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Transgenic silkworms expressing human insulin receptors for evaluation of therapeutically active insulin receptor agonists

Yasuhiko Matsumoto [a], Masaki Ishii [a], Kenichi Ishii [a], Wataru Miyaguchi [a], Ryo Horie [a], Yoshinori Inagaki [a], Hiroshi Hamamoto [a], Ken-ichiro Tatematsu [b], Keiro Uchino [b], Toshiki Tamura [b], Hideki Sezutsu [b], Kazuhisa Sekimizu [a,*]

[a] Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 111-0033, Japan
[b] Transgenic Silkworm Research Unit, National Institute of Agrobiological Sciences, 1-2 Owashi, Tsukuba, Ibaraki 305-8634, Japan

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ABSTRACT

We established a transgenic silkworm strain expressing the human insulin receptor (hIR) using the GAL4/UAS system. Administration of human insulin to transgenic silkworms expressing hIR decreased hemolymph sugar levels and facilitated Akt phosphorylation in the fat body. The decrease in hemolymph sugar levels induced by injection of human insulin in the transgenic silkworms expressing hIR was blocked by co-injection of wortmannin, a phosphoinositide 3-kinase inhibitor. Administration of bovine insulin, an hIR ligand, also effectively decreased sugar levels in the transgenic silkworms. These findings indicate that functional hIRs that respond to human insulin were successfully induced in the transgenic silkworms. We propose that the humanized silkworm expressing hIR is useful for *in vivo* evaluation of the therapeutic activities of insulin receptor agonists.

1. Introduction

Understanding the mechanisms of pathogenicity and exacerbation in human disease is important toward establishing prophylaxis and therapy. Although many animal models have been developed for preclinical studies, inconsistencies between humans and model animals are a major stumbling block. To address this issue, humanized mice, i.e., mice expressing human genes, have been constructed and used in many studies [1]. Humanized mice are categorized into three groups, in which either (i) human genes are introduced, (ii) human cells are transplanted, or (iii) human tissues or organs are transplanted [1–5]. Humanized mice expressing human receptor proteins required for the infection of pathogens such as poliovirus have been established [6,7]. Immunodeficient mice transplanted with human immune cells or cancer cells are used for immunologic and cancer therapy studies [1,8]. In recent studies, humanized mice receiving transplantation of human tissues established from human embryonic stem cells or induced pluripotent stem cells were used as animal models to study the exacerbation mechanisms of various diseases and for vaccine development [1,9–11]. The large number of mice needed for drug screening, however, is both costly and associated with ethical problems in terms of animal welfare. Invertebrates, on the other hand, are less costly to rear and house, and are associated with fewer ethical problems compared to mammals; therefore, the development of alternative invertebrate animal models would be highly advantageous.

We previously proposed using silkworms as an invertebrate animal model for screening drug candidates for therapeutic efficacy and toxicity. The silk industry has a long history with established methods for handling a large number of silkworms. Silkworms cost much less and occupy less space than mammals such as mice and rats. Silkworms move slowly and their large body size is suitable for sample injection by syringes. Moreover, drug metabolism is similar between silkworms and mammals. We compared the toxic effects of various chemical compounds between both silkworm and mammalian models, and demonstrated that the LD_{50} values of toxic compounds were similar between models [12]. Furthermore, we previously established various silkworm infection models using bacteria, fungi, and viruses, and reported that the ED_{50} values of medicines were comparable to those in mammals [13–15]. These findings suggest that the pharmacokinetics of various medicines in silkworms is similar to those in mammals, including humans.

Abbreviations: hIR, human insulin receptor; PI3, phosphoinositide 3; UAS, upstream activating sequence.

* Corresponding author.
E-mail address: sekimizu@mol.f.y-tokyo.ac.jp (K. Sekimizu).

