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# Decrease of Plasma Glucose by Allantoin, an Active Principle of Yam (*Dioscorea* spp.), in Streptozotocin-Induced Diabetic Rats

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The effect of allantoin, an active component of yam, on plasma glucose of streptozotocin-induced diabetic rats (STZ-diabetic rats) is investigated. Allantoin decreased plasma glucose levels in a dose-related manner, which was reduced by pretreatment with naloxone or naloxonazine. A concomitant increase in plasma $\beta$-endorphin, detected by enzyme-linked immunosorbent assay, was observed. Moreover, allantoin enhanced $\beta$-endorphin release from the isolated adrenal medulla of STZ-diabetic rat in a dose-related manner. However, its plasma glucose lowering action was reduced but not totally abolished by bilateral adrenalectomy. Furthermore, allantoin directly increased radioactive glucose uptake in isolated skeletal muscle, and repeated administration for 3 days increased GLUT4 mRNA and protein levels in muscle. This effect was markedly reduced in STZ-diabetic rats with bilateral adrenalectomy. This study suggests that allantoin increases GLUT4 gene expression in muscle by increasing $\beta$-endorphin secretion from the adrenal gland in STZ-diabetic rats.

**KEYWORDS: Allantoin; blood glucose; $\beta$-endorphin; diabetes; opioid $\mu$-receptor**

## INTRODUCTION

Allantoin is mainly a botanical extract of the comfrey plant (*1*) and green tea (*2*). It is identified as an abundant and active component in yam (*Dioscorea* spp.) (*3*). Yam is an important plant that is widely used in the pharmaceutical industry, whereas *Dioscorea* rhizome contains ureides, including allantoin, used for the prevention of inflammation and ulcers (*4*). Moreover, the *Dioscorea* species accumulate allantoin mainly in tubes, and their herbs have been shown to aid in improving diabetic disorders (*5*). Shan-Yaw (*Dioscorea opposita*) has also been effective in improving insulin resistance in animals (*5*). It is one of the six herbs in the combined prescription of Di-Huang-Wan that has been applied to treat diabetes for a long time in Chinese traditional medicine (*6*). Die-Huang-Wan formula with Shan-Yaw removed shows no effect on blood glucose, suggesting that Shan-Yaw is the major herb for its blood lowering activity (*5*). However, its main component for the regulation of blood glucose remains unclear.

Activation of opioid $\mu$-receptors by endogenous $\beta$-endorphin or agonists reportedly exerts a blood glucose lowering effect (*7*−*9*). Loperamide activates opioid $\mu$-receptors to increase glucose uptake in $C_2C_{12}$ cells (*10*, *11*). In addition, stevioside increases glycogen synthesis though an opioid $\mu$-receptor-dependent pathway (*12*). However, the role of opioid $\mu$-receptors in allantoin-induced blood glucose lowering is unknown.

The present study administered allantoin into streptozotocin-induced diabetic rats (STZ-diabetic rats) to evaluate its effects on plasma glucose and investigate possible mechanisms in relation to the role of opioid $\mu$-receptors.

## MATERIALS AND METHODS

**Animals.** Male Wistar rats weighing 200−250 g were obtained from the Animal Center of National Cheng Kung University Medical College. These were housed in a temperature-controlled room (25 ± 1 °C) and kept on a 12:12 light/dark cycle (light on at 6:00 a.m.) with freely available water and standard laboratory diet. The STZ-diabetic rats were induced by intravenous (iv) injection of 65 mg/kg STZ (Sigma-Aldrich, St. Louis, MO). Rats with plasma glucose concentrations of >18 mmol/L were considered as type 1-like diabetes mellitus in addition to polyuria and other diabetic features (*13*). Experiments in rats were conducted 2 weeks after STZ injection. Because allantoin was reportedly degraded in the gastrointestinal tract (*14*) with marked loss after oral administration (*15*), it was administered intravenously. All animal procedures were performed according to the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and the guidelines of the Animal Welfare Act.

