2 カイコにおける薬物の体内動態

2-1　実験動物としてのカイコの利点

医薬品の候補化合物の治療効果を評価する上で動物モデルの使用が必要不可欠である．なぜなら，試験管内での探索により選別されたほとんどの候補化合物は，体内動態の問題のため治療効果を示さず，医薬品とはならないからである．化合物の体内動態を知るためには，動物に投与し，動物体内での安定性を調べなければならない．そのために動物を犠牲にして実験することが必要である．その際の問題点として，動物飼育施設の維持に必要な高い経費，並びに，ほ乳動物の使用に伴う動物愛護の観点からの問題がある．後者については，近年，医薬品の開発をはばむまでとなっている．特に，ヨーロッパ諸国では医薬品開発にほ乳動物を用いることを制限する法律が施行されている．これらの問題を解決するために，線虫，昆虫のショウジョウバエ，原生動物のアメーバなどの無脊椎動物を使用する事が提案されている．特に線虫やショウジョウバエは遺伝子の破壊や導入ができ，様々な遺伝子破壊系統が作出されているため，自然免疫系に関わる宿主動物側の因子を同定するのに極めて有効である．しかしながら，これらの動物は手で扱うには小さすぎ，正確な量のサンプルを体の中に注射する目的には適さない．この注射技術は細菌の病原性と抗生物質の治療効果を定量的に評価するには必要である．

カイコは医薬品の治療効果を評価する上でいくつかの利点を有する．蚕糸業の長い歴史のおかげで，私たちはいつでも何千匹もの蚕を手に入れる事ができる．どんなに多くのカイコを使用しても，倫理的な問題を生じる事はない．これらの点に加えて，カイコは他の無脊椎動物のモデルにはない大きな利点を有する．彼らの体の大きさは手で扱うのに十分大きく，注射器を使って，体液中，及び腸管中にサンプルを打ち込む事ができる．もし，ショウジョウバエや線虫でやろうとしたら，顕微鏡下でこの作業を行わなければならない．カイコの体液中に赤い色素を打ち込めば，カイコの体は赤色に変わる．これは，カイコが開放血管系を有するからだ．一方，カイコの腸管中に赤い色素を打ち込めば，赤い色素は腸管の中に入って行き，体の色は変わらない．この注射技術により，私たちは細菌の病原性を定量的に評価できる．さらに，私たちは病原性細菌を注射したカイコの組織や体液を取り出し，組織中，及び体液中の生菌数を測定できる．そして，細菌の感染プロセスを把握することができる．

近年，日本と中国のグループがカイコのゲノムプロジェクトを終了させている．また，カイコにおけるRNA干渉法も確立されている．ゲノム情報とRNA干渉法は感染に関与する宿主側の因子の研究に役立つと考えられる．さらに，外来遺伝子を導入したトランスジェニックカイコの作出も可能となっている．これらの新しい技術を駆使することにより，従来はほ乳動物でしか評価することが困難であった医薬品の評価がカイコを使って可能となると期待される．

2-2　カイコにおける薬物の体内動態

カイコは，ほ乳類と同様に，薬物動態に関与する腸管・肝臓・腎臓と相同する臓器を有する．カイコは開放血管系であり，血管に相当する臓器はないが，血液を攪拌する心臓と類似した臓器も有する．薬物の体内動態は，腸管からの透過後，主に肝臓における薬物の代謝（シトクローム P 450 による水酸化と抱合），及び腎臓からの排泄，組織への薬物の分布などの要因が影響する．以下に示すように，カイコにおいても，これらの要因について解析を行い，薬物体内動態がカイコ幼虫とほ乳類で類似していることを見いだした．

(i) 腸管透過性

受動拡散によって吸収される薬物の腸管透過性には，化合物の脂溶性及び分子量が影響することが知られている．それらの要因について検討した結果，カイコ幼虫の腸管にも分子量の障壁が存在すること，及び物質の脂溶性が影響することを見いだした．また，抗生物質の腸管内（経口）投与における治療効果の有無も，腸管透過性と一致することを明らかにしている．

(ii) 代謝及び排泄

薬物代謝はシトクローム P450（CYP450）による水酸化に続き，糖（グルクロン酸やグルコース）などによる抱合が起こり，水溶性が高まる方向へ修飾される．カイコにおいても，CYP450 による代謝に引き続き，グルコースによる抱合が起こることを見いだした（図 2）．また，カイコにおいては代謝された薬物は，腎臓に相当するマルピーギ管を介して糞へ排泄することが知られている．カイコに薬物を投与すると，代謝され抱合化された薬物が糞に排泄されることを私たちは見いだした．従って，カイコ幼虫においても薬物の排泄をほ乳類同様に検討することが可能であると考えられる．

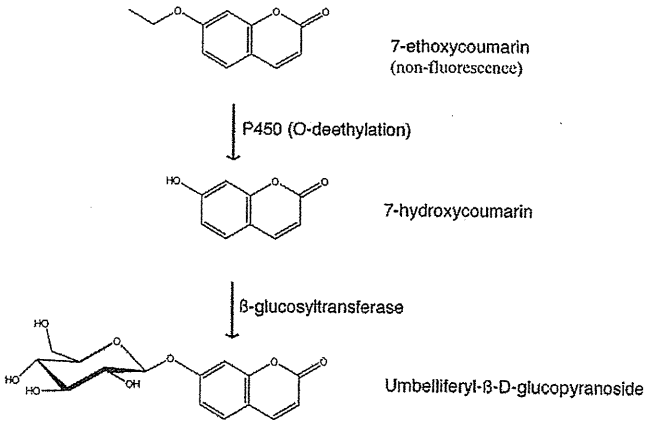

図 2　カイコにおける薬物代謝

(iii) 毒性評価

カイコは，医薬品の候補化合物の毒性を評価する上にも有用である．カイコに様々細胞毒性を示す化合物を投与し，毒性試験を実施した．得られたLD_{50}値は哺乳類における値と一致した．従って，カイコを用いて細胞毒性を示す化合物の毒性評価を行うことが可能である．

3 カイコの感染モデルを用いた細菌の病原性遺伝子の同定

3-1　カイコの細菌感染モデル

ヒトに対する病原性細菌である黄色ブドウ球菌をカイコの血液内に注射すると，カイコは数日以内に死亡する．この黄色ブドウ球菌のカイコに対する殺傷効果はヒトの臨床で抗菌治療薬として用いられる抗生物質により抑圧される．カイコに対する殺傷効果は，A群レンサ球菌，緑膿菌，リステリア菌，セラチア菌，サルモネラ菌，コレラ菌など様々な病原性細菌で観察される．細菌の毒素はカイコに対する殺傷効果を有する．黄色ブドウ球菌のα溶血毒素，β溶血毒素，緑膿菌の外毒素A，ジフテリア毒素のカイコにおける半数殺傷容量（LD_{50}）はマウスにおける値とよく一致している．従って，カイコは細菌毒素のヒトを含む多細胞生物に対する効果を研究する上でも有効である．

3-2　黄色ブドウ球菌の病原性に必要な遺伝子 *cvfA*，*cvfB*，*cvfC* の発見

次に，私たちが，カイコを用いて細菌の病原因子を同定した研究例を紹介したい．黄色ブドウ球菌は免疫力の低下した患者に対して日和見感染を引きおこす．病原性の発現に至る分子メカニズムの理解は，本病原体に対する有効治療法の開発に役立つと考えられる．本病原性細菌のゲノムプロジェクトは終了しており，病原性細菌間で保存されているが，機能がわかっていない遺伝子が589個存在することが明らかとなっている．

私たちはこれらの機能未知遺伝子のうち，100種類について遺伝子破壊株を作出した．これらの遺伝子破壊株をそれぞれカイコの体液中に注射し，カイコを殺傷する能力の低下した遺伝子破壊株を3株見出した．親株が半数のカイコを殺傷するのに36時間がかかるのに対し，これらの遺伝子破壊株は80時間以上が必要であった．私たちはこれらの遺伝子を *cvfA*，*cvfB*，*cvfC*（conserved virulence factor A, B, C）と命名した．

cvfA，*cvfB*，*cvfC* 遺伝子は様々な細菌に保存されている．特に *cvfA* 遺伝子は多くの細菌のゲノムに存在するが，その機能は明らかとなっていない．他の病原性細菌における *cvfA* 遺伝子の機能の保存性を知るために，私たちはA群レンサ球菌の *cvfA* 遺伝子破壊株を作出し，カイコにおける病原性を検討した．その結果，A群レンサ球菌の *cvfA* 遺伝子破壊株はカイコに対する殺傷能力を減弱していることが明らかとなった．従って，*cvfA* 遺伝子は黄色ブドウ球菌だけでな

く，他の細菌においても病原性に必要である．

次に，私たちは黄色ブドウ球菌の *cvfA*，*cvfB*，*cvfC* 遺伝子の破壊株がマウスに対する病原性を低下するかどうかを検討した．その結果，半数のマウスを殺傷するのに必要な菌数（LD_{50}）はそれぞれの変異株で野生株よりも高い数値を示すことが分かった．従って，これらの遺伝子はほ乳類に対する病原性にも必要である．

黄色ブドウ球菌の *cvfA*，*cvfB*，*cvfC* 遺伝子破壊株は野生株に比べて産生する毒素の量が少なかった．β溶血毒素の基質となる羊の赤血球を含む寒天培地上で，それぞれの遺伝子破壊株は，野生株に比べ赤血球を破壊した領域が狭かった（図3，白い矢印で示す部分が毒素活性を表す）．この表現型はそれぞれの遺伝子を導入すると相補された．*cvfA* 遺伝子破壊株においては，細胞外に放出される毒素である DNA 分解酵素，タンパク質分解酵素の量の低下も見られた．これらの結果から，私たちは *cvfA*，*cvfB*，*cvfC* 遺伝子が細胞外毒素の発現制御因子をコードしているかもしれないと考えた．

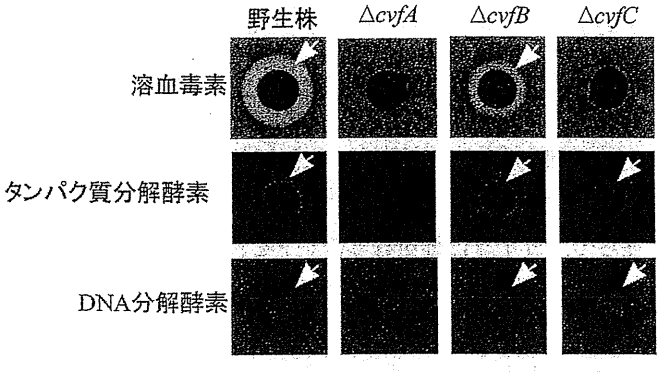

図3　*cvfA*，*cvfB*，*cvfC* 遺伝子破壊株の毒素量低下

3-3　CvfA タンパク質の機能

黄色ブドウ球菌の *agr* 遺伝子座は細胞外毒素の発現制御に必須である事が分かっている．私たちは *cvfA* 遺伝子の破壊が *agr* 遺伝子座の発現に影響を与えるかを検討した．ノーザンブロット解析の結果，*cvfA* 遺伝子破壊株においては *agr* 遺伝子座の転写産物である RNAII 及び RNAIII の量が著しく低下していることが明らかとなった．従って，*cvfA* 遺伝子は溶血毒素，タンパク質分解酵素，DNA 分解酵素などの細胞外毒素の発現に必要な *agr* 遺伝子座の発現に必要であると考えられる．