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Transgenic techniques to express exogenous genes in silkworms are established [16]. We previously generated a transgenic silkworm line expressing GFP in a tissue-specific manner by applying the GAL4/UAS system [17]. Silkworm strains expressing human genes have been established [18]. These reports provide examples of the use of silkworms as efficient machinery for recombinant protein production. To date, however, the *in vivo* functions of human gene products have not been assessed in live transgenic silkworms. Based on previous studies using silkworms as disease models, we considered the transgenic technique applicable for generating humanized silkworms for *in vivo* evaluation of drugs that target human proteins. In the present study, we constructed humanized silkworms expressing the human insulin receptor (hIR). We previously reported that silkworms fed a glucose-containing diet exhibited hyperglycemia, and the increased sugar levels in hyperglycemic silkworms were decreased by intra-hemolymph injection of human insulin [19]. Furthermore, administration of human insulin stimulated Akt phosphorylation in fat body cells, which was inhibited by wortmannin, a pharmacologic inhibitor of the upstream phosphoinositide 3 (PI3) kinase. These findings indicate that the intracellular insulin-signaling pathway, which is well elucidated in mammals, is conserved in silkworms. In our previous study, the concentration of human insulin required for therapeutic effects in silkworms was 100-fold higher than that used clinically for human patients. This could be explained by the lower affinity of the silkworm insulin receptor for human insulin. We therefore considered that transgenic expression of a human-type insulin receptor in silkworms could decrease the amount of drug required and make the silkworm model more accurately reflect the activity of human insulin. Here, we examined the utility of the transgenic hIR-expressing silkworm as a model animal to evaluate the *in vivo* therapeutic effects of insulin receptor agonists. This is the first report of the use of transgenic silkworms expressing a receptor gene of human origin to evaluate the activities of various therapeutic compounds.

2. Materials and methods

2.1. Insects

The silkworm strain w1-pnd, *actin A3*-GAL4/3×P3-DsRed2 (w1) [20], is maintained at the Transgenic Silkworm Research Unit of National Institute of Agrobiological Sciences. Silkworms were reared with an artificial diet, SilkMate 2S (Nihon Nosan Kogyo, Yokohama, Japan), at 27 °C.

2.2. Chemicals

Recombinant human insulin and recombinant bovine insulin were purchased from Wako and Sigma, respectively, and dissolved in 0.9% NaCl containing 0.1% acetic acid. Wortmannin was purchased from Wako and dissolved in DMSO for stock solution at 10 mM.

2.3. Construction of the hIR expression vector

The full-length cDNA of the hIR cloned in pCR-XL-TOPO was purchased as a Funakoshi IMAGE clone from Open Biosystems (clone ID: 40125723). The ORF region was amplified by PCR using a primer set with a cleavage site for *Xba* I (Supplementary Table 1). This PCR product was ligated into a pZErO-2 vector (Invitrogen), and transformed into a competent *Escherichia coli* strain. Plasmid pZErO-2-hIR was extracted from a drug-resistant colony after overnight incubation at 37 °C and the DNA size of the insert was verified by electrophoresis. The DNA sequence of the *hIR* gene in the plasmid was further confirmed using primers, as shown in Supplementary Table 1.

Construction of the pBacMCS[UAS-SV40, 3×P3-EGFP] vector, encoding *upstream activating sequence* (*UAS*) and *egfp* genes, was previously described [21]. The *Xba* I fragment of pZErO-2-hIR, which contains the hIR ORF, was cloned into the *Bln* I site of pBacMCS[UAS-SV40, 3×P3-EGFP]. The resulting plasmid was named pBac-UAS-hIR-3×P3-GFP. The complete hIR sequence was analyzed and confirmed by DNA sequencing using primers, as shown in Supplementary Table 1.

2.4. Generation of transgenic silkworms expressing hIR

The method for generating transgenic silkworms was previously reported [21]. The plasmid constructed above (pBac-UAS-hIR-3×P3-EGFP) was micro-injected into silkworm eggs (w1-pnd strain), thus producing a line expressing the *hIR* gene under regulation of the UAS (UAS-*hIR*). Because the vector encodes GFP downstream of the 3×P3 promoter specific to compound eyes, transgenic silkworms could be selected by observation under a fluorescence microscope. This transgenic line was crossed with another line transformed by a plasmid, pBac-A3-GAL4-3×P3-DsRed, containing both the *GAL4* gene downstream of the cytoplasmic *actin A3* promoter and the *DsRed2* gene as an eye marker [20]. Silkworms expressing both EGFP and DsRed2 in the compound eyes (UAS-*hIR*; *actin A3*-GAL4) were selected as the "hIR-induced strain" under a fluorescence microscope. Western blotting analysis

