP) [30, 31]. It has been demonstrated to control mitochondria-dependent apoptosis in rat cardiomyocytes and coronary endothelial cells and could serve as a metabolic sensor modulating ATP generation in response to nutrient availability [32-34]. When mollusks are exposed to the elevated CO₂, the transport of H⁺ and HCO₃⁻ would be activated to maintain acid-base balance, resulting in accumulation of bicarbonate ions [35]. In our previous study, sAC was identified in oyster (CgsAC) and its involvement in the activation and regulation of physiological processes after CO₂ exposure had been evaluated [36]. The purposes of the present study were (1) to explore the effects of CO₂ exposure on apoptosis ratio of hemocytes and ATP allocation in gill; (2) to clarify the roles of CgsAC in the CO₂ exposure induced apoptosis and ATP allocation, to further understand the regulation mechanism and living strategy of oyster in response to OA.

2 Materials and methods

2.1. Oysters

Oysters *C. gigas* were collected from a local farm in Qingdao, China, with average shell length of 13.0 cm. The oysters were pre-punched near the muscle by the side of shell and then cultured in the aerated seawater at about 15 °C for one week before processing. The experiments were approved by the Qingdao Animal Care and

Use Committee with a permit number of SCXK (Shandong) 20090007, which complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. CO₂ exposure and inhibitor injection

Forty-five individuals were randomly divided into control group (pH=8.10±0.05) (9 oysters) and CO₂ exposure group (pH=7.50±0.05) (36 oysters). They were cultured in water tanks with volume of 100 L continuously bubbled with the ambient air or air-CO₂ mixtures as appropriate, and the water was changed every other day to ensure adequate water quality. The new water was pre-bubbled with air- CO₂ mixtures and the pH value was adjusted to 7.50. The pH value in the CO₂ exposure group was controlled using an acidometer (AiKB, Qingdao, China). Oysters in the control group and CO₂ exposure group were kept for 16 days under the same culture conditions except the pH value. During the treatment process, the temperature of the control group was controlled to be 14.9±0.5 °C and that of CO₂ exposure group was 15.0±0.3 °C; salinity of the control group was 32.9±0.1 and that of CO₂ exposure group was 32.7±0.1; the dissolved oxygen of the control group was 7.83±0.04 mg/L and that of CO₂ exposure group was 7.85±0.02 mg/L.

Twenty-seven oysters in the CO₂ exposure group were used for the inhibitor injection experiment, and they were pre-adapted to the CO₂ exposed environment for two days. One hundred microliter of sAC specific inhibitor (KH7, K3394, Sigma-Aldrich, St Louis, MO, USA) with concentration of 5 mM and 0.5 mM was injected into the muscle adductor of each oyster. The final concentrations of KH7 in

each oyster were calculated to be about $50 \mu\text{mol kg}^{-1}$ and $5 \mu\text{mol kg}^{-1}$, respectively (9 oysters in each treatment, the average wet weight of each oyster except the shell was about 10 g). The oysters received an injection of 100 μL DMSO were used as the negative control (9 oysters). After incubation for another 14 days, gills (including edge, and center) were cut from each individual, snap-frozen in liquid nitrogen, and then stored in -80°C for ATP determination. Fresh gill tissues were cut from oysters for mitochondria isolation. Hemocytes from the oysters in different groups were collected for the determination of apoptosis ratio and immunohistochemistry assay. The tissues and hemocytes from three oysters were pooled as one separate sample, and there are three samples for each tissue.

2.3. Apoptosis assay by flow cytometry

The hemolymphs were collected from three oysters in each group, and they were mixed immediately with equal volume of pre-chilled anticoagulant (8 g L^{-1} citric acid, 22 g L^{-1} sodium citrate, 24.5 g L^{-1} glucose), and then centrifuged at 800 g, 4°C for 10 min. The hemocytes were re-suspended in modified Leibovitz's L-15 mediums (M-L-15, Leibovitz's L-15 mediums with 20.2 g L^{-1} NaCl, 0.54 g L^{-1} KCl, 0.6 g L^{-1} CaCl_2 , 1 g L^{-1} MgSO_4 , 3.9 g L^{-1} MgCl_2) (Life Technologies, Carlsbad, CA, USA) and the cell concentration was adjusted to 10^6 mL^{-1} .