CvfA タンパク質のアミノ酸配列には，膜貫通領域，RNA 結合領域（KH 領域），金属依存リン酸加水分解領域（HD 領域）が存在する．CvfA タンパク質の KH 領域と HD 領域に変異を持つ株は，カイコ感染モデルにおける病原性を低下するので，これらの領域は CvfA の機能に必須である．CvfA タンパク質は KH 領域と HD 領域を両方持つタンパク質の中で最初に生理的役割

が明らかとなった例である．データベースに基づいた研究では，これらのKH領域とHD領域を両方持つタンパク質は核酸分解酵素として働く事が予想されている．

3－4　*cvfA* 遺伝子の欠損を抑圧する *sarZ* 遺伝子の発見

遺伝子変異株の表現型を抑圧する別の遺伝子を同定する方法は，遺伝学的な解析の基本的な方法である．黄色ブドウ球菌における *cvfA* 遺伝子と他の病原性遺伝子との関係を明らかにするために，私たちは *cvfA* 遺伝子破壊株の表現型を抑圧するマルチコピーサプレッサーを同定することを試みた．黄色ブドウ球菌のゲノムライブラリーから，*cvfA* 遺伝子破壊株の溶血毒素の産生量低下を回復させる遺伝子として，*sarZ* と命名されている機能未知遺伝子を同定した．

SarZタンパク質は大腸菌の転写因子であるMarRタンパク質と相同性を有する．*sarZ* 遺伝子が *cvfA* 遺伝子破壊株の表現型を抑圧したことから，私たちは *cvfA* 遺伝子が *sarZ* 遺伝子の発現を促進するのではないかと考えた．この考え方は，CvfAタンパク質の無い条件下でSarZタンパク質の大量発現が溶血毒素産生を回復させる現象を説明する．ノーザンブロット解析の結果，*cvfA* 遺伝子破壊株においては *sarZ* 遺伝子の転写産物量が低下していることが明らかとなった．従って，*cvfA* 遺伝子は *sarZ* 遺伝子の発現を促進する．*sarZ* 遺伝子破壊株はカイコ感染モデル及びマウス感染モデルにおいて病原性を低下したので，*sarZ* 遺伝子それ自身は *cvfA* 遺伝子によって活性化を受ける病原性遺伝子である．

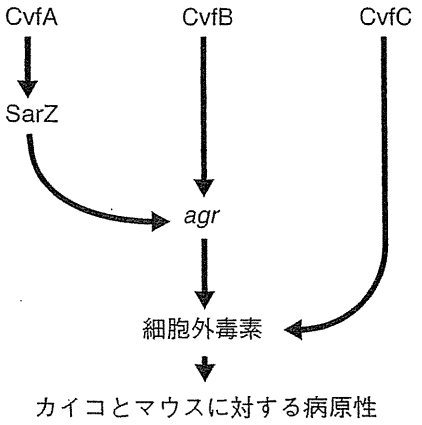

図4　病原性遺伝子の制御モデル

私たちはカイコ感染モデルを用いて3つの黄色ブドウ球菌の病原性遺伝子を同定した．私たちは，カイコが病原性細菌の病原性遺伝子の同定と評価に適したモデルである事を提唱したい．私たちの研究から，黄色ブドウ球菌の *cvfA*，*cvfB*，*sarZ* 遺伝子は様々な細胞外毒素の産生制御に働く *agr* 遺伝子座の発現の上位に有る事が明らかになっている（図4）．これらの遺伝子は病原性細菌間で保存されているため，これらの遺伝子は様々な細菌の病原性制御において，根幹となる経路に働いていると考えられる．また，最近，我々はメチシリン耐性黄色ブドウ球菌に特異的

に存在する新規遺伝子 *fudoh* が MRSA の病原性を変化させる事を見出している．我々は，これらの新規の遺伝子の機能を明らかにすることによって，なぜ病原性細菌がヒトに病原性を示すかを理解したいと考えている．

4 カイコの感染モデルを用いた新規抗生物質の探索方法

4-1 抗生物質の治療効果の定量的評価

私たちはカイコ細菌感染モデルを用いて，抗生物質の定量的評価が可能であり，その体重あたりの治療に必要な薬剤量が，マウスと一致することを見いだしている（表1）．私たちは，独立行政法人医薬基盤研究所の「保健医療分野における基礎研究推進事業」において，この系を利用した天然物及び合成化合物のライブラリーから，治療効果を示す新規抗菌薬の探索を実施している．これまでに1万株以上の土壌細菌を分離し，黄色ブドウ球菌に対し抗菌活性を示す培養上清の中から，カイコ黄色ブドウ球菌感染モデルにおいて治療効果を示すものを得ている．それらの培養上清から，カイコモデルにおける治療効果を指標に抗生物質を精製し，マウスモデルで治療効果を示す新規抗生物質を得ることに成功している．

また細菌・真菌だけではなく，ヘルペスウイルスに有効な抗ウイルス薬が，ヘルペスウイルスと同様なカイコに感染する DNA ウイルスであるバキュロウイルスに対して治療効果を示すことを明らかにしている．この系を用いて，ヘルペスウイルスにも作用する抗ウイルス薬を探索，同定が可能である．

表1 抗生物質の治療効果のカイコ幼虫及びマウスとの比較

抗生物質	ED_{50}（μg/g・animal）	
	カイコ幼虫	マウス
テイコプラニン	0.3	0.1
バンコマイシン	0.3	1
ミノサイクリン	4	1
フロモキセフ	0.2	0.3
リネゾリド	9	4

4-2 カイコの感染モデルを用いた新規抗生物質の探索

以上のように，カイコにおける薬物の体内動態は，哺乳類と基本的な部分は類似しており，カイコを用いて細胞毒性の評価が可能である．私たちは，様々な疾病モデルを構築しており，これまでに述べたような細菌感染モデルや，ウイルス感染モデルの他に，以下に詳細に述べる糖尿病モデルや，カイコの自然免疫活性化経路についても解析を行い，自然免疫活性化物質の探索系を確立している[2]．このようなカイコ疾病モデルを用いて，治療効果を指標とした新規薬剤の探索

が可能であり，本方法は従来の創薬の手法を大きく変えることが期待される．

5 カイコの高血糖モデル

5-1　糖尿病治療薬の探索の重要性

糖尿病は慢性の高血糖状態の持続，及び耐糖能の異常を示す疾患であり，腎症，末梢神経障害，網膜症などの様々な臓器障害を引き起こす原因となる．日本において，予備群を含めて糖尿病患者数は1800万人以上存在し，さらに増加傾向にあることから，現在社会的な問題となっている（2008年，厚生労働省，糖尿病実態調査）．日本以外の先進諸国においても，インスリン抵抗性が原因となるII型糖尿病の患者数は増加傾向にある．インスリン，及びインスリン抵抗性改善作用により血糖値を低下させる医薬品が糖尿病の治療に用いられているが，薬剤抵抗性や副作用を引き起こすという問題があり，新たな治療薬の開発が望まれている．

5-2　高血糖値を示すカイコの作出

血糖値は，インスリン，グルカゴンなどの様々なホルモンの作用により，全身の組織における糖の取り込み，代謝により調節されている．よって，糖尿病治療薬の効果を評価するためには，動物個体を用いる必要がある．そのため，マウスやラットなどの哺乳動物による糖尿病の病態モデルを用いた研究が行われている．しかし，多数の哺乳動物を用いて糖尿病治療薬をスクリーニングすることは，高い飼育コストばかりでなく，動物愛護の観点からも問題があると指摘されている．

私たちは，無脊椎動物であるカイコを用いて，病態モデルを構築し，医薬品の治療効果を評価できることを提唱している．すでに私たちは，上記の通りカイコ感染モデルを用いて，細菌感染治療薬，及び抗ウイルス薬の評価を行えること，及び抗生物質の体内動態において哺乳動物とカイコで共通した面があることを報告している．無脊椎動物であるカイコは，容易に多数の個体を用いて治療薬の評価を行うことができる．また，カイコは，哺乳動物より安価で，狭いスペースで大量の個体を飼育することが可能である．さらに，カイコは線虫やショウジョウバエのような小型無脊椎動物では困難な注射器を用いた定量的な薬物の血液内注射が容易に実施できる．また，比較的大量の血液を採取することが可能である．

さらにカイコ幼虫は，体液中の糖（以後，血糖と記載する）を人の肝臓，及び脂肪組織に相当する脂肪体という臓器，または筋肉にグリコーゲンとして貯蔵することが知られている（図5）．そこで，私たちはカイコを用いて，糖尿病治療薬の評価，及び同定のための無脊椎動物糖尿病モデルの構築を試みた．

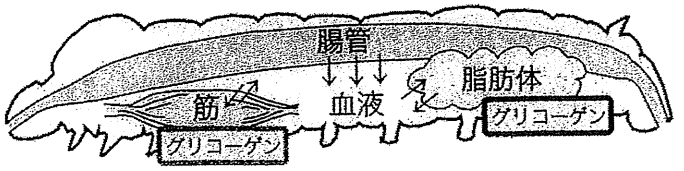

図5　カイコ幼虫の糖分布

我々は，カイコを高血糖状態にするための条件を検索した．カイコにグルコースを添加した人工餌を食べさせることにより，カイコの血糖値を上昇させることができた．この高血糖カイコに，哺乳動物の血糖値を低下させる物質であるヒトインスリン，及びAMPK（AMP依存的プロテインキナーゼ）活性化作用を持つ化合物であるAICAR（5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside）を投与するとカイコの血糖値が低下した．さらに，摘出したカイコの脂肪体にヒトインスリンを処理すると，脂肪体細胞内の糖含量の上昇，及びAktのリン酸化の促進が見られた．このとき，PI3キナーゼの阻害剤であるワートマニンは，ヒトインスリンによるこれらの反応を抑制した．一方，哺乳動物細胞においてAMPKのリン酸化による糖取り込みの促進を導くAICARは，カイコの脂肪体細胞内においてもAMPKのリン酸化を亢進させた．したがって，カイコはインスリンシグナル伝達経路やAMPKの活性化を介した血糖値調節機構を有している（図6）．