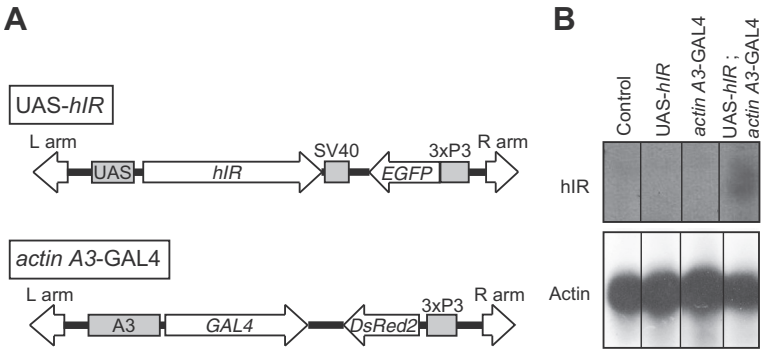

Fig. 1. Development of transgenic silkworms expressing the human insulin receptor using the GAL4/UAS system. (A) Structures of UAS-*hIR* and *actin A3*-GAL4 for expressing hIR in silkworms. "UAS-*hIR*" represents the genotype for UAS-*hIR* with the green fluorescent protein marker, and "*actin A3*-GAL4" represents the genotype for *actin A3*-GAL4 with the red fluorescent protein marker. Note that the "UAS-*hIR*; *actin A3*-GAL4" strain possesses both UAS-*hIR* and *actin A3*-GAL4, which enable the induced expression of hIR in whole body. hIR: human insulin receptor. (B) Detection of human insulin receptors in the fat body of transgenic silkworms. Fat bodies of the silkworms, (Control), (UAS-*hIR*), (*actin A3*-GAL4) and (UAS-*hIR*; *actin A3*-GAL4), were isolated. Human insulin receptor and ß-actin were determined by Western blot analysis using each antibody.