**Adrenalectomy.** Bilateral adrenalectomy was performed in the rats after sterostactic surgery under pentobarbital anesthesia (30 mg/kg, ip) as

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described previously (*16*). Bilaterally adrenalectomized rats were fed standard rat chow and 0.9% NaCl drinking water ad libitum. They were allowed to recover for 2 weeks after the operation, and diabetes was induced by STZ injection as described above. Blood samples were collected to determine levels of plasma glucose and $\beta$-endorphin.

**Laboratory Determinations.** Plasma glucose levels were measured by the glucose oxidase method using an automatic analyzer (Quik-Lab, Ames, Miles Inc., Elkhart, IN), whereas plasma $\beta$-endorphin level was determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Penisula Lab, Torrance, CA).

**Determination of Glucose Uptake.** Assay of glucose uptake in skeletal muscle was conducted according to a previous study (*17*). Briefly, the soleus muscle isolated from diabetic rats was incubated with allantoin or porcine insulin monocomponent (Novo Industries, Bagsvaerd, Denmark) as positive control at indicated concentrations for 30 min at 37 °C under continuous shaking at 40 cycles/min. Subsequently, the muscle strips were further incubated with 1 $\mu$Ci/mL 2-[$^{14}$C]-deoxy-D-glucose (2-DG) (PerkinElmer Life Sciences, Boston, MA) for 5 min. Uptake was terminated with the addition of ice-cold phosphate buffer solution.

Radioactivity was determined by lysis of samples in 1 N NaOH, and the aliquots were neutralized for estimation in a scintillation counter (Beckman LS5000TA, Fullerton, CA). Nonspecific uptake was obtained by parallel determinations in the presence of 20 $\mu$mol/L cytochalasin B (Sigma-Aldrich). Specific 2-DG uptake was expressed as picomoles per milligram of protein over 5 min. Protein content was determined using the Bio-Rad protein dye binding assay (Richmond, CA).

**Northern Blot Analysis.** Total RNA was extracted from the soleus muscle of experimental animals using the Ultraspec-II RNA extraction system (Bioteck, Houston, TX) based on the manufacturer's instructions. For Northern blot analysis, RNA (20 $\mu$g) was denatured in a solution containing 2.2 mmol/L formaldehyde and 50% formamide (v/v) at 55 °C for 15 min. Aliquots of total RNA were size-fractionated in 1.2% agarose/formaldehyde gel, and the RNA was transferred to a Hybond-N membrane (Amersham, Bucks, U.K.).

Plasmid containing cDNA of GLUT4 and $\beta$-actin cDNA were prepared as probes, which were labeled with [$\alpha$-$^{32}$P]dCTP using the Megaprime labeling system kit (Amersham). GLUT4 mRNA levels were detected by random prime-labeled full-length cDNA under stringent hybridization conditions. Band intensity was quantified by Gel-Pro analyzer software 4.0 (Media Cybernetics, Silver Spring, MD). $\beta$-Atin was used as internal standard.

**Western Blot Analysis.** After homogenization of the soleus muscle using a glass/Teflon homogenizer, protein content was determined by Bio-Rad protein dye binding assay. Protein lysates (50 $\mu$g) were separated by 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis using the Bio-Rad Mini-Protein II system (80 and 100 V for the stacking and separation gels, respectively). Protein was transferred to expand the polyvinylidene difluoride membrane using a Bio-Rad Trans-Blot system (70 min at 90 V in 25 mmol/L Tris, 192 mmol/L glycine,and 25% MeOH). Following the transfer, the membrane was washed with PBS-T and blocked for 1 h at room temperature with 10% (w/v) skim milk powder in PBS-T.

Western blot analysis was done using anti-rat GLUT4 antibody (1:1000) (Millipore, Billerica, MA). The intensity of the blots incubated with goat polyclonal antibody (1:5000) to bind the actin (Millipore) was used as control to ensure that the amount of protein loaded into each lane of the gel was constant. After removal of the primary antibody, the blots were extensively washed with PBS-T. Blots were incubated with appropriate peroxidase-conjugated secondary antibodies.

