Palliative effect of abalone insulin-related peptide 2 in pacific abalone (*Haliotis discus hannai*) on the hyperglycemia induced by emersion stress.

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Introduction

Pacific abalone (*Haliotis discus hannai*), widely distributed along East Asia's coast, is the main species for abalone aquaculture in Korea and is considered a highly valuable seafood. Since the early 2000s, farmed abalone production in Korea has exponentially increased, growing from 20 tons in 2000 to 22,078 tons in 2022 due to the widespread use of the sea-based net cage culture system (KOSIS, 2022). Despite this remarkable progress, repeated cultivation of abalone in limited areas has become a serious issue in the Korean aquaculture. Moreover, physiological damage resulting from high water temperatures during summer and air exposure occurring while handling the abalones during the sorting, selection, and transportation processes is also considered a major issue that decreases efficiency and productivity in the abalone aquaculture. Among the various physiological roles reported in the insulin superfamily in both vertebrates and invertebrates, the most prominent role is recognized as a key regulator in maintaining plasma glucose concentrations. In general, when vertebrates are exposed to stressful conditions, they secrete glucose from their liver into the bloodstream, i.e., stress-induced hyperglycemia. This acute elevation of plasma glucose can cause hyperglycemia-induced oxidative stress in mitochondria, leading to dysfunction in a broad range of biological processes. In response to elevated blood glucose levels, insulin secretion is triggered to normalize glucose concentrations by promoting glycogen synthesis and glucose uptake, which helps alleviate acute oxidative stress due to physiological stress. Although some IRPs and insulin-like growth factor binding proteins (IGFBPs) have been found in the Pacific abalone, their specific role remains unclear. This study aimed to understand the regulatory mechanism of elevated hemolymph glucose (hyperglycemia) following environmental stress (high temperature and air exposure) and the specific role of IRPs in the alleviation of cell damage by maintaining hemolymph glucose levels during stress-induced hyperglycemic states.

Results & Discussion

1. Changes in hemolymph glucose levels during recovery after high water temperature and emersion stress

Hemolymph glucose levels rapidly increased to 59.11 ± 13.17 µg/mL (2.5-fold, 0 h) immediately after exposure to emersion stress from pre-treatment (−1 h) levels of 23.5 ± 2.26 µg/mL (Fig. 1A), which kept in a recovery tank throughout experiment (Fig. 1A). This elevated hemolymph glucose due to emersion stress gradually decreased to 32.58 ± 5.88 µg/mL during the 24-hours recovery period. Animals exposed to heat stress in 30 °C seawater for 1 h showed gradual changes in hemolymph glucose, and an approximately 2-fold increase peak at 32.12 ± 7.58 µg/mL was observed after 12 h of recovery compared to the pre-treatment levels of 15.45 ± 0.88 µg/mL (Fig. 1B).