The apoptosis ratio of cells was measured by flow cytometry according to the instruction of Annexin V-Alexa Fluor 488/Propidium Iodide (PI) Apoptosis Assay Kit (KeyGEN BioTECH, Nanjing, China). One hundred microliter of hemocyte suspension was incubated with 5 μL Annexin V-Alexa Fluor 488 and 10 μL PI in the

dark for 20 min. The mixture was diluted with 400 μ L M-L-15 solution and analyzed by a flow cytometer BD FACS Aria II (BD Biosciences, Franklin Lakes, NJ, USA). The apoptotic percentage was determined by counting 10,000 cells.

2.4. Immuno-colocalization of CgsAC with mitochondria in hemocyte

The hemolymphs from six oysters in control and CO₂ exposure groups were collected and centrifuged at 800 g, 4 °C for 10 min to collect the hemocytes. The hemocytes were re-suspended in M-L-15 and the suspension was added onto positively charged slides. After incubated at room temperature for 3 h, the supernatant was discarded and 4% PFA (Paraformaldehyde diluted in PBS) was added to fix the hemocytes for 15 min. After three times of washing in PBST (0.1% Tween 20 in PBS, pH 7.40), the slides were blocked with 3% bovine serum albumin (BSA) in PBS at room temperature for 30 min. The supernatant was removed and the slides were incubated with polyclonal antibody of CgsAC (diluted by 1:500 in blocking buffer) and COX IV antibody (diluted by 1:2400 in blocking buffer, Cell Signaling Technology, Beverly, MA, USA) at 37 °C for 1 h, respectively. After another three times of washing, the slides were incubated with Alexa Fluor 488-labeled goat-anti-mouse antibody (diluted by 1:1000 in blocking buffer) and Alexa Fluor 594-labeled goat-anti-rabbit antibody (diluted by 1:1000 in blocking buffer) at 37 °C for 1 h. Finally, after three times of washing, 4',6-Diamidino-2'-phenylindole dihydrochloride (DAPI, 10 mg mL⁻¹, diluting in PBS by 1:2500, Roche, Basel, Switzerland) was added onto the slides to stain the nucleus. After the last three times of washing, 50% glycerol was added to seal the slides before observation under Laser

Scan Confocal Microscope (ZEISS, Oberkochen, Germany).

2.5. Mitochondria isolation from gill tissues

The isolation of mitochondria from gill tissues was performed using Tissue Mitochondria Isolation Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Protein concentrations were determined with the BCA (bicinchoninic acid) Protein Assay Kit (Beyotime, Shanghai, China) with BSA as standard. The purified mitochondria was used for ATP determination and western blotting.

2.6. Western blotting

The protein samples were loaded for SDS-PAGE assay according to previous report [4], and the proteins were electrophoretically transferred on to a nitrocellulose membrane. After blocked with 5% milk (dissolved in PBST) at room temperature for 1 h, the membrane was incubated with polyclonal antibody at 4 °C overnight, washed three times with PBST, and then incubated with HRP conjugated secondary antibody at room temperature for 1 h. After three times of washing with PBST, the membrane was visualized by Thermo Scientific SuperSignal kit (Life Technologies, , Carlsbad, CA, USA) and developed by X-ray films. The polyclonal antibodies of CgsAC and CgCaspase-3 were prepared according to our previous study [36, 37]. The secondary antibody of goat-anti-mouse IgG (AS003) and the polyclonal antibody of β -tubulin (AC008) were bought from ABclonal (Cambridge, MA, USA). The COX IV antibody was bought from Cell Signaling Technology (4844, Beverly, MA, USA), and the secondary antibody was goat-anti-rabbit IgG (AS014, ABclonal, Cambridge, MA,

USA).