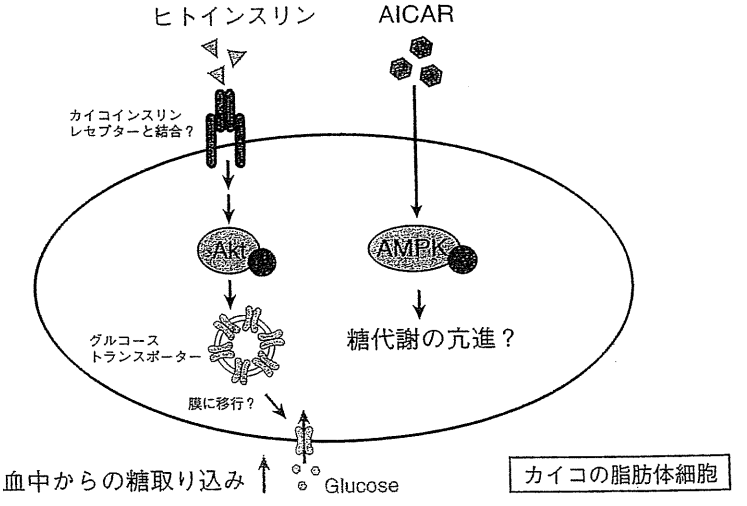

図6　ヒトインスリン，AICARのカイコの血糖調節に与える影響

5－3　カイコの高血糖モデルを用いた血糖降下作用を示す化合物の探索

私たちは，この高血糖カイコモデルを用いてヒトに対して効果を示す血糖降下物質を同定できるか検討している（図7）．具体的には，ヒトに対して血糖降下作用が報告されているが有効成

分が不明な生薬（ニンジン，ソウジュツ，ブクリョウなど），及び食品（玄米，納豆など）からカイコに対する血糖降下活性を指標に化合物を精製する．その後，カイコ幼虫の血糖値を低下させる活性を有する化合物が糖尿病モデルマウスにおいても血糖降下活性を有するか検討する．そして，血糖降下物質が，哺乳類の血糖調節において重要なインスリン経路，AMPKの活性化を介する経路，もしくはそれ以外の経路により血糖調節に関与するのか明らかにする．

高血糖カイコを用いた糖尿病治療薬の開発法は，倫理的な問題の少ないカイコ幼虫を用いて，個体の血糖降下活性を指標に血糖降下物質を精製，同定することを目指している．このような，血糖降下薬の評価系として，無脊椎動物を用いたモデルは初めてである．この研究が完遂されることにより，血糖調節機構など，内分泌代謝疾患に関する研究に無脊椎動物を用いたモデルが有用であるという新たな概念が提案される．今後，様々な昆虫を用いた内分泌代謝疾患モデルが登場し，治療薬の開発のツールとして利用されるのではないかと期待される．この概念は，動物実験等の国際原則の一つであるReplacement（代替法の開発）において，革新的な提案になり得る可能性がある．

また，本研究において，血糖降下物質が未同定な生薬，及び食品から活性物質を同定することから，新規の構造の血糖降下薬が同定できる可能性があると考えられる．その新規の構造の血糖降下物質が糖尿病患者に対して血糖降下作用を有する場合，臨床において新規の糖尿病治療薬として使うことができると考えられる．

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An Invertebrate Hyperglycemic Model for the Identification of Anti-Diabetic Drugs

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Abstract

The number of individuals diagnosed with type 2 diabetes mellitus, which is caused by insulin resistance and/or abnormal insulin secretion, is increasing worldwide, creating a strong demand for the development of more effective anti-diabetic drugs. However, animal-based screening for anti-diabetic compounds requires sacrifice of a large number of diabetic animals, which presents issues in terms of animal welfare. Here, we established a method for evaluating the anti-diabetic effects of compounds using an invertebrate animal, the silkworm, *Bombyx mori*. Sugar levels in silkworm hemolymph increased immediately after feeding silkworms a high glucose-containing diet, resulting in impaired growth. Human insulin and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an AMP-activated protein kinase (AMPK) activator, decreased the hemolymph sugar levels of the hyperglycemic silkworms and restored growth. Treatment of the isolated fat body with human insulin in an *in vitro* culture system increased total sugar in the fat body and stimulated Akt phosphorylation. These responses were inhibited by wortmannin, an inhibitor of phosphoinositide 3 kinase. Moreover, AICAR stimulated AMPK phosphorylation in the silkworm fat body. Administration of aminoguanidine, a Maillard reaction inhibitor, repressed the accumulation of Maillard reaction products (advanced glycation end-products; AGEs) in the hyperglycemic silkworms and restored growth, suggesting that the growth defect of hyperglycemic silkworms is caused by AGE accumulation in the hemolymph. Furthermore, we identified galactose as a hypoglycemic compound in jiou, an herbal medicine for diabetes, by monitoring its hypoglycemic activity in hyperglycemic silkworms. These results suggest that the hyperglycemic silkworm model is useful for identifying anti-diabetic drugs that show therapeutic effects in mammals.

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Introduction

The number of individuals diagnosed with type 2 diabetes mellitus, which is caused by insulin resistance and/or abnormal insulin secretion, is increasing worldwide [1], creating a strong demand for the development of more effective anti-diabetic drugs. Blood glucose levels are regulated by hormones such as insulin that regulate glucose uptake and metabolism in tissues throughout the body. Evaluation of the effects of anti-diabetic drugs thus requires the use of an animal model. The use of mammalian animals to screen for anti-diabetic drugs, however, is not only very expensive from an animal husbandry perspective, but also presents ethical problems in terms of animal welfare.

We previously reported that a silkworm infection model can be utilized to evaluate antibacterial and antiviral agents, and that there are a number of similarities in the pharmacokinetics of antibiotics between silkworms and mammals [2,3,4,5,6]. It is far less costly to rear silkworms than mammals, and a large number of larvae can be maintained in a small space. Screening of therapeutic agents can be easily performed with a large number of individual silkworms without the same ethical concerns involved in the use of mammals. Thus, we aimed to establish a method for evaluating the anti-diabetic effects of compounds using silkworms (Figure S1). Here we propose an invertebrate animal model of the disease utilizing the silkworm to evaluate the therapeutic effects of drugs.

Results

Immediate increase in sugar concentration in hemolymph of silkworms fed a high-glucose diet

To establish a hyperglycemic silkworm model, we first evaluated the conditions required to induce hyperglycemia in silkworms. Silkworms fed a high-glucose diet (10% glucose-containing diet) for 1 day had a greater than 4-fold increase in the hemolymph sugar level compared with silkworms fed a normal diet (Figure 1A). The hemolymph sugar level of fasted silkworms was less than half that of silkworms fed a normal diet. The amount of sugar in the fat body, which corresponds to liver and adipose tissue in mammals, was also higher in silkworms fed a high-glucose diet than in silkworms fed a normal diet (Figure 1B). Increased sugar in the muscle and in the malpighian tubule, which corresponds to the

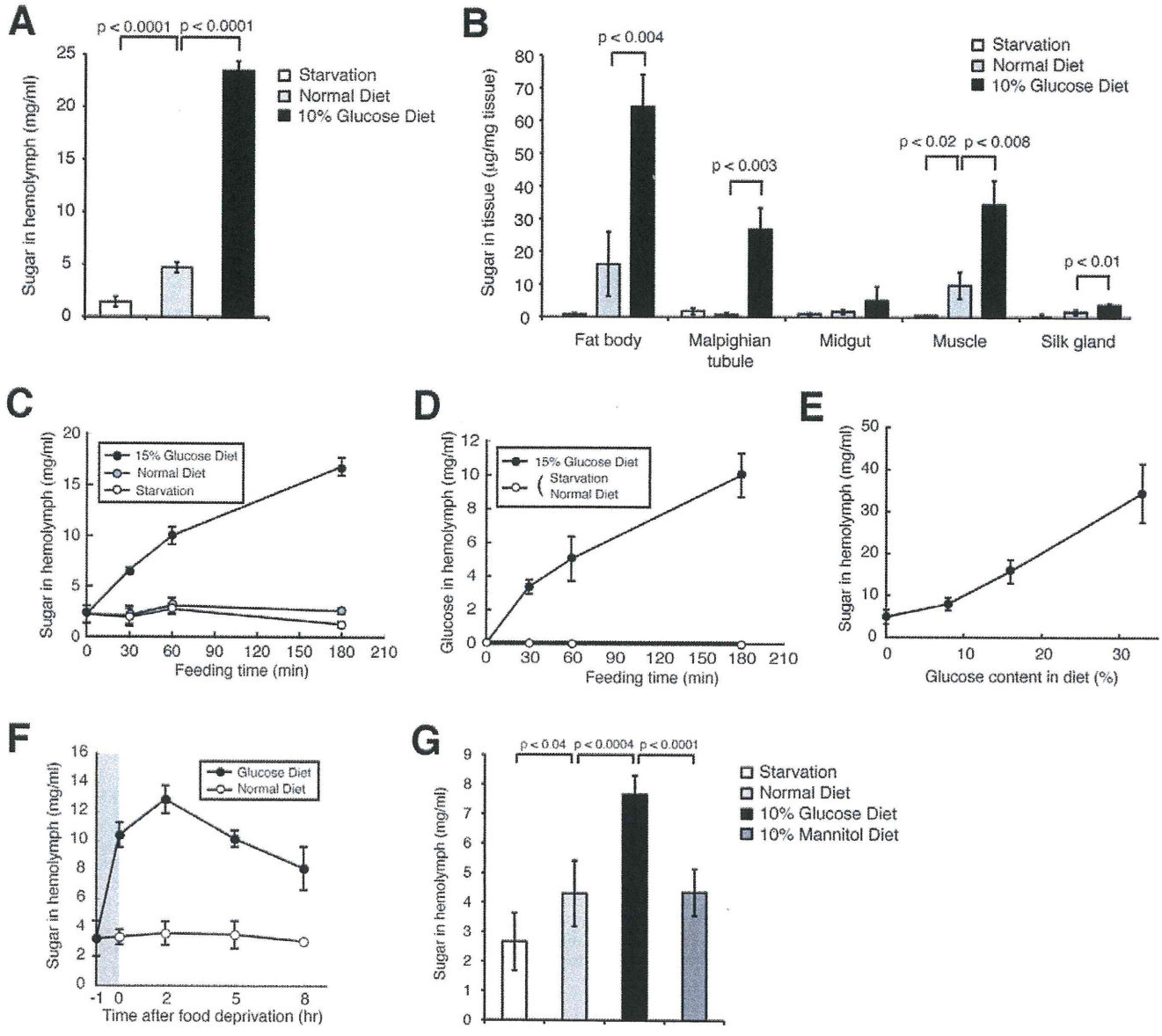

Figure 1. A high-glucose diet in silkworms increased hemolymph sugar levels. (A, B) Silkworms were fed a normal diet or 10% (w/w) glucose diet or fasted for 24 h, and then the hemolymph sugar level was determined (A). n = 5 per group. The sugar level in the fat body, malpighian tubule, midgut, muscle, and silk gland was determined (B). n = 3 per group. Data represents means ± SD. (C, D) Silkworms were fed a normal diet, 15% (w/w) glucose diet, or fasted for 0, 30, 60, or 180 min. Total sugar level (C) and glucose level (D) in the hemolymph were determined. n = 4–5 per group. (E) Hemolymph sugar levels in silkworms fed a normal diet, or an 8%, 16%, or 33% (w/w) glucose diet for 60 min. n = 3 per group. (F) Silkworms were fed a normal diet or 12% (w/w) glucose diet for 1 h (shown in gray) then fasted. Sugar level in hemolymph before feeding and at 0, 2, 5, or 8 h after fasting was determined. n = 5 per group. (G) Silkworms were fed a normal diet, 10% (w/w) glucose diet, 10% (w/w) mannitol diet, or fasted for 60 min, and hemolymph sugar levels were measured. n = 5 per group.
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mammalian kidney, was also observed in silkworms fed a high-glucose diet, although the amount of sugar was lower than that in the fat body (Figure 1B). The amount of sugar in the fat body of fasted silkworms was less than one-tenth that in silkworms fed a normal diet. Therefore, hemolymph and fat body sugar levels could be manipulated in silkworms by either feeding them a high-glucose diet or by fasting them.