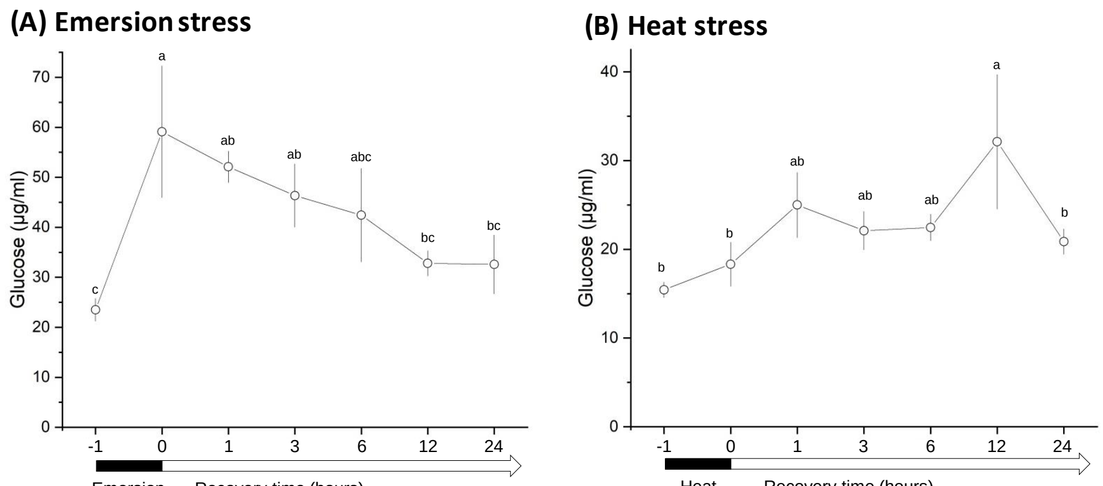


Fig. 1. Changes in hemolymph glucose levels in Pacific abalone (without injection) during recovery after 1-h emersion (A; exposure to air) and heat stress (B; 30 °C) treatments(mean ± S.E., $n = 6$, $p < 0.05$).

2. Effect of bovine insulin injection on hemolymph glucose clearance during recovery after hyperglycemia induced by emersion and heat stress

To investigate the presence of glucose regulatory mechanisms in abalone, changes in hemolymph glucose levels after bovine insulin and MS injection were measured during recovery following 1 h of exposure to emersion and high water temperature. In the experiment with emersion stress, pre-treatment groups kept in seawater (acclimation tank or recovery tank) showed a basal level of glucose (19.96 ± 0.59 µg/mL, Fig. 2A). After 1 h of emersion stress, bovine insulin and MS were injected into the ventral hemolymph vessel of abalones, and then immediately transferred into normal seawater for recovery (recovery tank). The highest levels of hemolymph glucose after bovine insulin (52.62 ± 1.6 µg/mL) and MS (50.61 ± 3.68 µg/mL) injections were observed immediately after 1 h of emersion (hour 0 in Fig 2A). A gradual decrease in glucose levels was observed after 3 h of recovery, with no significant difference between the two groups. From hours 6 to 24, hemolymph glucose levels decreased, with the bovine insulin-injected group showing a more rapid clearance of hemolymph glucose than the MS-injected group ($p < 0.05$). After 24 h of recovery, the final hemolymph glucose levels of the bovine insulin- and molluscan saline-injected group were 15.57 ± 2.78 µg/mL and 24.19 ± 2.66 µg/mL, respectively. In the heat stress experiment, significant differences in hemolymph glucose were seen between the bovine insulin- and MS-injected groups at 12 h of recovery time, with 25.54 ± 1.23 µg/mL and 33.01 ± 1.23 µg/mL, respectively (Fig. 2B, $p < 0.05$). After 24 h of recovery, the final values of glucose returned to near the basal level, falling to 16.43 ± 1.87 µg/ml in the bovine insulin-injected group and 20.11 ± 2.0 µg/ml in the MS-injected group.