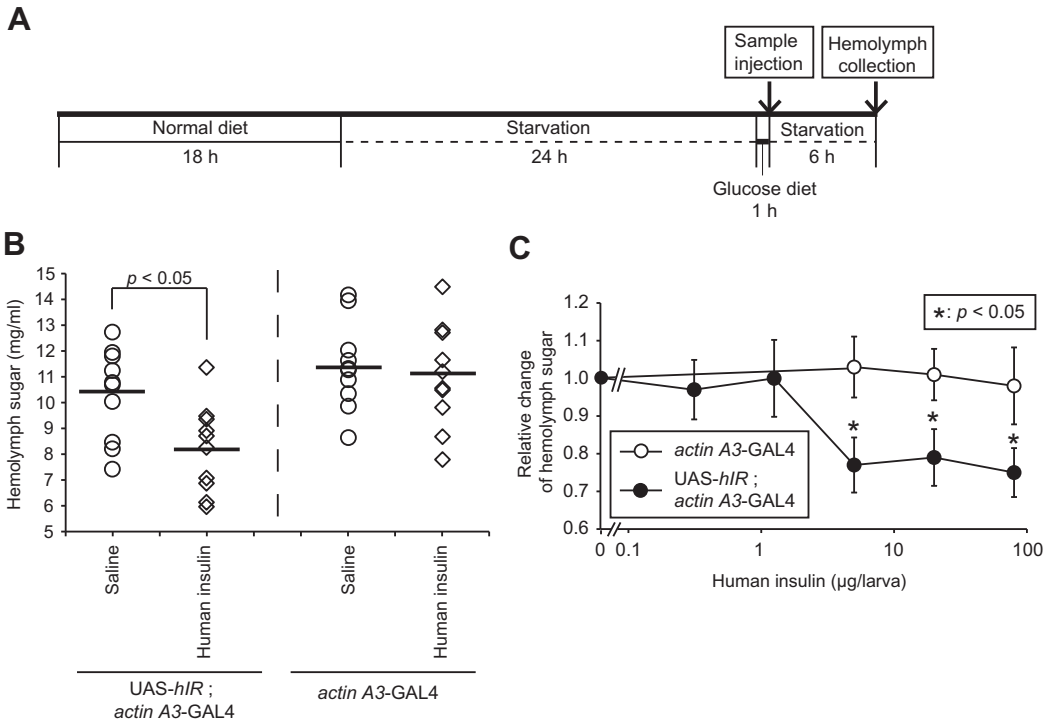

Fig. 2. Human insulin-induced decrease in hemolymph sugar levels of transgenic silkworms expressing human insulin receptors. (A) Experimental design. (B) Human insulin (5 μg/larva) or saline (0.9% NaCl + 0.1% acetic acid) was administered into the hemolymph of the hIR-induced silkworms (UAS-*hIR*; *actin A3*-GAL4) or control siblings (*actin A3*-GAL4). Hemolymph sugar levels were measured 6 h after injection (n = 9–10 per group). Student's t-test was used to evaluate significant differences between groups. (C) Serially-diluted human insulin (0.3–80 μg/larva) was administered into the hemolymph of the hIR-expressing transgenic silkworms (UAS-*hIR*; *actin A3*-GAL4) or control silkworms (*actin A3*-GAL4). Hemolymph sugar levels were measured 6 h after injection. Relative changes in the hemolymph sugar levels compared with that of saline-injected silkworms were calculated (n = 6 per group). Data represent mean ± SEM. Asterisks indicate significant differences based on Student's t-test ($p < 0.05$).

was performed to assess hIR expression in the silkworm fat body, as described below.

2.5. Determination of the hemolymph sugar concentration

Sugar levels in the silkworm hemolymph were determined by the method described previously [19]. Hemolymph (5 μl) was collected by cutting the first proleg with scissors, mixing with 45 μl of 0.6 N $HClO_4$, and centrifuging at 3000 rpm for 10 min to settle out proteins. The amount of sugar in the supernatant was quantified using the phenol–sulfuric acid method [22] as follows: 5 μl of each supernatant was diluted with 95 μl of distilled water, and 100 μl of 5% (w/v) phenol solution was added. Samples were mixed with 500 μl of concentrated sulfuric acid, incubated for 20 min at room temperature, and absorbance at 490 nm was measured. A series of glucose solutions was used as a standard.

2.6. In vitro culture of the fat body

The *in vitro* culture system for fat bodies isolated from the silkworms was previously reported [19]. Fat bodies were isolated from the silkworms, washed with insect saline (10 mM Tris/HCl pH 7.9, 130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$), and incubated in 200 μl of Grace's insect medium at 27 °C, with or without 10 μM wortmannin. After pre-incubation for 30 min, 50 μl of 0.5 mg/ml insulin solution was added and the mixture was incubated for another 3 h.

2.7. Western blotting analysis

The fat bodies were washed with insect saline, mixed with 250 μl of NP-40 lysis buffer (10 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1% Nonidet P-40, 10 mM NaF, 1 mM Na_3VO_4), and sonicated for 20 s. Proteins precipitated by trichloroacetic acid were electrophoresed, transferred to a polyvinylidene difluoride membrane (Millipore), probed with antibody, and the signals were detected using Western Lightning Plus chemiluminescence reagent (Perkin-Elmer Life Sciences). The following antibodies were used for immunoblot analysis: rabbit polyclonal antibodies to total Akt, phosphorylated Akt, and ß-actin from Cell Signaling Technology, and mouse monoclonal antibody to insulin receptor (ß-subunit) from Calbiochem. Quantification of the amount of phosphorylated Akt was performed by densitometric scanning with Image Gauge software (ImageJ 1.43u). The relative amount of phosphorylated Akt to total Akt was determined.

2.8. Statistical analysis

All experiments were performed at least twice and the data are shown as the mean ± SD or SEM. The significance of differences was calculated using Student's t-test. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Generation of transgenic silkworms expressing hIR

To establish silkworm strains expressing hIR, we first constructed a plasmid that contained the hIR gene under control of the UAS promoter (Fig. 1A). We then selected transgenic silkworms possessing this construct by monitoring the enhanced GFP marker expressed in compound eyes (Supplementary Fig. 1). This line was crossed with another silkworm strain that expressed GAL4 in the whole body together with the DsRed2 marker in the compound eyes (Supplementary Fig. 1). We next selected silkworms

