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Gene Expression of Early Growth Response Protein 1 in INS-1 Pancreatic β-cells Treated with *Allomyrina dichotoma* Hemolymph

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We have investigated the expression of early growth response protein 1 (EGR1) in INS-1 pancreatic β-cells treated with *Allomyrina dichotoma* hemolymph. The Korean rhinoceros beetle, *A. dichotoma* (Coleoptera: Scarabaeidae), is important in the insect industry for medical applications. We have already established a method for purification of *A. dichotoma* hemolymph that can be used in many experiments. EGR1 is reported as a multifunctional transcription factor that is implicated in virus infections. EGR1 has therefore been revealed as a major mediator and regulator in the physiological and pathological conditions of several cell and tissue types. New findings in this study are that *A. dichotoma* hemolymph, which promotes a dose- and time-dependent upregulation of EGR1 gene expression, shows an enhancement of this gene expression when combined with hypothermia or endoplasmic reticulum (ER) stress. These results suggest that *A. dichotoma* hemolymph may provide clues to EGR1-associated disease therapies involving gene regulation of EGR1.

Key words : *Allomyrina dichotoma,* early growth response protein 1 (EGR1), hemolymph, INS-1 pancreatic β-cells

Introduction

In insects, hemolymph is a circulation fluid within the body cavity, functionally analogous to the blood and lymph in vertebrates [12]. It comprises certain kinds of hemocytes and many proteins including hormones and small peptides, providing various biologically crucial properties such as protection against invading microorganisms, wound therapy, regulation of developmental timing, metamorphosis, metabolism, growth, reproduction, and determination of behavior patterns [10, 11]. There is a growing interest, especially among pharmaceutical companies, in using insect hemolymph with various functional substances in the development of new drugs to improve human health and possibly the health of other mammals [21]. The Korean rhinoceros beetles (*Allomyrina dichotoma*) used in this study are regarded as a traditionally medicine for liver-related diseases in Korea, and is one of the most economically significant insect species [5, 13]. We have recently established the collection of mass hemolymph without contamination and melanization for the development of new drugs [12, 15].

Early growth response protein 1 (EGR1) is one of the mammalian transcription factors, which also known as NGFI-A, Krox-24, TIS8, and Zif268. EGR1 has the common regions of three cysteine2-histidine2 (C_2H_2) zinc fingers DNA-binding domains [1, 8]. EGR1 shows essential functions for several physiological processes including synaptic plasticity, wound repair, inflammation, and differentiation [3, 17]. Disorders for the EGR1 gene are involved in various diseases such as acute myeloid leukemia, chronic myelogenous leukemia, chronic lymphocytic leukemia, multiple myeloma, and B cell lymphoma (https://www.omim.org/entry/128990?search=EGR1&highlight=egr1) [9]. Here, we have tested the upregulation of EGR-1 gene in *A. dichotoma* hemolymph-treated INS-1 pancreatic β-cells.

Materials and Methods

Hemolymph used in this experiment was extracted from a healthy third-instar larva of *A. dichotoma*. The collected hemolymph was incubated for five minutes with 5 ml of thrombin to 500 ml of hemolymph at room temperature. To remove the insoluble matters including several kinds of

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blood cells, the hemolymph was centrifuged for five minutes at 11,000 × g at 4℃. After filtering through 0.22 mm syringe filter, the supernatant was divided and stored at -70℃ until the next experiment. The concentration of final protein was 2.5 mg/ml measured by Bio-Rad protein assay kit. INS-1 pancreatic β-cells were cultured in RPMI-1640, supplemented with 10% fetal calf serum (Gibco BRL, Gaithersburg, MD), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM/ml L-glutamine, 10 mM/ml HEPES, 1 mM/ml sodium pyruvate, and 50 mM/ml 2-mercaptoethanol, in a humidified atmosphere (5% CO_2, 37℃). After 5 days of culture, cells were used for the various experiments. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The estimation of total RNA quality was by Agilent's 2,100 Bioanalyzer System (Santa Clara, CA, USA). Analysis of identifies genes that are differentially expressed was by Agilent's Gene Expression Hybridization Kit, Scan and image analysis was by Agilent's DNA microarray scanner and DNA analysis was by Agilent's GeneSpring Software. All processes were done according to the manufacturer's instructions. Total RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA). The mRNA in the samples was reverse-transcribed using a SuperscriptII ™ First Strand Kit (Invitrogen Carlsbad, CA, USA). The resulting cDNA was amplified by reverse transcription polymerase chain reaction (RT-PCR) using the primer pairs mouse actin F (5′-GAAATCCACCAAAGCTCAC-3′) and R (5′-TCTCGGTCAAGTTCAACATC-3′) and EGR1 F (5′-AAC ACTTTGTGGCCTGAACC-3′) and EGR1 R (5′-AGGTCTC CCTGTTGTTGG-3′). RT-PCR conditions for 30 cycles were: 95℃ for 30 seconds; 56℃ for 30 seconds; and 72℃ for 2 minutes (10 minutes in the final cycle), using both primers mentioned above with Taq DNA polymerase.