2.7. Caspase activity analysis of hemocytes samples

The caspase-3/8/9 activities of hemocytes samples were detected using caspase-3/8/9 activity assay kits (Beyotime, Shanghai, China) according to the manufacturer's instructions.

2.8. ATP measurement

The amount of ATP was measured by the luciferin-luciferase method following the protocols of ATP detection kit (Beyotime, Shanghai, China). Twenty milligram tissue samples were ground with 200 μ L lysis buffer and centrifuged at $12,000 \times g$, 4 $^{\circ}\text{C}$ for 5 min to collect the supernatants. The luminescence of 100 μ L supernatant sample was assayed in a SYNERGY H1 microplate reader (BioTek, Winooski, VT) together with 100 μ L ATP detection buffer. The standard curve of ATP concentration was prepared from a known serial concentrations (1 nM to 1 μ M). The purified mitochondria were ground in the lysis buffer, and the ATP content as well as protein concentration were determined as described above.

2.9. Statistical analysis

Results from three technical replicates were expressed as means $\pm$ SD (N=3) and analyzed by Statistical Package for Social Sciences (SPSS) 16.0. The significant differences among groups were tested by one-way analysis of variance (ANOVA) and multiple comparisons and the assumptions of ANOVA (normality and homogeneity of variances) were met. The relationship was analyzed by Pearson test. Differences were considered significant at $p < 0.05$.

3 Results

3.1. Apoptosis ratios of hemocytes

The mean apoptosis ratio in CO₂ exposure group was $34.6 \pm 0.6\%$ (28.3% apoptosis at early stage and 6.3% apoptosis/necrosis at late stage), which was significantly higher ($p < 0.05$) than that in control group with ratio of $23.7 \pm 1.2\%$ (19.8% apoptosis at early stage and 3.9% apoptosis/necrosis at late stage) (Fig. 1). Such increased apoptosis ratio was significantly inhibited ($p < 0.05$) by the specific inhibitor of sAC, KH7. The apoptosis ratio of the group injected with 50 $\mu\text{mol/kg}$ KH7 was $12.3 \pm 1.6\%$ (9.1% apoptosis at early stage and 3.2% apoptosis/necrosis at late stage), which was much lower than that in the CO₂ exposure group and even lower than that in the control group. Although the ratio of the group injected with 5 $\mu\text{mol/kg}$ KH7 ($22.2 \pm 0.8\%$) was higher than that in the group injected with 50 $\mu\text{mol/kg}$ KH7, it was still lower than that in the CO₂ exposure group and the control group. For oysters received an injection with DMSO, the apoptosis ratio was $31.6 \pm 2.2\%$ (21.5% apoptosis at early stage and 10.1% apoptosis/necrosis at late stage) which was not significantly different from that in the CO₂ exposure group.

3.2. Immunohistochemistry of CgsAC and mitochondria in hemocytes

Immunohistochemistry of CgsAC and mitochondria in hemocytes was observed by fluorescence confocal (Fig. 2). CgsAC was labeled by polyclonal antibody of CgsAC and Alexa Fluor 488-labeled goat-anti-mouse antibody, which was observed in green. The positive immunoreactivity of CgsAC (green signal) was observed in both nuclei and cytoplasm and distributed separately in the control group. After CO₂

exposure, the immunoreactivity of CgsAC was also found in nuclei and cytoplasm, but most of them were clustered together rather than dispersed from each other (Fig. 2). Mitochondria was labeled by polyclonal antibody of COX IV and Alexa Fluor 594-labeled goat-anti-rabbit antibody, which was observed in red. In the control group, the signal of mitochondria distributed separately in the cytoplasm and did not overlap with the signal of CgsAC. After CO₂ exposure, the mitochondria seemed to be more concentrated and clustered with CgsAC.