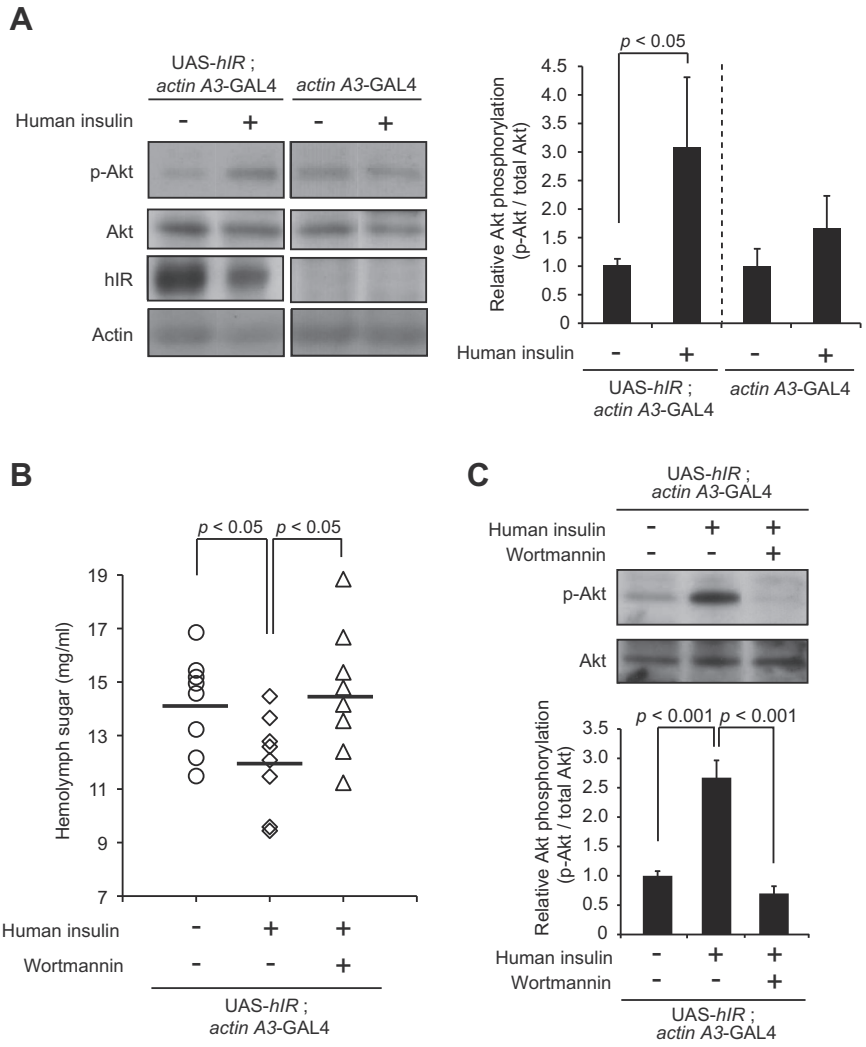

Fig. 3. Wortmannin-induced inhibition of the effects of human insulin in transgenic silkworms expressing human insulin receptors. (A) Human insulin (5 μg/larva) or saline (0.9% NaCl + 0.1% acetic acid) was administered into the hemolymph of the transgenic silkworms (UAS-*hIR*; *actin A3*-GAL4). After 1 h, the silkworm fat bodies were isolated. Phosphorylated Akt, total Akt, human insulin receptor, and ß-actin levels were determined by Western blot analysis using each antibody ($n = 3$ per group). Data represent mean ± SD. Student's *t*-test was used to evaluate significant differences between groups. (B) Control solvent (0.9% NaCl + 2.5 % DMSO), human insulin (100 μg/larva), or human insulin and wortmannin (human insulin: 100 μg/larva and wortmannin: 12.5 nmol/larva) were administered into the hemolymph of the transgenic silkworms UAS-*hIR*; *actin A3*-GAL4). Hemolymph sugar levels were measured 6 h after injection ($n = 8$ per group). Student's *t*-test was used to evaluate significant differences between groups. (C) Fat bodies isolated from the transgenic silkworms (UAS-*hIR*; *actin A3*-GAL4) were cultured in Grace's insect medium with or without wortmannin (10 μM) for 30 min and further cultured after the addition of human insulin (final 0.1 mg/ml) for 3 h. Phosphorylated Akt, total Akt, human insulin receptor, and ß-actin levels were detected by Western blot analysis using each antibody ($n = 3$ per group). Data represent mean ± SD. Student's *t*-test was used to evaluate significant differences between groups.

expressing both eye markers of enhanced GFP and DsRed2 (UAS-*hIR*; *actin A3*-GAL4) as the "hIR-induced strain", and expression of the hIR protein in the silkworm fat bodies was confirmed by Western blotting analysis (Fig. 1B).