After removal of the secondary antibody, blots were washed and developed by autoradiography using the ECL−Western blotting system (Amersham Corp., Braunschweig, Germany). Densities of the obtained immunoblots at 55 kDa for GLUT4 and 43 kDa for actin were quantified using Gel-Pro analyzer software 4.0 (Media Cybernetics, Silver Spring, MD).

**Statistical Analysis.** Data were expressed as mean ± SEM for the number ($n$) of animals in the group. Repeated measures analysis of variance (ANOVA) was used to analyze the experiments, and Dunnett range post hoc comparisons were used to determine the source of significant differences when appropriate. A $p$ value of $< 0.05$ was considered to be statistically significant.

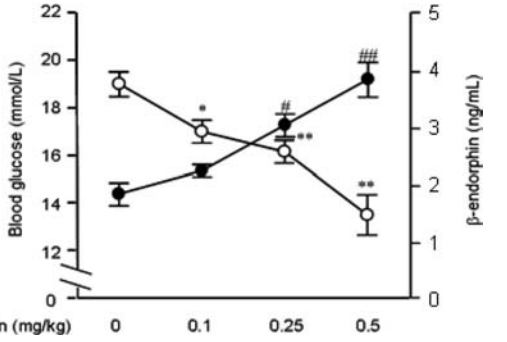


**Figure 1.** Allantoin administration decreased blood glucose and $\beta$-endorphin release. Plasma glucose lowering activity (open circles) and plasma $\beta$-endorphin increment (solid circles) was noted after iv injection of allantoin in STZ-diabetic rats. Values (mean ± SEM) were obtained from each group ($n = 8$). The saline vehicle used to dissolve allantoin was given in the same volume. ∗, $p < 0.05$; ∗∗, $p < 0.01$, versus data from animals treated with the vehicle for blood glucose determination. #, $p < 0.05$; ##, $p < 0.01$, versus data from animals treated with the vehicle in $\beta$-endorphin levels.

**Table 1.** Effects of Opioid $\mu$-Receptor Antagonists on Allantoin-Induced Plasma Glucose Lowering Effect in STZ-Diabetic Rats

| | plasma glucose[a] (mmol/L) |
|---|---|
| *n* | 8 |
| basal | 18.88 ± 2.4 |
| allantoin (0.5 mg/kg, iv) | |
| + vehicle | 12.54 ± 2.8** |
| + naloxone (mg/kg, iv) | |
| 0.5 | 13.52 ± 1.8** |
| 1.0 | 15.66 ± 2.1* |
| 2.0 | 15.91 ± 0.8* |
| + naloxonazine (mg/kg, iv) | |
| 0.5 | 12.87 ± 2.0** |
| 1.0 | 14.29 ± 1.1* |
| 2.0 | 15.81 ± 0.9* |
| naloxone (2.0 mg/kg, iv) | 19.19 ± 3.4 |
| naloxonazine (2.0 mg/kg, iv) | 19.83 ± 2.9 |

[a] Data are mean ± SEM. Basal level shows the value from fasted animal without treatment. The saline vehicle used to dissolve naloxone or naloxonazine was given in the same volume. *, $p < 0.05$; **, $p < 0.01$ compared to basal value.

## RESULTS

**Effects of Allantoin on Plasma Glucose and $\beta$-Endorphin Levels.** After intravenous injection of allantoin in STZ-diabetic rats, plasma glucose was markedly reduced at 120 min. Allantoin dose-dependently decreased plasma glucose by 29.1 ± 3.7% ($n = 8$) (**Figure 1**, open circles) and reached a maximal effect at 0.5 mg/kg. Increasing the dose of allantoin to 1 mg/kg showed no further plasma glucose lowering activity by 26.7 ± 2.4%. Plasma glucose was not modified in normal rats after 0.5 mg/kg allantoin injection compared to the vehicle-treated group (5.3 ± 0.7 vs 5.8 ± 0.4 mmol/L; $n = 8$). There was a dose-dependent increase of plasma $\beta$-endorphin level in STZ-diabetic rats after intravenous injection of allantoin (**Figure 1**, solid circles). Increased plasma $\beta$-endorphin-like immunoreactivity in STZ-diabetic rats by allantoin was not different from 1 and 0.5 mg/kg ($p > 0.05$), and there was no further increase of plasma $\beta$-endorphin level at higher doses of allantoin. Therefore, 0.5 mg/kg of allantoin was used in subsequent experiments.