3.3. Cleavage of Cgcaspase-3 after CO₂ exposure and CgsAC inhibition

The western blotting of Cgcaspase-3 was conducted after the treatments of CO₂ exposure and CgsAC inhibition and tissues from three oysters were pooled as one sample (N=1) (Fig. 3). The result showed that a proenzyme form of Cgcaspase-3 with the molecular weight of 40 kD was revealed in all the groups including the control group, CO₂ exposure group, DMSO injection group and KH7 injection group. The cleaved forms of Cgcaspase-3, two subunits of active endogenous Cgcaspase-3 with the molecular weight of about 17 kD, were revealed in the control group, CO₂-exposure group, DMSO injection group and 5 µmol/kg KH7 injection group. But the cleaved forms of Cgcaspase-3 was not detected in the 50 µmol/kg KH7 injection group.

3.4. Caspase-3/8/9 activity of hemocytes after the treatments of CO₂ exposure and CgsAC inhibition

The homogenates of hemocyte were incubated with the caspase substrates for human caspase-3 (Ac-DEVD-pNA, acetyl-Asp-Glu-Val-Asp p-nitroanilide),

caspase-8 (Ac-IETD-pNA, acetyl-Ile-Glu-Thr-Asp-nitroanilide) and caspase-9 (Ac-LEHD-pNA, acetyl-Leu-Glu-His-Asp p-nitroanilide) to determine the caspase-3/8/9 activities. It was 27.2 U/mg protein for caspase-3 (Fig. 4 A) in the CO₂ exposure group, which was higher than that in the control group (22.6 U/mg protein) ($p < 0.05$). The caspase-3 activity in the 50 μ mol/kg KH7 injection group (19.0 U/mg protein) decreased significantly relative to that in the CO₂ exposure group ($p < 0.05$), and the treatment with 5 μ mol/kg KH7 also reduced the caspase-3 activity. The caspase-9 activity in the CO₂ exposure group (9.99 U/mg protein) also increased significantly ($p < 0.05$) relative to that in the control group (7.62 U/mg protein). The caspase-9 activity in the 50 μ mol/kg KH7 injection group decreased to 6.13 U/mg protein which was lower ($p < 0.05$) than that in the CO₂ exposure group. In the 5 μ mol/kg KH7 injection group, the caspase-9 activity decreased to 9.27 U/mg protein (Fig. 4 B). The changes of caspase-9 activity (Fig. 4 B) in different groups were similar to that of caspase-3, and there were positive relationships between caspase-3 and caspase-9 activity ($r = 0.980$, $p < 0.05$). However, the caspase-8 activity in CO₂ exposure group and KH7 injection group did not change significantly compared with that in the control group (Fig. 4 C).

3.5. The distribution of CgsAC in the mitochondria and gill

The distributions of CgsAC, COX IV, and β -tubulin in mitochondria and tissues of gill were determined by western blotting (Fig. 5). There were two CgsAC bands of 126 kD band and 45 kD revealed in the gill samples, while only one band of 45 kD identified in the purified mitochondria. A β -tubulin band of 55 kD was only detected

in the tissue samples but not in the mitochondria samples (Fig. 5). For the COX IV, a band of 20 kD was detected in both the tissue samples and the mitochondria samples.

3.6. ATP content in the gill tissue and mitochondria samples

The contents of ATP was 3.15 $\mu\text{mol/g}$ wet tissue in the gill of the CO₂ exposure group, which was 1.88-fold higher ($p < 0.05$) than that in the control group (1.68 $\mu\text{mol/g}$ wet tissue) (Fig. 6 A). After treatment with 50 $\mu\text{mol/kg}$ KH7, the ATP content was reduced significantly from 3.15 $\mu\text{mol/g}$ wet tissue to 1.94 $\mu\text{mol/g}$ wet tissue, while the injection of 5 $\mu\text{mol/kg}$ KH7 and DMSO did not change ATP content significantly in gill.