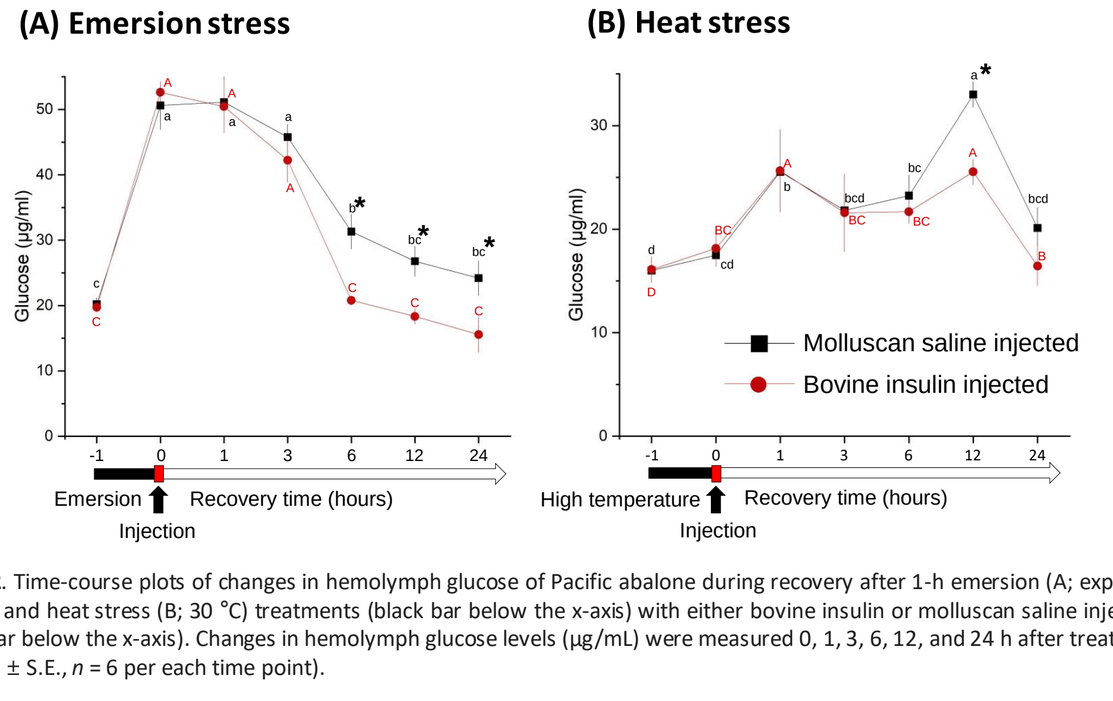


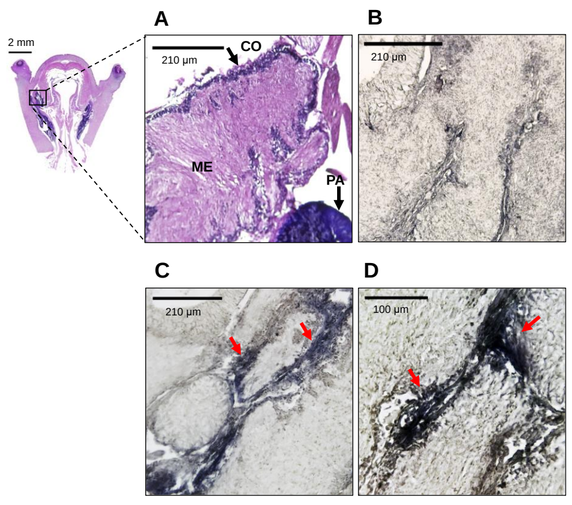
Fig. 2. Time-course plots of changes in hemolymph glucose of Pacific abalone during recovery after 1-h emersion (A; exposure to air) and heat stress (B; 30 °C) treatments (black bar below the x-axis) with either bovine insulin or molluscan saline injection (red bar below the x-axis). Changes in hemolymph glucose levels (µg/mL) were measured 0, 1, 3, 6, 12, and 24 h after treatment (mean ± S.E., $n = 6$ per each time point).

3. Changes in three AIP gene expression during recovery after emersion and heat stress

In order to explore the functions of AIPs following hyperglycemia, relative expression of AIP2 in the cerebral ganglion and hepatopancreas were examined during recovery after 1 h of exposure to emersion and high water temperature treatments. After emersion stress, the relative expressions of AIP2 in cerebral ganglia and hepatopancreases were significantly downregulated from 0 to 1 h after recovery ($p < 0.05$, Fig. 3A). From 3 to 12 h, both AIP2 expression in cerebral ganglia gradually increased, and the highest expressions were detected at 12 h after recovery, reaching 1.71-fold that of pre-treatment levels (−1 h). The relative expression of AIP2 in the hepatopancreas maintained at a relatively constant expression level throughout the recovery period (Fig. 3A). Abalones exposed to heat stress also showed the highest expression level of AIP 2 in cerebral ganglia, with 2.97-fold increases, after 12 h of recovery ($p < 0.05$, Fig. 3B). AIP2 was upregulated at 1 h, with 2.4-fold of that in the pre-treatment group (Fig. 3B). By comparing the results from the time-course glucose concentration and AIP gene expression experiments, the overall tendency of AIP2 expression, in particular, seemed closely correlated with hemolymph glucose regulation following stress-induced hyperglycemia.