3.2. Decrease in hemolymph sugar levels of transgenic silkworms promoted by human insulin

We tested whether the transgenic silkworms expressing hIR showed an enhanced response to human insulin compared with their control siblings (Fig. 2A). The hemolymph sugar levels in the hIR-induced silkworms (UAS-*hIR*; *actin A3*-GAL4) were decreased by administration of 5 μg human insulin (Fig. 2B). On the other hand, administration of the same amount of insulin did not decrease hemolymph sugar levels in *actin A3*-GAL4 siblings not expressing hIR (Fig. 2B). We further examined the amount of human insulin necessary for the hypoglycemic action in the transgenic silkworms. Hemolymph sugar levels in the hIR-induced silkworms (UAS-*hIR*; *actin A3*-GAL4) were significantly decreased with by 5, 20, and 80 μg of human insulin, but not by 0.3 and 1.3 μg (Fig. 2C). Hemolymph sugar levels in silkworms not expressing hIR (*actin A3*-GAL4) were not decreased by injection of 5, 20, or 80 μg of human insulin (Fig. 2C). The results indicated that 5 μg of human insulin was needed to decrease hemolymph sugar levels in the transgenic silkworms. In our previous report, we demonstrated that the effective dose of human insulin for hyperglycemic silkworms was greater than 45 μg/larva [19]. The apparent discrepancy with the above findings of the control siblings (*actin A3*-GAL4) is likely due to the background genetic differences between silkworm strains; one was a hybrid strain Hu Yo × Tsukuba Ne and the other an inbred line w1-pnd. In the UAS-*hIR*; *actin A3*-GAL4 line, transgenic hIR induction very clearly promoted insulin activity.

3.3. Activation of the insulin-signaling pathway in the transgenic silkworms

Next, we tested whether human insulin stimulates the insulin-signaling pathway in the fat bodies of hIR-expressing transgenic

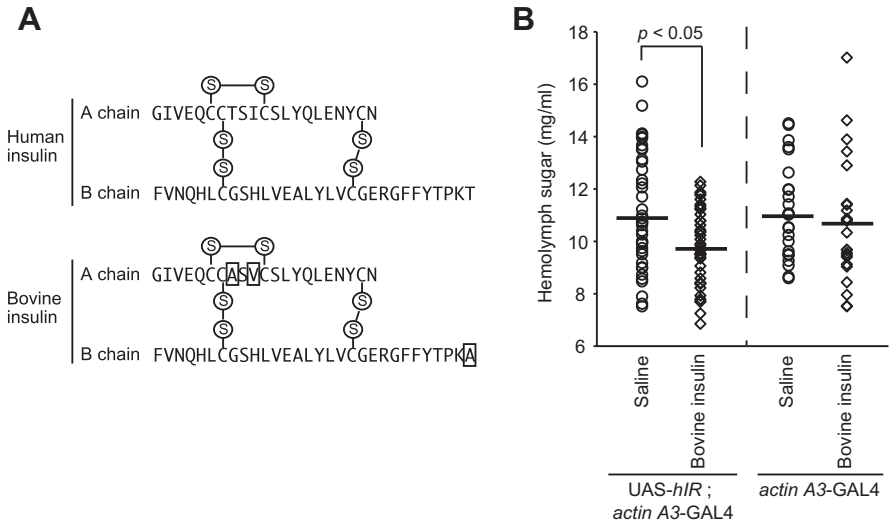

Fig. 4. Hypoglycemic effect of bovine insulin in transgenic silkworms expressing human insulin receptors. (A) Structures of human and bovine insulin. Amino acids that differ between human and bovine insulin are indicated by the square. (B) Bovine insulin (80 μg/larva), or saline (0.9% NaCl + 0.1% acetic acid) was administered into the hemolymph of the transgenic silkworms (UAS-*hIR*; *actin A3*-GAL4) or control silkworms (*actin A3*-GAL4). Hemolymph sugar levels were measured 6 h after injection. $n = 23–42$ per group. Student's *t*-test was used to evaluate significant differences between groups.