**Effects of Opioid $\mu$-Receptor Antagonists on Allantoin-Induced Plasma Glucose Lowering Effect. Table 1** shows the concentration-dependent action of naloxone or naloxonazine in blocking

**Table 2.** Effects of Adrenalectomy on the Allantoin-Induced Changes of Plasma Concentrations in Glucose and $\beta$-Endorphin in STZ-Diabetic Rats

| | sham[a] | adrenalectomized[a] |
|---|---|---|
| *n* | 8 | 8 |
| plasma glucose (mmol/L) | | |
| basal | 19.8 ± 2.4 | 22.4 ± 3.5 |
| vehicle | 18.4 ± 1.7 | 20.5 ± 2.1 |
| allantoin (0.5 mg/kg, iv) | 13.1 ± 0.9** | 16.8 ± 1.2* |
| plasma $\beta$-endorphin (ng/mL) | | |
| basal | 1.57 ± 0.05 | 0.43 ± 0.01 |
| vehicle | 1.72 ± 0.09 | 0.38 ± 0.03 |
| allantoin (0.5 mg/kg, iv) | 3.85 ± 0.12** | 0.51 ± 0.09 |

[a] Data are means ± SEM. Basal level shows the value from fasted animal without treatment. The saline vehicle used to dissolve allantoin was given in the same volume. **, $p < 0.01$ vs basal value in each group.

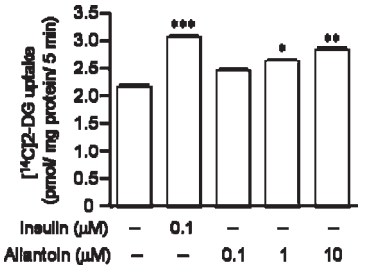


**Figure 2.** Effects of allantoin on glucose uptake in isolated skeletal muscle of STZ-diabetic rats. Glucose uptake was determined using a $\beta$-counter. Results were obtained from three independent experiments and expressed as mean ± SEM. ∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$ compared to control group.

the plasma glucose lowering effect of allantoin (0.5 mg/kg, iv) in STZ-diabetic rats. After pretreatment with naloxone, the plasma glucose level was markedly reduced, but the effect of allantoin was not totally abolished. There were similar results in STZ-diabetic rats pretreated with naloxonazine. Moreover, naloxone and naloxonazine at the highest dose showed no effect on basal plasma glucose levels in STZ-diabetic rats.

**Bilateral Adrenalectomy Partially Abolished the Effect of Allantoin on Plasma Glucose and $\beta$-Endorphin Levels.** Plasma glucose concentrations were slightly deceased 2 weeks after bilateral adrenalectomy, and basal plasma $\beta$-endorphin levels were unchanged in adrenalectomized STZ-diabetic rats compared to the sham-operated group (**Table 2**). However, allantoin-induced plasma glucose decrement was markedly reduced in STZ-diabetic rats with bilateral adrenalectomy. Moreover, allantoin failed to affect the plasma $\beta$-endorphin levels in STZ-diabetic rats with bilateral adrenalectomy. The effects of allantoin on plasma glucose decrement and plasma $\beta$-endorphin increment persisted in sham-operated STZ-diabetic rats (**Table 2**).

**Direct Effects of Allantoin on Glucose Uptake in Isolated Skeletal Muscle.** The direct effect of allantoin on glucose uptake in isolated skeletal muscle of STZ-diabetic rats was investigated. 2-DG uptake in isolated soleus muscle strips was markedly raised by allantoin in a concentration-dependent manner (**Figure 2**). Increased 2-DG uptake by allantoin at maximal concentration (10 $\mu$M) was less effective than that induced by insulin (0.1 $\mu$M) (**Figure 2**).