We then examined the time course of the increase in total sugar in the silkworm hemolymph during feeding with a high-glucose diet. Hemolymph sugar levels in silkworms fed a high-glucose diet increased 2-fold by 30 min, 4-fold by 60 min, and 6-fold by 180 min after feeding, respectively (Figure 1C). Hemolymph sugar levels in silkworms either fasted or fed a normal diet did not increase for up to 180 min. Glucose levels in the hemolymph were also measured using the glucose oxidase method. Glucose levels in the hemolymph of silkworms fed a high-glucose diet increased rapidly, whereas no glucose was detected in the hemolymph of silkworms either fed a normal diet or fasted (Figure 1D). We next tested whether hemolymph sugar levels increased according to the glucose content in the diet. Hemolymph sugar levels increased following intake of up to a 33% glucose diet without saturation (Figure 1E). These findings indicated that silkworms can be made hyperglycemic by feeding them a high glucose-containing diet. Hemolymph sugar levels in silkworms fed a high-glucose diet for 1 h began to decrease after fasting for the subsequent 2 h (Figure 1F). Hemolymph sugar

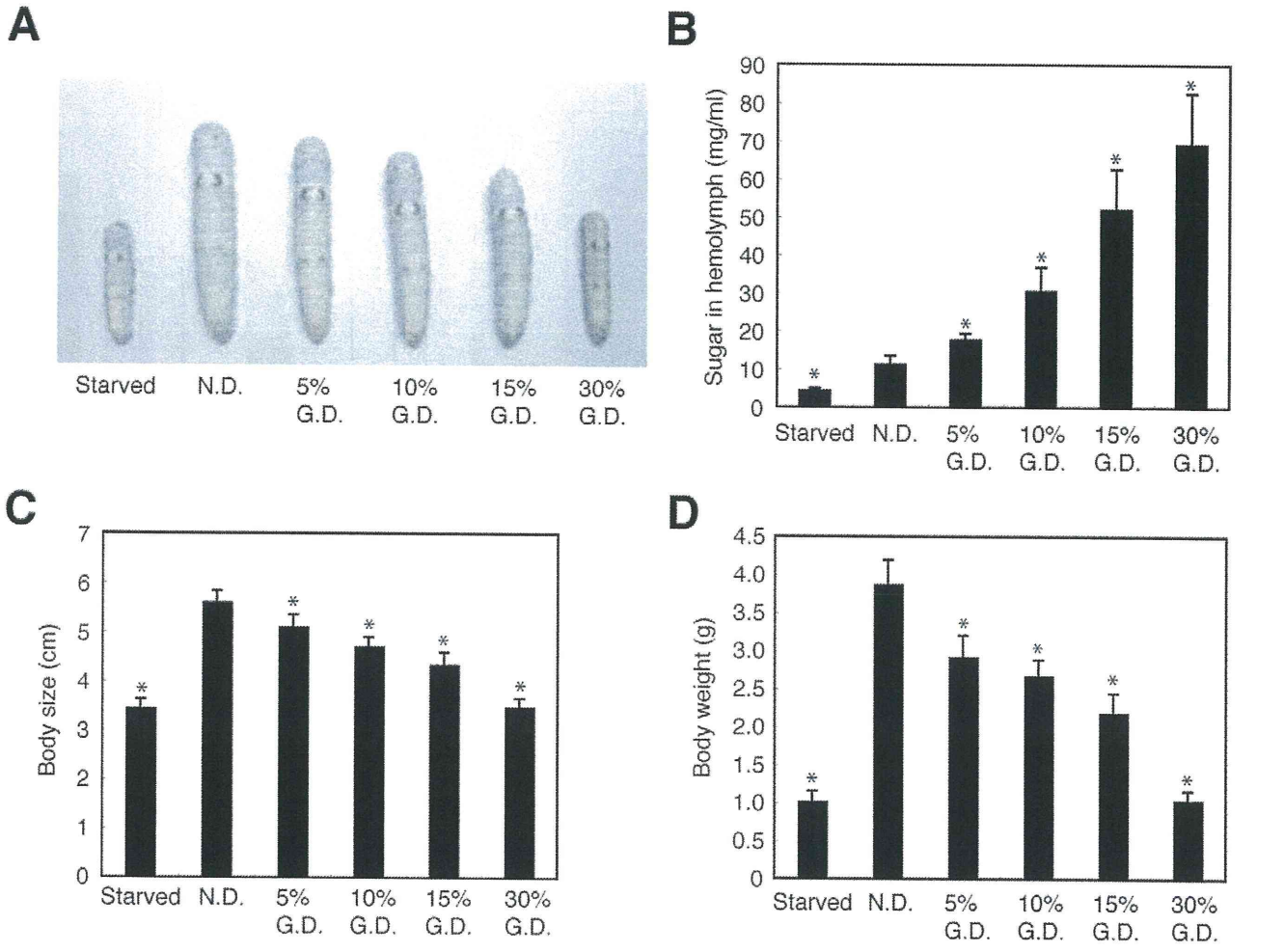

Figure 2. A high-glucose diet in silkworms inhibited growth. (A–D) Female silkworms were fed a normal diet (N.D.), a 5%, 10%, 15%, or 30% (w/w) glucose diet (G.D.), or fasted for 3 days. Sugar level in hemolymph (B), body size (A, C), and body weight (D) were determined. n = 8–15 per group. *$p<0.0001$ versus saline injected silkworms fed a normal diet (N.D.). Data are represented as means ± SD. In all panels, the statistical significance of the difference was evaluated using Student's *t* test.
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levels were increased in silkworms fed a normal diet for 24 h. Fasting for the subsequent 12 h induced a drop in the hemolymph sugar level to that of continuously fasted silkworms (Figure S2). Silkworms fed a 10% mannitol diet did not show the increase in hemolymph sugar levels observed in silkworms fed a 10% glucose diet (Figure 1G). Thus, we assume that glucose is taken up in the silkworm midgut by a specific transporter-mediated system, thereby increasing the hemolymph sugar level. Together, these findings suggest that silkworms have a regulatory system for maintaining hemolymph sugar levels. Hemolymph sugar levels in silkworms fed a high glucose-containing diet increased more than 2-fold, indicating that we established a hyperglycemic model with silkworms. Diabetic patients generally suffer from several disorders due to hyperglycemia. We investigated whether hyperglycemia induced by feeding silkworms a high-glucose diet caused disorders. A high-glucose diet for 3 days increased hemolymph sugar levels (Figure 2B). Growth, in terms of body size and weight, was inhibited in both male and female hyperglycemic silkworms (Figure 2A, C, D, and Figure S3). Furthermore, administration of glucose into the silkworm hemolymph also increased sugar levels in the hemolymph and impaired growth (Figure 3).

Human insulin and AICAR decrease sugar levels in silkworm hemolymph

We next examined whether the hypoglycemic effect of anti-diabetic drugs can be evaluated using hyperglycemic silkworms. Insulin is a major therapeutic agent for patients with type I diabetes. The administration of recombinant human insulin decreased the hemolymph sugar level in silkworms fed a high-glucose diet (Figure 4A, and Figure S4A, B). In mammals, insulin enhances glucose uptake via Akt phosphorylation in tissues such as adipose tissue [7]. We tested whether human insulin enhanced glucose uptake into the fat body, the silkworm organ that corresponds to mammalian adipose tissue, in an *in vitro* culture system using isolated fat bodies. The amount of sugar in cultured fat bodies increased in a time-dependent manner after adding glucose to the medium (Figure 4B), indicating that isolated fat bodies have the capacity to take up glucose from the culture medium. Moreover, the addition of human insulin to the medium led to an increase in the total sugar and phosphorylated Akt in the fat bodies (Figure 4C–E). These effects by human insulin were inhibited by wortmannin, an inhibitor of phosphoinositide 3 (PI3) kinase (Figure 4C, E). Furthermore, the hypoglycemic effect of human insulin was blocked by the administration of wortmannin (Figure 4F). These results suggest that human insulin induces glucose uptake via