The ATP content in the mitochondria purified from fresh gill using mitochondria purification kit also changed significantly after CO₂ exposure and treatment with KH7 (Fig. 6 B), which was negatively correlated to the ATP changes in gill tissues. It decreased significantly ($p < 0.05$) from 35.60 nmol/mg protein to 8.80 nmol/mg protein (decreased by 75%) after CO₂ exposure. The treatment with 50 $\mu\text{mol/kg}$ KH7 significantly increased the ATP content in gill mitochondria sample (40.05 nmol/mg protein) compared to that in the CO₂ exposure group (8.80 nmol/mg protein) ($p < 0.05$). There was no significant effect of 5 $\mu\text{mol/kg}$ KH7 and DMSO on ATP content in gill mitochondria.

4 Discussion

Extensive evidences have demonstrated the impacts of OA on the physiological and biochemical processes of mollusk, such as metabolism, acid-base status, immune

response, calcification and protein synthesis [35, 38]. Recently, OA was reported to increase the apoptosis ratio and reactive oxygen species (ROS) production in hemocytes of oyster [11]. Furthermore, OA could also disturb the energy metabolism which thereupon affected the immune competence of bivalves [5, 23]. The information and underlying mechanisms are necessary to further illustrate the long term adaptation of bivalves. Soluble adenylyl cyclase is a conserved bicarbonate sensor and the source of cAMP, which is involved in the regulation of a series of downstream pathways. CgsAC has been identified in oyster, and it could regulate the physiological status of oyster under CO₂ exposure, such as intracellular pH, ROS, phagocytic rate [36], but its roles in regulating apoptosis and ATP metabolism remain unclear.

In the present study, the apoptosis ratio was observed to increase after CO₂ exposure for 16 days, indicating that apoptosis pathway could be activated after CO₂ stress, and apoptosis might be an adaptive response of oyster to CO₂ induced acid stress. In order to understand the role of CgsAC in apoptosis induced by CO₂, the apoptosis ratio was measured after the treatment with KH7, a specific sAC inhibitor, during the CO₂ exposure. The increased apoptosis ratio induced by elevated CO₂ was significantly inhibited ($p < 0.05$) by KH7, suggesting a key role of CgsAC in the CO₂ stress-induced apoptosis of hemocytes. After CO₂ exposure, the signals of CgsAC and mitochondria became more concentrated and were clustered in the hemocytes. Previously, sAC participated in the mitochondrial pathway of apoptosis induced by oxysterol treatment in human vascular smooth muscle cells and mediated

mitochondrial apoptosis of adult rat cardiomyocytes under simulated ischaemia/reperfusion [33, 34]. In these studies, mitochondrial translocation of sAC was found under anoxic stress which would then lead to a selective activation of protein kinase A (PKA) localized within mitochondrial microdomains. Therefore, the overlap of the signal of CgsAC and mitochondria suggested that CgsAC might enter into the mitochondria after CO₂ exposure and the mechanism of CO₂ effects in apoptosis was proposed to be the mitochondrial apoptosis pathway.

Caspases are a group of cysteine proteases playing crucial roles in apoptosis. They are sub-classified into initiator caspases (caspase-2, 8, 9, 10) and executioner caspases (caspase-3, 6, 7) based on their known roles in apoptosis [37]. The executory step of all the apoptosis pathways is initiated by the cleavage of caspase-3 [15]. Pro-caspase-3 can be activated by upstream caspase-8 [39] and caspase-9 [40], and then mediates death receptor pathway and mitochondrial pathway, respectively. According to previous study [15], these components of the apoptotic pathways are conserved throughout evolution including mollusks. Therefore, the cleavage of caspase-3 and the activity of caspase 3/8/9 were detected. The result showed that caspase-3 is activated by CO₂ exposure and thus likely to be involved in the observed increase of apoptosis. After CO₂ exposure treatment, pro-caspase-3 in oyster was cleaved into two small fragments as previous report [37], and such cleavage was inhibited by KH7 in a concentration-responsive manner (N=1). Caspase-9 is the key protease in the mitochondria-induced apoptosis pathway [18], while Caspase-8 is the key element in the death-receptor pathway [18]. The activation of caspase-8 or