Results and Discussion

In this study, we have first tested the DNA chip experiment to determine what kinds of genes are differentially regulated by *A. dichotoma* hemolymph in INS-1 pancreatic β-cells. Everything used in the microarray analysis was Agilent company's system described above. As shown in Fig. 1A, the gene expression of EGR-1 is approximately five folds up-regulated on the transcriptional level in the cDNA microarray experiment. In the same condition, treatment of 5% hemolymph for two hr, EGR1 gene expression was confirmed by RT-PCR, also the accolated resulting of EGR1 gene expression is shown in Fig. 1B. And then we chose EGR1 as a target gene of the following experiments. As a next step, we tested the effect of dialysis on the regulation of EGR1 gene expression by hemolymph. One dialyzed-hemolymph (5% hemolymph for 2 hr) enhanced more upregulation of EGR1 gene expression than raw hemolymph (Fig. 1C). Especially, when the treatment times of 1% and 5% hemolymph were treated for 2 hr and 4 hr, respectively, dialyzed hemolymph that increased the expression of EGR1 gene (Fig. 1D). We suggesting that adjust the acid-base of hemolymph to be suitable for culture cells by dialysis. Dialyzed-hemolymph was used for the following experiments.

Gene expression of EGR1 was dose-dependently upregulated on the *A. dichotoma* hemolymph for 2 hr exposure. EGR1 gene expression showed higher expression than its control from the addition of 3% hemolymph (Fig. 2A). Conversely, the treatment time of 5% *A. dichotoma* hemolymph was observed to enhance EGR1 gene expression from 0.5 hr (Fig. 2B). The results of Fig. 2A and Fig. 2B indicate that gene expression of ERG1 was upregulated in dose- and time-dependent of hemolymph treatment. It is recently recognized that hypothermia is effective therapy for clinical treatment. To understand the possibility of hypothermia treatment through the regulation of EGR1 gene expression, INS-1 pancreatic β-cells were incubated at 32℃ for one, two, and four hours with 5% *A. dichotoma* hemolymph. As shown in Fig. 2C, remarkable upregulation of EGR1 gene expression by hypothermia treatment was observed through the all treatment time. Thus, the effect of hypothermia was strongly suggested to be mainly associated with the regulation of EGR1 gene expression, which may provide a clue to the development of EGR1 gene associated disease therapies. It was reported that induction of EGR1 expression is stimulated by endoplasmic reticulum (ER) stress through extracellular regulated kinase arm of the mitogen-activated protein kinase pathways [19]. Another interesting result is that EGR1 improves the insulin/ Akt signal, protecting pancreatic β-cells from ER stress and apoptosis [4]. We have studied the regulation of EGR1 gene expression by ER stress using ER stress inducible drug of calcium ionophore A23187. The result is that EGR1 gene expression is only upregulated by relatively weak (1 mg/ml) ER stress rather than strong (5 mg/ml) (Fig. 2D). In many recent studies, natural products have shown potential in regulating ER stress in different cancer cell lines. Reconceptualization of the molecules which modulates ER stress associated factors will lead to advances in unfolded

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Gene Symbol	Normalized	Flags	HL-treated	Control
Egr1	5.100	P	2403.2947	471.2519
Egr1	3.179	P	471.7508	148.39679
Junb	2.648	P	7889.687	2979.5376
Gpx1	2.310	P	65.972176	28.561157
Smad1	2.137	P	967.5001	452.79898
Dlx5	2.106	P	121.3256	57.604065
F2rl1	2.069	P	30.08854	14.543163
Bmp2	1.983	P	799.6886	403.33124
Met	1.982	P	48.6062	24.520956
Wnt10b	1.959	P	352.2784	179.80377
Epha2	1.909	P	621.91797	325.77115
Sp2	1.907	P	104.7206	54.90733
Irf6	1.902	P	266.9631	140.35484
Ddit4	1.842	P	47.90924	26.008236
Prpf19	1.681	P	941.0245	559.7431
Tspan1	1.598	P	414.4647	259.3653
Fst	1.570	P	33.08904	21.075077
Prkcd	1.551	P	1859.8666	1198.858
Xbp1	1.532	P	4108.665	2682.0364
Hook3	0.665	P	248.8313	374.1081
Zfp503	0.614	P	169.5756	276.04837
RGD1309079	0.614	P	31.73346	51.704956
Gpc3	0.611	P	18.25379	29.887259
Ogfod1	0.588	P	9.892606	16.822424
Mcm10	0.587	P	10.65558	18.164865
Foxc1	0.559	P	97.194435	173.99608
Zfp37	0.557	P	23.85865	42.870396
Myc	0.533	P	142.75356	267.74533
Nkx6-1	0.460	P	580.8571	1261.8254
Tnf	0.456	P	537.8365	1178.7849
Tacc2	0.438	P	16.08163	36.685482
Id4	0.433	P	611.5859	1413.7999
Ihh	0.425	P	408.9994	962.10406
Pou3f2	0.397	P	50.55869	127.30558