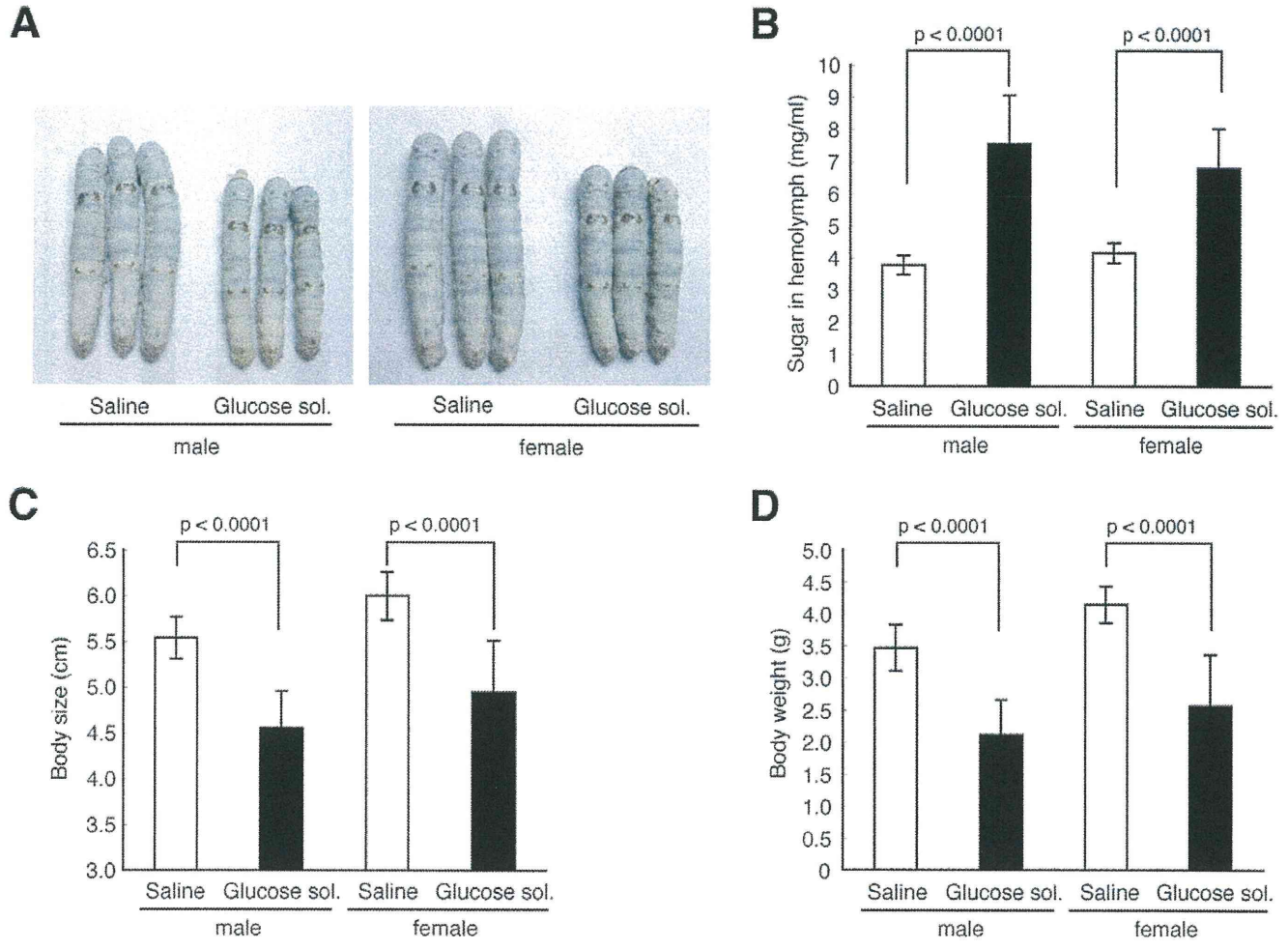

Figure 3. Growth inhibition of silkworms after injection of glucose. (A–D) Silkworms were fed a normal diet and injected with glucose solution (40%) or saline into the hemolymph every 12 h for 3 days. Hemolymph sugar level (B), body size (A, C), and body weight (D) were determined. n = 10–20 per group. Data represents means ± SD.
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the activation of phosphoinositide 3 kinase in the silkworm fat body, as in insulin-stimulated mammalian adipose tissue. Activation of the AMPK signaling pathway decreases blood glucose levels in mammals [8]. We examined whether AICAR, which activates AMPK, lowers sugar levels in silkworm hemolymph. AICAR injection decreased the hemolymph sugar level in hyperglycemic silkworms (Figure 5A). Moreover, the amount of phosphorylated AMPK was increased by the addition of AICAR to an *in vitro* culture system using isolated silkworm fat bodies (Figure 5B, and Figure S5). These results suggest that AICAR activates the AMPK pathway in the fat body and lowers sugar levels in silkworm hemolymph. We next examined whether human insulin or AICAR can restore the growth defect of hyperglycemic silkworms. In silkworms fed a 10% glucose diet for 4 days, both body size and weight were reduced compared to silkworms fed a normal diet. Under this condition, injection of human insulin or AICAR into the hemolymph of the hyperglycemic silkworms increased body size and weight compared to saline-injected controls (Figure 6). This finding suggests that human insulin and AICAR reverse the growth defect in hyperglycemic silkworms by lowering total sugar levels in the hemolymph. Therefore, the anti-diabetic effects of candidate drugs that activate the insulin signaling pathway and/or the AMPK signaling pathway can be evaluated using a silkworm hyperglycemic model.

Increase in the amount of AGEs in the hemolymph of hyperglycemic silkworms

The Maillard reaction is a series of nonenzymatic reactions, where carbonyl groups of reducing-sugars and amino groups of proteins form Schiff bases, which subsequently undergo Amadori rearrangements and oxidative modifications. The end result of these complex reactions is the formation of advanced glycation end-products (AGEs), which are considered to cause disorders in the tissues and blood vessels of diabetic patients. Recent studies suggest a correlation between the accumulation of AGEs and diabetic nephropathy [9,10]. We examined whether AGEs are present in the hemolymph of hyperglycemic silkworms with impaired growth. A 120-kDa protein was detected in silkworm hemolymph using an anti-AGEs antibody by Western blot analysis (Figure 7A). The amount of the 120-kDa protein detected by anti-AGEs antibody was higher in silkworms fed a high-glucose diet (Figure 7A). Aminoguanidine, an inhibitor of the Maillard reaction, has therapeutic effects against cardiac hypertrophy and albuminuria in a diabetic rat model [11,12]. Injection of aminoguanidine inhibited the increase of the 120-kDa AGEs in hyperglycemic silkworms (Figure 7A). We further tested whether aminoguanidine reverses/ameliorates the growth defect of hyperglycemic silkworms. Repeated injections of aminoguanidine in

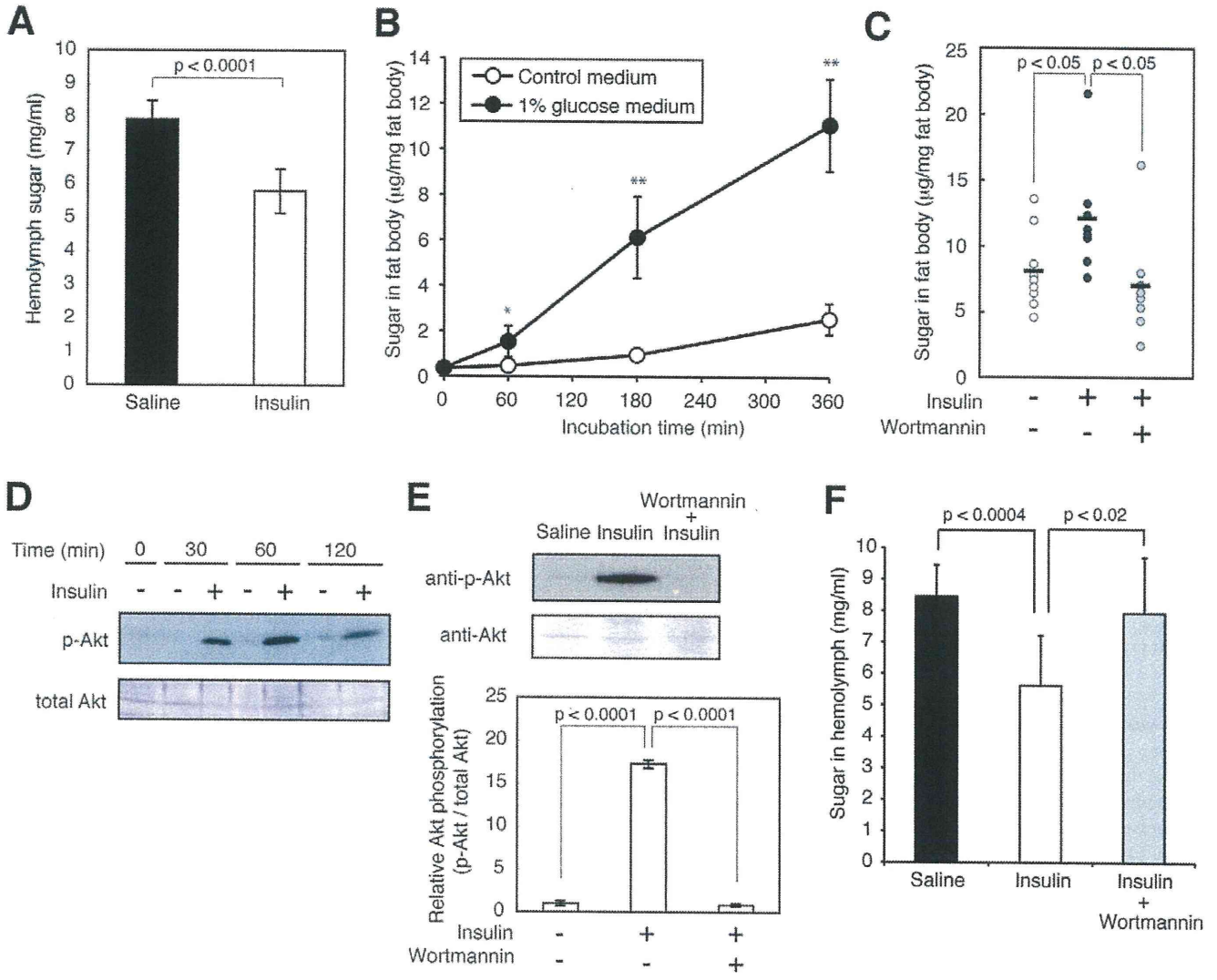

Figure 4. Decrease in the hemolymph sugar level of hyperglycemic silkworms and increase in Akt phosphorylation in the fat body by human insulin. (A) Silkworms were fed a 10% (w/w) glucose diet for 60 min. After removal of the diet, 50 μl of human insulin (3.5 mg/ml) was administered into the hemolymph of the hyperglycemic silkworms. Hemolymph sugar level was measured 6 h after injection. n = 6–7 per group. Data are shown as means ± SD. (B) Increase in total sugar in fat bodies cultured in insect medium containing 1% glucose. Isolated fat bodies from silkworm were cultured in Grace's insect medium containing 1% glucose for 0, 1, 3, or 6 h, and the amount of sugar in fat body was measured. n = 4–5 per group. *p<0.05, **p<0.001 versus control medium samples. (C) Isolated fat body from silkworm was cultured in Grace's insect medium containing 1% glucose with or without wortmannin (0.01 mM) for 30 min and further cultured with or without human insulin (final conc. 0.7 mg/ml) for 3 h. Total sugar in fat body was determined. n = 8–9 per group. (D) Isolated fat bodies from silkworm were cultured with human insulin (final conc. 0.6 mg/ml) in Grace's insect medium for 0, 30, 60, or 120 min. Fat bodies were homogenized and extracts were prepared. Total Akt and phosphorylated Akt were detected by immunoblot analysis. (E) Isolated fat body from silkworm was cultured in Grace's insect medium with or without wortmannin (0.01 mM) for 30 min and further cultured after adding human insulin (3 mg/ml) for 3 h. Immunoblots of total Akt and phosphorylated Akt (Top) and calculation of relative Akt phosphorylation (Bottom). n = 3 per group. Bottom data are shown as means ± SD. (F) Cancellation of the hypoglycemic effect of human insulin by co-administration of wortmannin. Silkworms were fed a 10% (w/w) glucose diet for 60 min. After removal of the diet, 50 μl of human insulin (3.5 mg/ml) with or without wortmannin (0.5 mM) was administered into the hemolymph of the hyperglycemic silkworms. Hemolymph sugar level was measured 6 h after injection. n = 9–10 per group. Data are shown as means ± SD. Statistical significance between groups was evaluated using Student's *t* test.
doi:10.1371/journal.pone.0018292.g004

silkworms fed the high-glucose diet resulted in an increase of both body size and weight (Figure 7B–D). These results suggest that aminoguanidine ameliorates the growth defect of hyperglycemic silkworm by inhibiting AGE production in the hemolymph.