caspase-9 could further trigger the downstream executioner caspase-3 and induce cell apoptosis [15, 16]. In the present study, the caspase-8 activity in the CO₂ exposure group and KH7 injection group did not change significantly compared to that in control group. It seems that the effect of the pH and KH7 on the activation of the apoptotic pathway is not dependent of the death receptors. The positive correlation between caspase-3 and caspase-9 activation ($r = 0.980$, $p < 0.05$) indicated that CO₂ exposure might induce apoptosis via the mitochondrial pathway. These results were in agreement with the previous report about the apoptosis in hepatopancreas of crab induced by Cd exposure [18]. In oyster, the increased activities of caspase-3 and caspase-9 was inhibited by KH7, suggesting that the mitochondrial pathway of apoptosis induced by CO₂ exposure could be regulated by CgsAC. During exposure to elevated CO₂, the active transport of H⁺ and HCO₃⁻ would be stimulated and intracellular HCO₃⁻ ions would increase [35], which could lead to the activation of CgsAC. Then downstream PKA might be activated and the CgsAC mediated mitochondrial apoptosis pathway would be stimulated to maintain internal homeostasis. This regulation mechanism should be one of the living strategies of oyster in such complex environment.

A growing number of evidences have been documented about the effects of elevated CO₂ exposure on the energy metabolism of marine and estuarine mollusks [5, 22, 23, 41]. In the present study, the ATP content in the gill tissue increased significantly ($p < 0.05$) after CO₂ exposure. Similarly, tissue ATP production in *Sabellia spallanzanii* also increased significantly after it was exposed to elevated CO₂

conditions for 5 days [42]. In sea urchin, ion transport accounted for 11% to 30% of the metabolic rate under normal condition, while this ratio increased to a range of 20-46% under acidification [20]. In most bivalve mollusks, there was an increase in standard metabolic rate following exposure to elevated CO₂, which would increase the ATP levels [35]. Therefore, the increase of ATP in gill tissue demonstrated that the ATP production was accelerated in oyster to cover the increased energy expenditure of ion transport after 16 days' CO₂ exposure. ATP is primarily produced by the energy machinery (mitochondria) and then it could be rapidly redistributed according to the energy charge [43, 44]. In the present study, the ATP content in the purified mitochondria sample decreased significantly ($p < 0.01$) after CO₂ exposure, indicating that the increasing ATP demand of ion-exchange in the cytoplasm after CO₂ exposure accelerated the transport of ATP from the mitochondria to the cytoplasm. Collectively, the present results suggested that the gill tissue of oyster could stabilize energy charge by adjusting the ATP synthesis and allocating after CO₂ exposure. As a result, more ATP produced inside the mitochondria would be transported to the cytoplasm to meet the demand of increasing ion transport.

In the present study, one CgsAC isoform of 45 kD was confirmed to exist in mitochondria of oyster gill, which was suspected to be the truncated form of the 126-kD band when it entered into the mitochondria according to our previous results [36]. The similar result has also been reported that sAC was truncated in the mitochondria of mouse liver (approximately 48 kD) [32]. In previous study, elevated CO₂ was demonstrated to promote the accumulation of HCO₃⁻ ions in marine bivalves

[35] and the elevation of bicarbonate could activate CgsAC and promote its truncation to increase cAMP synthesis in oyster gill tissue [36]. Moreover, sAC-dependent cAMP elevation in mitochondria promoted ATP production in mouse liver [45]. In the present study, the increase of ATP content in gill tissues and decrease in gill mitochondria caused by CO₂ exposure could be both significantly inhibited by KH7, suggesting that CgsAC might not only play essential roles in promoting ATP synthesis or oxidative phosphorylation [32], but also in ATP translocation from mitochondria to cytoplasm. It indicated that the mitochondria-CgsAC signaling pathway might participate in resetting and optimizing the proper energy balance between enhanced ion transport and ATP metabolism.