silkworms. After injection of human insulin into the hemolymph, fat bodies were isolated and a homogenate was prepared for further analysis. We observed increased Akt phosphorylation after injecting human insulin in the hIR-induced silkworms (UAS-*hIR*; *actin A3*-GAL4), but not in the control silkworms (*actin A3*-GAL4) (Fig. 3A). PI3-kinase is a key factor acting upstream of Akt in the insulin-signaling pathway in mammals [23]. Wortmannin, a PI3-kinase inhibitor, suppresses the insulin-dependent stimulation of the insulin-signaling pathway in silkworms [19]. In the hIR-expressing transgenic silkworms injected with human insulin, the decrease in the hemolymph sugar levels was inhibited by co-injection of wortmannin and human insulin (Fig. 3B). Furthermore, the stimulation of Akt phosphorylation observed in the *in vitro*-cultured fat bodies of the transgenic silkworms was inhibited by wortmannin treatment 30 min prior to insulin supplementation (Fig. 3C). These findings suggest that the insulin-dependent Akt phosphorylation in the fat bodies followed by the decrease in the hemolymph sugar levels in silkworms expressing hIR was dependent on PI3-kinase activity.

3.4. Evaluation of hypoglycemic activity in bovine insulin using the transgenic silkworms

Bovine insulin, which differs from human insulin in three amino acid residues (Fig. 4A), has an affinity for hIR comparable to that of human insulin [24]. We tested whether the hypoglycemic activity of bovine insulin is observed in transgenic silkworms expressing hIR. Hemolymph sugar levels in hIR-induced silkworms were decreased by bovine insulin injection (Fig. 4B). This finding suggests that the hIR-expressing transgenic silkworms are suitable for assessing the activities of IR agonists, including human and bovine insulin.

4. Discussion

We constructed a strain of transgenic silkworms expressing the hIR. We demonstrated that the human-type receptor expressed in silkworms was functionally active by examining the whether a decrease in the hemolymph sugar was promoted and whether signaling molecules were activated in response to human insulin. We also demonstrated that injection of bovine insulin led to a decrease in the silkworm sugar levels. Our results suggest that the therapeutic effects of hIR agonists could be evaluated in hIR-expressing transgenic silkworms.

Silkworms have a number of advantages for evaluation of the therapeutic effects of drug candidates. Evaluation of ligands in the nervous system or endocrine system with silkworms, however, has yet to be achieved as silkworms and mammals possess different receptor proteins in these systems. Establishing transgenic silkworm strains that express human-type receptor proteins would be useful to reduce the difficulties in the use of silkworms as a model to evaluate drugs targeting the nervous and endocrine systems. Here, we demonstrated that injection of human insulin had hypoglycemic effects in hIR-expressing transgenic silkworms. There are few reports in which the responses to drugs using transgenic mice were increased by the expression of human receptor genes, probably because there is little difference in the ligand affinity of receptors between these two mammalian species. Compared to mice, structures of silkworm-type receptors functioning in the nervous and endocrine systems may significantly differ from those in humans. This feature allowed us to obtain clear results reflecting the function of human-type receptors expressed in transgenic silkworms without background effects of endogenous silkworm-type receptors. Our findings suggest that transgenic silkworms expressing human-type receptors could be applied for the evaluation of other drugs used to treat a wide range of nervous and endocrine-related human diseases.

We previously reported that the stability of chemical compounds in the silkworm hemolymph is quite consistent with that in the mammalian plasma [25]. The present study demonstrated that human insulin and bovine insulin, both possessing similar affinities for the hIR, also showed therapeutic activities in the hIR-expressing transgenic silkworms. This finding suggests that this hIR-expressing transgenic silkworm is suitable for evaluating the therapeutic activities of insulin analogs against hyperglycemia. The use of humanized silkworms for evaluating drug candidates may allow us to efficiently eliminate drug candidates with apparent activities *in vitro* but not *in vivo* due to problems in pharmacokinetics. We expect that our study, in which we used humanized silkworms for drug evaluation for the first time, will provide a new approach for *in vivo* drug evaluation in alternative model animals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.143.

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