Identification of galactose as an effective compound to decrease blood sugar levels

We next tested whether hyperglycemic silkworms are useful for identifying hypoglycemia-inducing compounds. Jiou, an herbal medicine, is considered to be effective for diabetic patients. Jiou has therapeutic effects in diabetic mouse and rat models [13]. A hot water extract of jiou reportedly has hypoglycemic activity in the streptozotocin induced-diabetic mouse model [13]. The active compound for the hypoglycemic effect in jiou, however, was not previously identified. We attempted to identify the hypoglycemia-inducing compound in jiou by using our silkworm diabetic model. Injection of the jiou extract decreased hemolymph sugar levels in hyperglycemic silkworms (Figure 8A, B). We presumed that the

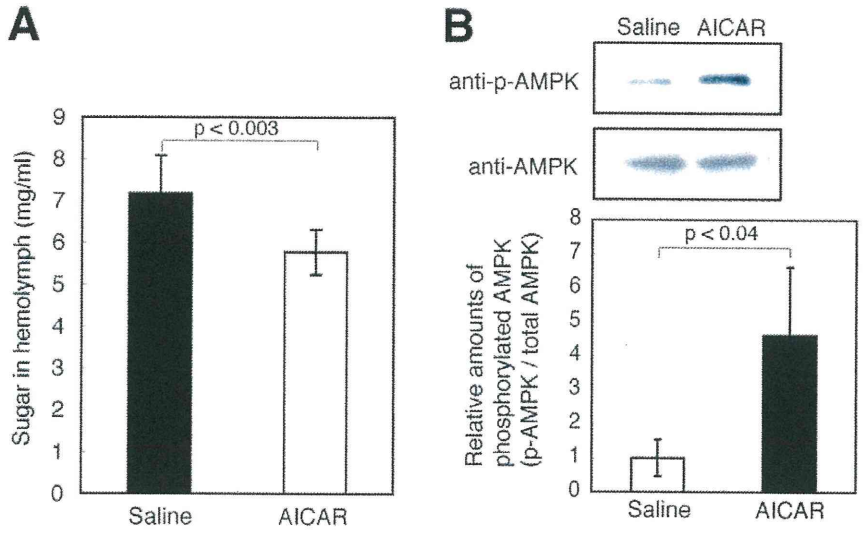

Figure 5. Decrease in the hemolymph sugar level of hyperglycemic silkworms and increase in AMPK phosphorylation in the fat body by AICAR. (A) Silkworms were fed a 10% (w/w) glucose diet for 60 min. After cessation of the diet, 50 μl of AICAR (4 mg/ml) was administered into the hemolymph of the hyperglycemic silkworms. Hemolymph sugar levels were measured 6 h after injection. n = 8–10 per group. Data are shown as means ± SD. (B) Western blot analysis of phosphorylated AMPK in fat body. Isolated fat body from silkworm was cultured with addition of AICAR (final conc. 0.8 mg/ml) for 2 h. Immunoblots of total AMPK and phosphorylated AMPK (Top) and calculations of relative AMPK phosphorylation (Bottom). n = 3 per group. Data at the bottom of the figure are shown as means ± SD.
doi:10.1371/journal.pone.0018292.g005

jiou extract contains a polysaccharide for two reasons; (i) the estimated sugar weight in the jiou extract was approximately half that of the dry weight, and (ii) the extract formed a precipitate after the addition of ethanol (Figure 8A). Therefore, we assumed that the polysaccharide in the jiou extract possessed a hypoglycemic effect. Thin layer chromatography (TLC) analysis of trifluoroacetic acid (TFA)-hydrolyzed materials from jiou revealed a single spot, which migrated to the same position as galactose. This spot was not observed when TFA-treatment was omitted (Figure 8C). Under this TLC condition, glucose, mannose, fructose, xylose, and arabinose migrated faster than galactose (Figure 8C, D). This finding suggests that the jiou extract contains galactose polymers. We next tested whether galactose shows hypoglycemic activity in hyperglycemic silkworms. Injection of galactose decreased the hemolymph sugar levels in hyperglycemic silkworms (Figure 8E). On the other hand, glucose, talose, and mannose, which are structural isomers of galactose, did not show this hypoglycemic effect (Figure 8E, F). We next tested whether galactose exerts hypoglycemic activity in a mammalian diabetic model. Intraperitoneal injection of galactose decreased the blood glucose levels in streptozotocin induced-diabetic mice (Figure 8G). Therefore, the hypoglycemic effect of galactose was demonstrated in hyperglycemic silkworms and in diabetic mice. To explore the molecular mechanism of the blood glucose reducing effect of galactose, we analyzed the expression level of glucose transporter 2 (GLUT2) in the liver of streptozotocin-induced diabetic mice. GLUT2 is expressed in the liver and facilitates glucose uptake [14]. GLUT2 levels in the membrane fraction prepared from the liver of diabetic mice were increased by galactose administration (Fig. 8H).

Discussion

The findings of the present study demonstrated that hyperglycemia can be induced in silkworms by feeding a diet containing glucose. The total amount of sugar increased in the fat bodies of the hyperglycemic silkworms. An increase in hemolymph sugar in silkworms may lead to the uptake and accumulation of sugar in the fat bodies, similar to what is observed in the liver and adipose tissue in mammals. Trehalose, a dimer of two glucose molecules, is a major sugar in insect hemolymph and glucose is generally not detected in insect hemolymph. We detected glucose in the hemolymph of silkworms fed a high-glucose diet.

To examine whether glucose uptake is mediated by a specific transporter, we examined the dose response of glucose in the medium on sugar accumulation in the fat body. Excess glucose in the medium resulted in saturation of the sugar accumulation in the fat body (Figure S6). This finding suggests that at least in the fat body, sugar does not passively diffuse into the organ but is transported by a specific uptake system. Moreover, silkworms have a trehalose and glucose transporter, Tret1 (Trehalose transporter 1) [15]. In silkworms fed a normal diet, higher levels of Tret1 are expressed in muscle and in the fat body compared to the midgut, silk gland, or malpighian tubules [15]. The data we present here show that under normal diet conditions, sugar accumulation per unit weight of tissue is higher in muscle and in the fat body than in the midgut, silk gland, or malpighian tubules (Fig. 1B). The high sugar accumulation detected in organs expressing high levels of Tret1 indicates the possibility that sugar uptake is regulated by sugar transporters.

The administration of human insulin or AICAR decreased the hemolymph sugar level in hyperglycemic silkworms. This study is the first report demonstrating the possibility of evaluating the therapeutic effect of anti-diabetic drugs in an invertebrate hyperglycemic animal model. We also demonstrated that human insulin enhances the uptake of sugar into the fat body of silkworms by Akt phosphorylation via the activation of phosphoinositide 3 kinase. Therefore, the hypoglycemic effect of human insulin in hyperglycemic silkworms is due to activation of the insulin signaling pathway in silkworms, similar to mammals. Silkworms have bombyxin, a peptide hormone with structural similarity to human insulin [16]. Bombyxin increases phosphorylated Akt in silkworms [17]. Moreover, injection of glucose promotes the

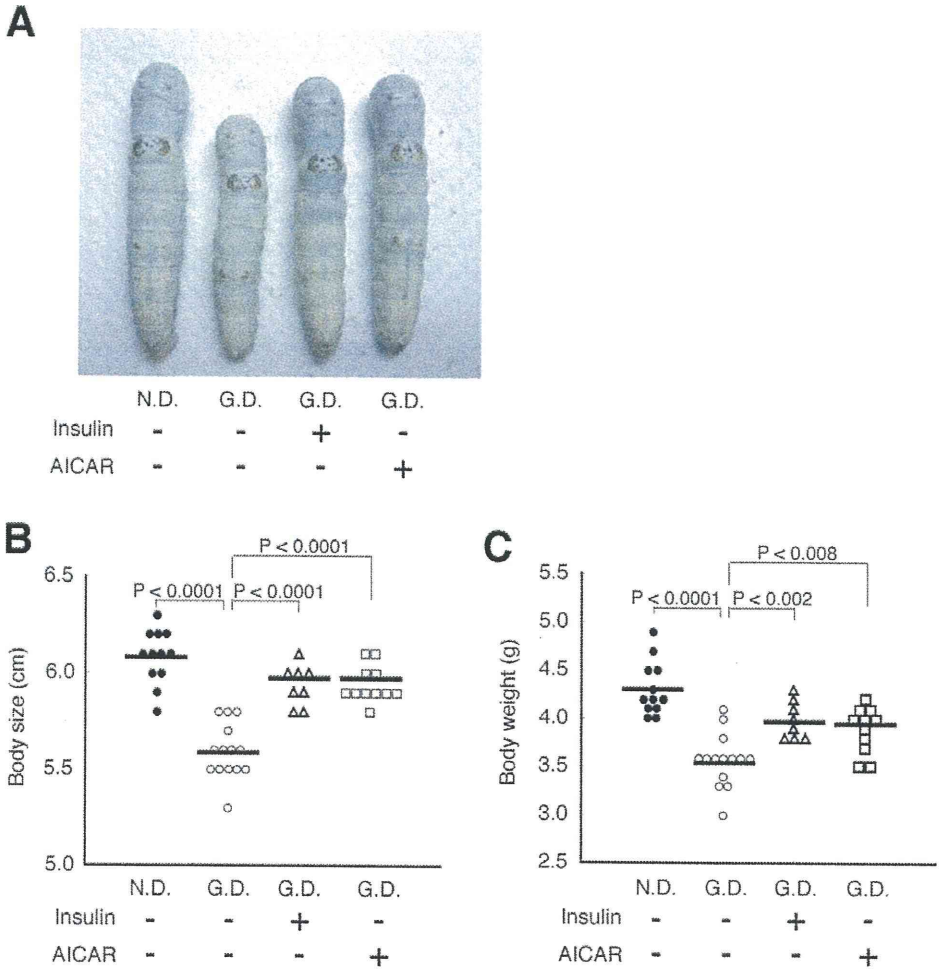

Figure 6. Repeated injections of human insulin and AICAR ameliorated growth defect in silkworm fed with high-glucose diet. (A–C) Silkworms were fed a normal diet (N.D.) or a 10% (w/w) glucose diet (G.D.) for 4 days. During the 4 days, silkworms were injected with 50 μl of human insulin (3.5 mg/ml), AICAR (4 mg/ml), or saline into the hemolymph every 12 h. Body size (A, B) and body weight (C) were measured. n = 8–14 per group. Bar represents mean. In all panels, the statistical significance of the difference was evaluated using Student's *t* test.
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release of bombyxin into the hemolymph [18]. Silkworms might control the hemolymph sugar level by activating the insulin-signaling pathway with bombyxin. Activation of AMPK by AICAR also decreased hemolymph sugar levels in silkworms. Therefore, the anti-diabetic effects of candidate drugs that activate the insulin signaling pathway and/or the AMPK signaling pathway can be evaluated using a silkworm hyperglycemic model.