In summary, elevated CO₂ activated mitochondria-CgsAC pathway of apoptosis and ATP metabolism in oyster. As a bicarbonate regulated enzyme, CgsAC entered mitochondria and regulate apoptosis pathway and energy allocation. During CO₂ exposure, accumulation of HCO₃⁻ ions in oyster activated CgsAC and trigger downstream mitochondria-CgsAC pathway in regulation of apoptosis and ATP metabolism to maintain internal balance in response to CO₂ exposure and adapt to OA and it should be a strategy for oyster to live in such complex environment.

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Figure captions:

Figure 1. Induction of apoptosis in hemocytes of oysters by different treatments. A-E: Representative dot plots for Annexin V-Alexa Fluor 488/Propidium Iodide (PI) staining in hemocytes of the control group (pH=8.10) (A), CO₂ exposure group (pH=7.50) (B), DMSO injection group (pH=7.50) (C), 50 μ mol/kg KH7 group (pH=7.50) (D) and 5 μ mol/kg KH7 group (pH=7.50) (E). F: Quantitative graph of the data shown in A-E, indicating the changes of the proportion of early apoptotic and late apoptotic cells in hemocytes of different treatments. The results shown in F are means $\pm$ SD from three technical replicates. Bars with lower-case letters indicated significantly different ($p < 0.05$).

Figure 2. Immunohistochemistry of CgsAC and mitochondria in oyster hemocytes of the control group (pH=8.10) and CO₂ exposure group (pH=7.50). The nucleus of hemocytes was stained with DAPI (blue), immunoreactive signal of CgsAC was visualized by Alexa 488-labeled secondary antibody (green), immunoreactive signal of COX IV was visualized by Alexa 594-labeled secondary antibody (red) and the overlap of CgsAC with COX IV signal was visualized with yellow.

Figure 3. Western blotting of Cgcaspase-3 and β -tubulin in hemocytes of oysters under different treatments. 1: the control group (pH=8.10), 2: CO₂ exposure group

(pH=7.50), 3: DMSO injection group (pH=7.50), 4: 50 $\mu\text{mol/kg}$ KH7 group (pH=7.50), 5: 5 $\mu\text{mol/kg}$ KH7 group (pH=7.50).

Figure 4. Effect of different treatments on hemocytes caspase-3/8/9 activity. A: caspase-3 activity; B: caspase-8 activity; C caspase-9 activity. The results shown in A, B, and C are means $\pm$ SD from three technical replicates. Bars with lower-case letters indicated significantly different ($p < 0.05$).

Figure 5. Western blotting of CgsAC, β -tubulin and COX IV in gill tissue and mitochondria samples. 1: gill tissue sample, 2: gill mitochondria sample.

Figure 6. ATP content in gill tissue sample (A) and gill mitochondria sample (B). The samples were from oysters under different treatments: the control group (pH=8.10), CO₂ exposure group (pH=7.50), DMSO injection group (pH=7.50), 50 $\mu\text{mol/kg}$ KH7 group (pH=7.50) and 5 $\mu\text{mol/kg}$ KH7 group (pH=7.50). ATP values in the tissue are $\mu\text{moles ATP per gram tissue}$. ATP values in the mitochondria are $\text{nmoles ATP per milligram mitochondrial protein}$. The results shown in A and B are means $\pm$ SD from three technical replicates. Bars with * indicated significantly different ($p < 0.05$).

Figure 1

Figure 3

Figure 4

A **B** **C**

Figure 5 1 2

126kD-

CgsAC

45kD-

55kD-

20kD-

β -tubulin

COX IV

Figure 6