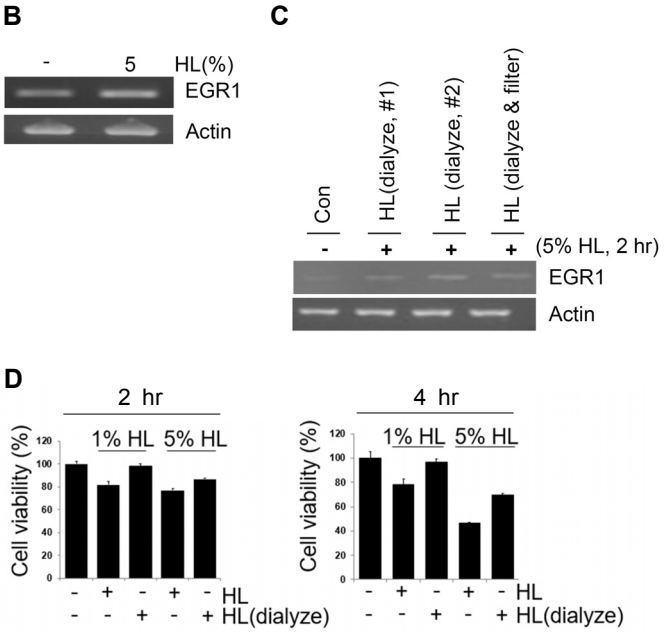

Fig. 1. *A. dichotoma* hemolymph enhances EGR1 gene expression in the INS-1 pancreatic β-cells. (A) The result of the microarray analysis. Typical examples of increasing/decreasing in gene expression are boxed, among them EGR1 was confirmed by the RT-PCR (B). (C and D) Dialysis effect for the EGR1 gene expression. Data were presented as means ± SD from at least three independent experiments.

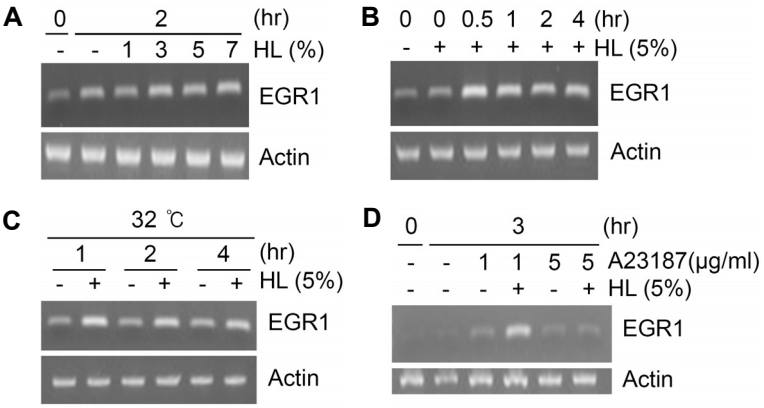

Fig. 2. EGR1 gene expression by different conditions of *A. dichotoma* hemolymph in the INS-1 pancreatic β-cells. (A) Dose dependent effect of *A. dichotoma* hemolymph for EGR1 gene expression. (B) Time dependent effect of *A. dichotoma* hemolymph for EGR1 gene expression. (C) Hypothermia effect on EGR1 gene expression. (B) ER stress effect on EGR1 gene expression.

protein response (UPR) therapy [6, 20]. These results suggest that mild ER stress regulates gene expression of EGR1, which may offer a possibility for the EGR1 associated disease therapies with *A. dichotoma* hemolymph using together.

In some recent results, edible insect extracts have been demonstrated to promote the activity of several factors that positively affect liver disease improvement [2, 10, 14, 16]. Our results also provide an insight to the development of EGR1-related disease therapies through gene expression regulation of EGR1 using *A. dichotoma* hemolymph combined with mild ER stress [4, 7, 18, 19]. *A. dichotoma* hemolymph may be considered one of the natural drugs for the UPR treatment.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 췌장 β-세포에서 *Allomyrina dichotoma* 혈림프 처리에 의한 EGR1유전자 발현

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INS-1 췌장 β 세포에서 *Allomyrina dichotoma* 혈림프 처리에 의한 early growth response protein 1 (EGR1) 유전자 발현을 조사되었다. 이 연구에서 새로운 발견은 EGR1 유전자 발현을 *A. dichotoma* 혈림프의 용량 및 시간 의존적으로 상향 조절하는것과 혈림프와 병행한 저체온효과 또는 소포체(endoplasmic reticulum, ER) 스트레스에 의해서도 유전자 발현이 상승하였다. *A. dichotoma* 혈림프가 EGR1의 유전자발현을 상승 조절을 할 수 있기 때문에, EGR1 관련 질병 치료 및 예방의 실마리를 제공 할 수 있을 가능성을 시사한다.