Fig. 3. Time-course plots of the relative of abalone insulin-related peptide 2 (AIP2) in the cerebral ganglion and hepatopancreas during recovery following 1-h emersion (expression levels A; exposure to air) and high-temperature (B; 30 °C) treatments (black bar below the x-axis).

4. Localization of AIP2 mRNA in the cerebral ganglion



To determine AIP2 cellular localization, DIG-labeled antisense and sense AIP2 probes were synthesized and hybridized using cerebral ganglion tissue together with conventional H&E staining (Fig. 4). The neural cortex (CO), medulla (ME), and posterior adductor (PA) were visualized by H&E staining. Comparing the H&E staining and positive hybridization signal from the antisense probe, it was noticed that AIP2 mRNA expression in the cerebral ganglion was mostly detected in cells of the neural cortex (McElwain and Bullard, 2014).

Fig. 4. In situ hybridization of AIP2 mRNA in the cerebral ganglia of Pacific abalone. (A) Histological transverse section of the cerebral ganglia stained with Mayer's H&E showing the neural cortex (CO), medulla (ME), and posterior adductor (PA). (B) In situ hybridization results showing the signal from AIP2 sense probes used as a negative control. (C and D) In situ hybridization of cerebral ganglia sections labeled with AIP2 antisense probes at 100× © and 200× (D) magnifications. Red arrows indicate positive hybridization signals from AIP2 antisense probes detected in the CO.

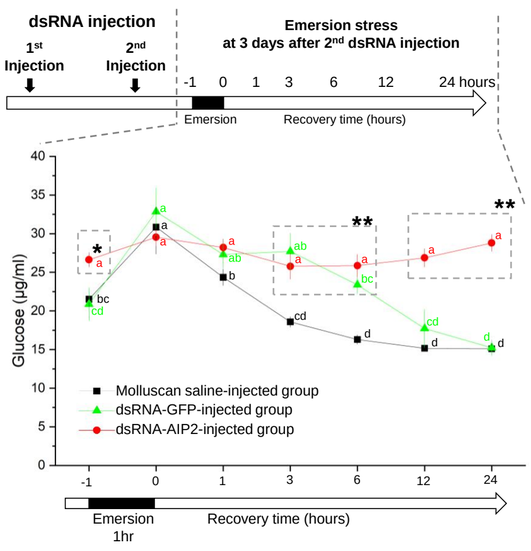
5. Interfering effect of AIP2 gene expression on hemolymph glucose

As a preliminary RNA interference experiment, the knockdown efficiency of dsRNA-AIP2 injection was tested. Three treatment groups were administered two doses of dsRNA-AIP2, dsRNA-GFP, and MS, respectively (once per day, n = 30 per treatment group). Changes in AIP2 expression levels in the cerebral ganglion, hepatopancreas, and hemolymph glucose collected at 1, 2, and 3 days after the final injection were examined to precisely compare the daily knockdown effect. At 2 and 3 days after dsRNA-AIP2 injection, AIP2 mRNA expression in cerebral ganglia significantly decreased to 55.65% (0.69 ± 0.08) and 49.64% (0.68 ± 0.1), respectively, compared to the relative expression of the MS-injected group (1.28 ± 0.17 and 1.37 ± 0.22) (Fig. 5A, $p < 0.01$). Abalones administered with MS and dsRNA-GFP showed no noticeable difference in AIP2 expression over the 3 days. In the hepatopancreas, the relative AIP2 expression following dsRNA-AIP2 injection fell to 21.37% at day 3 (0.2 ± 0.03) compared to that in the MS-injected group (0.95 ± 0.15) (Fig. 5B, $p < 0.05$). Hemolymph glucose levels in the dsRNA-AIP2-injected group on day 2 (16.4 ± 1.3 µg/mL) and day 3 (18.8 ± 1.1 µg/mL) were significantly higher than those in the MS- and dsRNA-GFP-injected groups (Fig. 5C, $p < 0.05$). This preliminary test revealed that two dsRNA-AIP2 injections, administered daily, induced a reduction in expression levels of AIP2 in the cerebral ganglion and hepatopancreas and increased hemolymph glucose, while injection of MS or dsRNA-GFP produced no noticeable effect in this study.