Impaired growth, a characteristic feature of hyperglycemic silkworms, may be due to the accumulation of AGEs. Injection of aminoguanidine, an inhibitor of the Maillard reaction, restored the impaired growth of hyperglycemic silkworms. There are established rodent models of diabetic complications, such as nephropathy, peripheral neuropathy, and retinopathy. Several months, however, are required to induce these complications. By comparison, the growth defect of hyperglycemic silkworms was observed within 3 days. Therefore, the hyperglycemic silkworm model may be highly useful for quickly evaluating the therapeutic effects of anti-diabetic drugs. Hemolymph sugar levels were not significantly increased in silkworms fed a diet with added olive oil or oleic acid (Figure S7A). Silkworms fed a high fat diet had low body weight and a low food intake (Figure S7B, C). These findings suggest that silkworms fed a high fat diet eat less, resulting in growth inhibition. Thus, compared to a high fat diet, a high glucose diet rapidly induces hyperglycemia in silkworms.

We previously reported similarities between silkworms and mammals with regard to drug toxicity and pharmacokinetics. 1) The therapeutic concentrations of antibiotics are similar in both a silkworm infection model and a mammalian infection model [2,4]. 2) Intestinal uptake of several compounds is similar between silkworms and mammals [19]. 3) Like humans, silkworms have drug excretion mechanisms such as oxidization mediated by P450 and conjugation [3]. 4) The LD50 of toxic compounds is similar between silkworms and mammals [3], 5). Compounds with a relatively long half-life in mammals are also stable in silkworms [20]. Thus, we assume that silkworms could be useful for evaluating the drug toxicity and pharmacokinetics of compounds *in vivo*.

We screened for anti-diabetic agents using the hyperglycemic silkworm model. We found that an extract of jiou, an herbal medicine used to treat diabetes, has hypoglycemic effects when administered to hyperglycemic silkworms. Moreover, we demonstrated that galactose, a major component of the polysaccharides in jiou, had hypoglycemic activity in the silkworm diabetic model. Structural isomers of galactose, such as glucose, talose, and

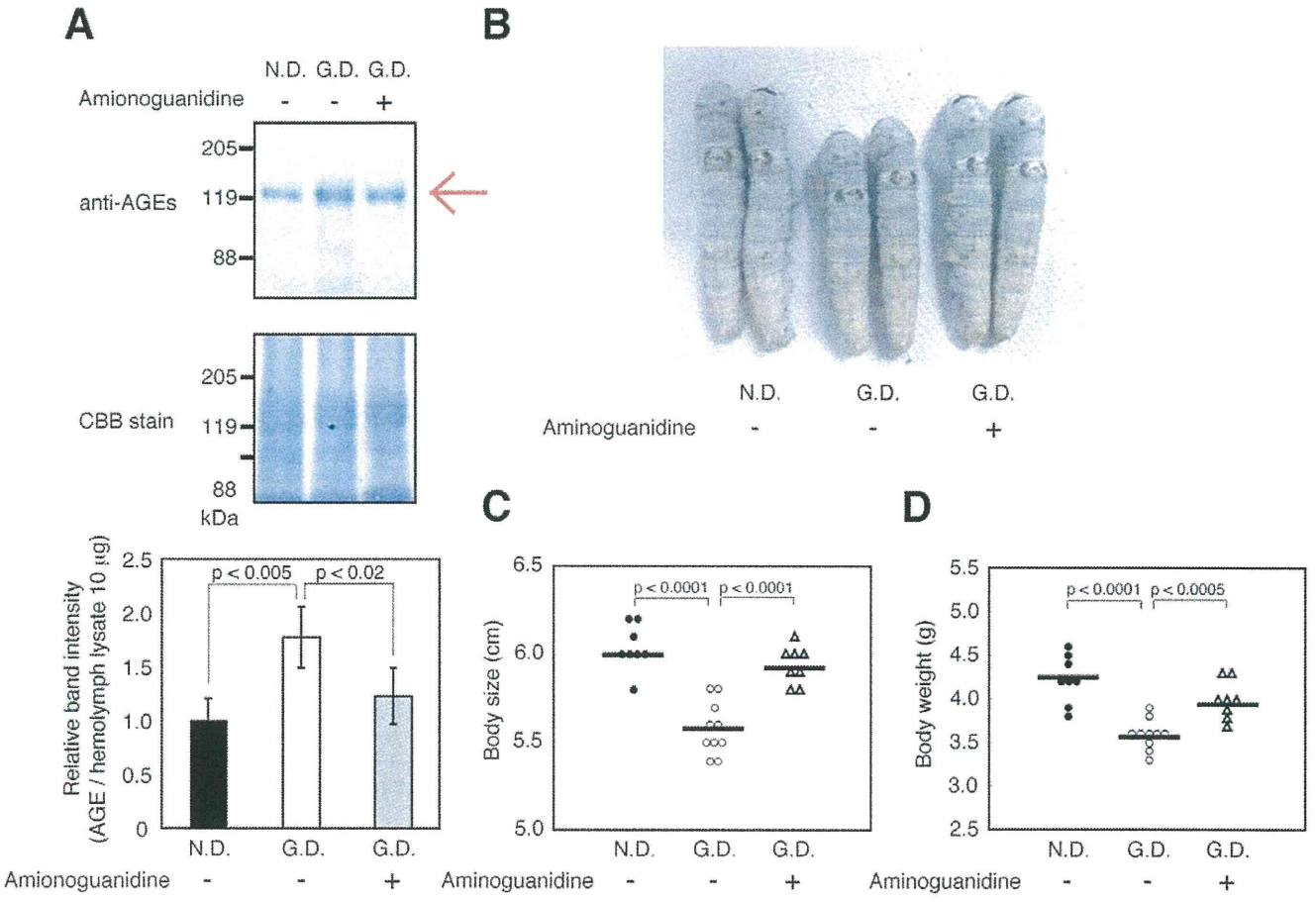


Figure 7. Increase in the amount of AGEs in the silkworm hemolymph after ingestion of a high glucose diet and decrease in AGEs after injection of aminoguanidine. (A) Western blot analysis of AGEs in the hemolymph. Silkworms were fed a normal diet (N.D.) or a 10% (w/w) glucose diet (G.D.) for 4 days. During the 4 days, silkworms were injected with 50 μl of aminoguanidine (10 mM) into the hemolymph every 12 h. The AGEs in hemolymph were determined by Western blot analysis with anti-AGE antibody. Hemolymph proteins were stained by Coomasie Brilliant Blue R-250. n = 3–4 per group. Top, immunoblots of AGEs. Middle, Coomasie Brilliant Blue staining. Bottom, calculations of relative band intensity. Bottom data are shown as means ± SD. (B–D) Body size (B, C) and body weight (D) were measured. n = 8–10 per group. Bar represents mean. In all panels, the statistical significance of the difference was evaluated using Student's *t* test.
doi:10.1371/journal.pone.0018292.g007

mannose did not have this hypoglycemic effect. In these galactose isomers, the position of the hydroxyl group(s) at C-4, C-2, or both differ from galactose. Therefore, the position of these hydroxyl groups in galactose is important for the hypoglycemic activity. Galactose also had a hypoglycemic effect in streptozotocin induced-diabetic mice. These findings suggest that the hyperglycemic silkworm model is useful for identifying anti-diabetic drugs that show therapeutic effects in mammals. To our knowledge, this is the first report that galactose has a hypoglycemic effect. Galactose is thought to have a hyperglycemic effect because it is isomerized to glucose in cells. The administration of excess amounts of galactose resulted in an increase in blood sugar levels in both mice and silkworms (data not shown). Accordingly, there is an optimal dose for galactose to exert its hypoglycemic activity. Some investigators reported that fructose and glucose have differential effects on food intake [21,22]. On the other hand, the effects of galactose on the maintenance of blood sugar levels may differ from those of glucose. GLUT2 levels in the membrane fraction prepared from the liver of streptozotocin-induced diabetic mice were increased by galactose administration compared to that after injection of PBS or glucose. This result suggests that the specific action induced by galactose, upregulation of the GLUT2 level in the membrane and corresponding upregulation of glucose uptake into the liver, accounts, at least in part, for the blood glucose lowering effect. Understanding the molecular mechanism of the hypoglycemic activity of galactose may pave the way for the development of galactose derivatives as candidate anti-diabetic drugs.

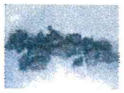
The use of animals in experimental research should follow the guiding principles proposed by Russell and Burch in 1959, referred to as the "three R's" (Replacement, Reduction, and Refinement) [23]. Thus, screening for anti-diabetic drugs using a large number of mammalian animal models such as mice and rats is difficult because of ethical issues, especially in terms of animal welfare. Russell and Burch also introduced the concept of relative replacement, which recommends using invertebrate models instead of mammalian animals. Our newly-developed invertebrate hyperglycemic model using silkworms matches this concept.

Materials and Methods

Silkworm rearing conditions, glucose diet preparation, and injection methods

Fertilized eggs of silkworm, *Bombyx mori* (Hu·Yo x Tukuba·Ne; Ehime Sanshu), were kept in disposable plastic containers at 27°C.

A



Jiou (238 g)

Extraction with 100% EtOH

100% EtOH precipitates

Extraction with hot 70% EtOH

70% EtOH precipitates

Extraction with hot water

Hot water extract
(A quarter of hot water extract was used to next step)

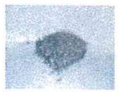
Dialysis against water

Internal solution

Extraction with 100% EtOH

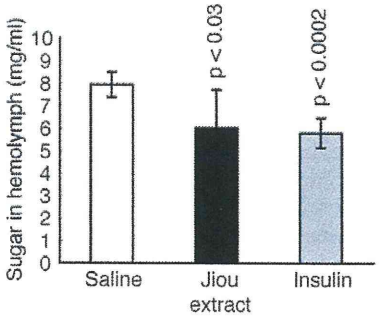
100% EtOH precipitation

Freeze drying

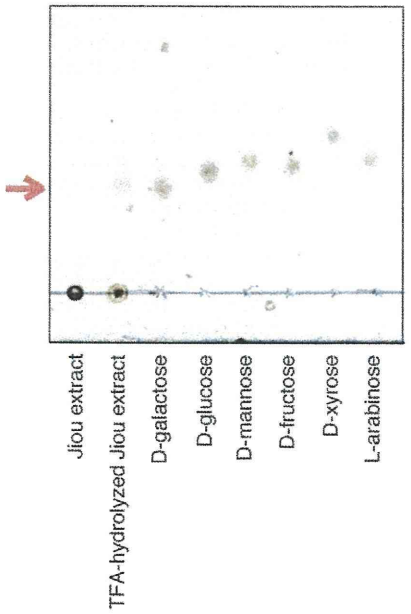


Jiou extract (1.0 g)

B



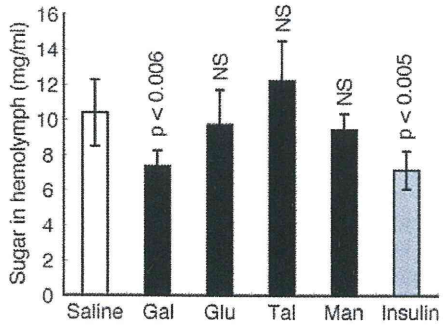
C



D

	Rf value
TFA-hydrolyzed Jiou extract (red arrow)	0.39
D-galactose	0.39
D-glucose	0.44
D-mannose	0.47
D-fructose	0.46
D-xyrose	0.56
L-arabinose	0.47

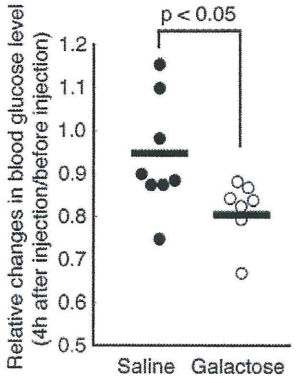
E



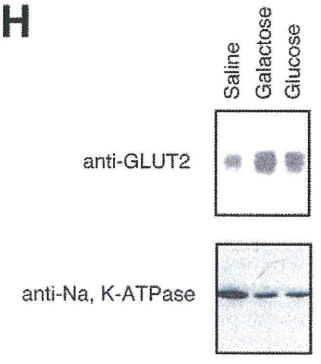
F

	Gal	Glu	Tal	Man
1	CHO	CHO	CHO	CHO
2	H–C–OH	H–C–OH	HO–C–H	HO–C–H
3	HO–C–H	HO–C–H	HO–C–H	HO–C–H
4	HO–C–H	H–C–OH	HO–C–H	H–C–OH
5	H–C–OH	H–C–OH	H–C–OH	H–C–OH
6	CH_2OH	CH_2OH	CH_2OH	CH_2OH
Activity	+	-	-	-

G



H



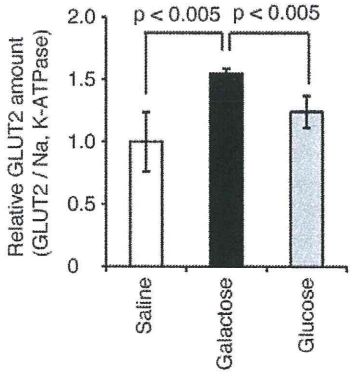


Figure 8. Identification of galactose as a hypoglycemic compound using the hyperglycemic silkworm model. (A) Preparation protocol for the jiou extract. (B) Silkworms were fed a 10% (w/w) glucose diet for 60 min. 50 µl of Jiou extract (1 mg/ml) or human insulin (3.5 mg/ml) was injected into the hemolymph of the hyperglycemic silkworms. The silkworms were fasted for 6 h and the sugar level in the hemolymph was determined. n = 6–7 per group. Data are shown as means ± SD. (C, D) The jiou extract was treated with TFA, and analyzed by TLC. Sugars were localized with 10% sulfuric acid solution. (E) Silkworms were fed a 10% (w/w) glucose diet for 60 min. 50 µl of D-Galactose (Gal), D-glucose (Glu), D-talose (Tal), D-mannose (Man), (1 mg/ml) or human insulin (3.5 mg/ml) was injected into the hemolymph of hyperglycemic silkworms. Silkworms were fasted for 6 h and sugar level in the hemolymph was determined. n = 6 per group. (F) Structure of sugar is shown by Fischer projection in the panel. Numbers shown on the left indicate the carbon positions of the sugar. Red hydroxyl group indicates the positions that differ from D-galactose. Activity represents the hypoglycemic effect. Data are shown as means ± SD. NS; not significant. (G) Galactose (10 mg/ml, 0.5 ml i.p.) was injected to streptozotocin induced-diabetic mice, and blood glucose level was determined after 4 h of fasting. Blood glucose levels in streptozotocin-induced hyperglycemic mice were measured (blood glucose 250–400 mg/dl) and then the mice were treated with either PBS or galactose solution. Four hours after administration and the removal of diet, the blood glucose levels were measured again. The data represent the blood glucose value after treatment divided by the blood glucose value before treatment of individual animals. In all panels, the statistical significance of the difference was evaluated using Student's *t* test. (H) Blood glucose levels in streptozotocin-induced hyperglycemic mice were measured (blood glucose 250–400 mg/dl) and then the mice were treated with either PBS, galactose (200 mg/kg mouse, i.p.), or glucose (200 mg/kg mouse, i.p.) solution. Two hours after administration and removal of the diet, the mice were killed and the membrane fraction in mouse liver was isolated. GLUT2 and Na, K-ATPase were detected by Western blot analysis with anti-GLUT2 antibody or anti-Na, K-ATPase antibody. Immunoblots of GLUT2 and Na, K-ATPase (Top) and calculations of relative GLUT2 (Bottom). n = 3–4 per group. Data at the bottom of the figure are shown as means ± SD. In all panels, the statistical significance of the difference was evaluated using Student's *t* test.
doi:10.1371/journal.pone.0018292.g008

Hatched larvae were reared to the fifth instar on an artificial diet, SilkMate 2S, which contains antibiotics (Nosan Corporation), at 27°C. All experiments were performed using fifth-instar male larvae (0.9–1.0 g) fasted overnight during the fourth ecdysis, unless otherwise mentioned.

The glucose diet was prepared by mixing Silkmate 2S and D-glucose at the amounts indicated as the percentage of glucose in the total diet.

Injection experiments were performed as follows[24]. Sample solution (50 µl) was injected into the hemolymph at the second abdominal segment of the larva. Syringes (1 ml) and needles (27G×3/4) were purchased from Terumo.

Sugar quantification

Hemolymph (20 µl) was collected from the larva through a cut on the first proleg ands mixed with 9 volumes of 0.6N perchloric acid. Precipitated proteins were removed by centrifugation at 3000 rpm for 10 min at 4°C. The supernatant (hemolymph extract) was diluted with the appropriate volume of distilled water for sugar quantification.

Total sugar in the hemolymph was determined using the phenol-sulfuric acid (PSA) method[25]. Hemolymph extract (100 µl) was mixed vigorously with 100 µl of 5% phenol aqueous solution, followed by vigorous mixing with 500 µl sulfuric acid, incubation at room temperature for 20 min, and absorbance at 490 nm was measured. Serially diluted glucose solution was used as a standard.

Glucose in the hemolymph was determined using the glucose oxidase method. Hemolymph extract (20 µl) was mixed with 400 µl of reaction solution (0.12 M sodium-phosphate buffer [pH 7.4] containing 4 U/ml glucose oxidase, 3 U/ml peroxidase, and 9 mM *o*-dianisidine), followed by vigorous mixing with 100 µl of 70% sulfuric acid solution, incubation at room temperature for 40 min, and absorbance at 530 nm was measured. Serially diluted glucose solution was used as a standard.

The fat body, isolated from the dorsolateral region of the larva, was rinsed in insect saline (130 mM NaCl, 5 mM KCl, and 1 mM $CaCl_2$), and weighed. The fat body (1~10 mg) was lysed in 50 µl of 30% KOH with heating at 90°C for 10 min. Distilled water (150 µl) and ethanol (300 µl; final 60%) were added and the mixture was incubated at 90°C for 10 min. The samples were incubated at 4°C overnight and centrifuged at 15,000 rpm for 3 min. The precipitate was dissolved in distilled water to give a concentration of 50~100 mg fat body/ml by heating at 90°C for 10 min. The resulting fat body extract was used for sugar quantification by the PSA method. The amount of sugar in 1 mg of fat body was calculated.

Chemicals

Recombinant human insulin was purchased from Wako and dissolved in 0.9% NaCl containing 0.1% acetic acid. Wortmannin was purchased from Calbiochem. AICAR was purchased from Toronto Research Chemicals Inc. Jiou was purchased from Uchida Wakanyaku. D-Glucose was purchased from Nacalai Tesque. D-galactose, D-mannose, and D-talose were purchased from Wako.

In vitro fat body sugar uptake assay

The fat body (wet weight 2~10 mg) was isolated from the dorsolateral region of the larva, rinsed in insect saline, and cultured in 200 µl Grace's insect medium supplemented with 1% glucose and antibiotics (penicillin and streptomycin) at 27°C for 30 min. Test sample solution (50 µl) was added to the culture medium, and the fat body was cultured and lysed and then the amount of sugar was determined using the PSA method.

Immunoblot analysis

The fat body (wet weight 1~10 mg) was isolated from the dorsolateral region of the larva, rinsed in insect saline, and cultured in 200 µl Grace's insect medium supplemented with 1% glucose and antibiotics (penicillin and streptomycin) at 27°C for 30 min with or without wortmannin. Test sample solution (50 µl) was added to culture medium and the fat body was cultured and then transferred to NP-40 lysis buffer (10 mM Tris/HCl [pH 7.5], 150 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 1% NP-40, 10 mM NaF, and 1 mM Na_3VO_4) and lysed by sonication. The samples were centrifuged at 15,000 rpm for 3 min and proteins in supernatants were precipitated by trichloroacetic acid followed by centrifugation at 15,000 rpm for 15 min. The precipitates were washed twice with ice-cold ethanol, dissolved in a buffer with sodium dodecyl sulfate, heat-treated, and electrophoresed in a 12.5% polyacrylamide gel according to the method of Laemmli[26]. Proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (Millipore), probed with antibody, and detected using Western Lightning (Perkin-Elmer Life Sciences). The following antibodies were used for immunoblot analysis: rabbit polyclonal antibodies to total Akt, phosphorylated Akt, total AMPK, phosphorylated AMPK, Na, K-ATPase from Cell Signaling Technology, GLUT2 from ALPHA DIAGNOSTIC; and mouse polyclonal antibody to AGEs from Cosmo Bio Co., LTD.