Fig. 5. RNA interference efficiency of the dsRNA-AIP2 injection treatment. Two doses each of dsRNA-AIP2, dsRNA-GFP, and molluscan saline were injected to examine their AIP2 gene knockdown efficiency. Effects of each injection on the expression levels of AIP2 in the cerebral ganglion (A) and hepatopancreas (B) and on hemolymph glucose levels (C) were investigated by sampling 1, 2, and 3 days after the last injection. Each value represents the mean ± S.E. (n = 8, *, $p < 0.05$ and **, $p < 0.01$). The control group consisted of abalones not receiving an injection.

6. Roles of AIP2 in hyperglycemia induced by emersion stress

With confirmation of the knockdown effect of AIP2 mRNA expression following dsRNA-AIP2 injection, the roles of AIP2 in the glucose regulation response to hyperglycemia induced by emersion stress were investigated after two daily dsRNA injections. Upon the 1-hour emersion treatment, abalones were placed in normal seawater (with the same conditions as the acclimation tank) to recover, and hemolymph samples were collected after 0 (immediately following treatment), 1, 3, 6, 12, and 24 h of recovery to measure glucose.



Prior to treatment (−1 h), the hemolymph glucose level of the dsRNA-AIP2-injected group was significantly higher than those of dsRNA-GFP- and MS-injected groups, as predicted by the knockdown effect on AIP2 mRNA expression found in the preliminary experiment (Fig. 6, $p < 0.05$). After emersion stress, hemolymph glucose levels in all three injection groups increased to nearly 30 µg/mL, and then the elevated glucose levels in MS- and dsRNA-GFP-injected groups returned to the basal level after 24 h of recovery time. However, even after 24 h, abalones receiving dsRNA-AIP2 still maintained a significantly high glucose level of 28.79 ± 1.10 µg/mL, approximately 1.9-fold of those in the two other groups ($p < 0.01$).

Fig. 6. Effect of *AIP2* knockdown on hemolymph glucose during recovery following a 1-h emersion treatment (black bar below the x-axis). Abalones administered with two doses of molluscan saline, dsRNA-GFP, or dsRNA-AIP2 were exposed to air (emersion stress) for 1 h. Hemolymph glucose levels were measured 0, 1, 3, 6, 12, and 24 h after the immersion treatment ended (mean ± S.E., n = 8, * $p < 0.05$ and ** $p < 0.01$).

Conclusion

In this study, we investigated the role of AIP2 on homeostasis in hyperglycemia following an acute stress response. Hyperglycemia was induced in abalone through exposure to stress via high water temperature and air (emersion). This stress-induced hyperglycemia was recovered by a bovine insulin injection, supporting the presence of a glucose regulatory system akin to vertebrates. Among the three insulin-related peptides studied, AIP2 mRNA expression in the cerebral ganglion correlated closely with high water temperature and emersion stress. Localization of AIP2 mRNA-expressing cells in the cerebral ganglion was characterized using in situ hybridization, revealing positive signals in neural cortex cells. Suppression of AIP2 mRNA expression through RNA interference resulted in the abalone maintaining high hemolymph glucose levels after recovery from emersion stress. These findings suggest that AIP2 affects the recovery rate from emersion stress-induced hyperglycemia by regulating hemolymph glucose.

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