**General Characteristics of STZ-Diabetic Rats Repeatedly Receiving Allantoin.** Plasma glucose levels of STZ-diabetic rats were reduced for 28.9 ± 2.3% after repeated intravenous injection of allantoin (0.5 mg/kg, tid) for 3 days. Elevated plasma $\beta$-endorphin was significantly increased in the same group of STZ-diabetic rats,

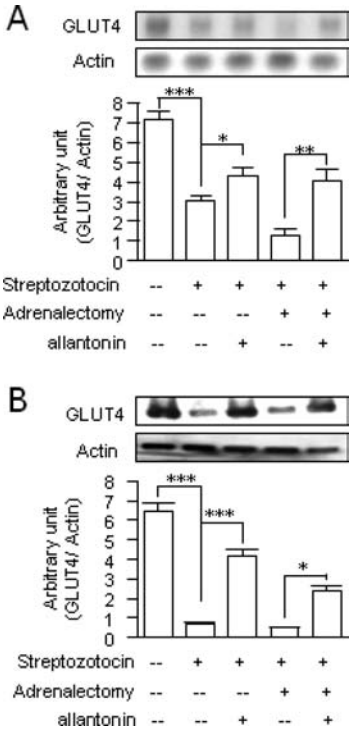


**Figure 3.** Effects of allantoin on GLUT4 expression in isolated skeletal muscle of STZ-diabetic rats illustrated as representative response of (**A**) GLUT4 mRNA or (**B**) protein levels in isolated soleus muscle from normal or STZ-diabetic rats receiving the repeated treatment with allantoin (0.5 mg/kg, iv) or the same volume of vehicle (saline) three times for 3 days. Quantification was obtained from three individual experiments. ∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$ compared to the normal rat group.

compared to vehicle-treated group ($p < 0.01$). The 3 day treatment with allantoin did not affect the feeding behavior and body weight of STZ-diabetic rats.

A similar allantoin treatment in bilateral adrenalectomized STZ-diabetic rats failed to increase plasma $\beta$-endorphin levels. The body weight of STZ-diabetic rats was also not changed by allantoin, although there was still reduced plasma glucose.

**Effects of Allantoin on GLUT4 mRNA and Protein Levels in Soleus Muscle of STZ-Diabetic Rats.** GLUT4 mRNA levels in soleus muscle isolated from STZ-diabetic rats was markedly reduced compared to those of normal rats (**Figure 3A**). Repeated allantoin treatment (0.5 mg/kg, iv, tid) for 3 days resulted in elevated GLUT4 mRNA levels in the soleus muscle (**Figure 3A**). Furthermore, the GLUT4 mRNA level was reduced in STZ-diabetic rats with bilateral adrenalectomy, but 3 days of allantoin treatment increased the GLUT mRNA level (**Figure 3A**).

The protein level of GLUT4 in the soleus muscle of STZ-diabetic rats was also significantly reduced compared to that of normal rats (**Figure 3B**), but repeated treatment with allantoin for 3 days elevated the protein level of GLUT4. The GLUT4 protein level was reduced in adrenalectomized STZ-diabetic rats (**Figure 3B**), but a 3 day allantoin treatment at 0.5 mg/kg increased the GLUT4 protein level (**Figure 3B**).

## DISCUSSION

The present study shows that allantoin has a dose-dependent ability to decrease plasma glucose and increase plasma $\beta$-endorphin levels in STZ-diabetic rats that is not observed in normal rats. This plasma glucose lowering effect is partially blocked by opioid receptor antagonists. Whereas $\beta$-endorphin is released by adrenocorticotrophic hormones from the pituitary gland (*19*), the

adrenal gland is also a source (*16*, *20*). This study demonstrates that the secretion of opioids from the adrenal gland is associated with decreased plasma glucose in STZ-diabetic rats (*18*), which is consistent with the view that pituitary gland-independent release of endogenous opioids is operative in other organs (*16*, *20*).

Bilateral adrenalectomy was used to confirm that the adrenal gland is the main source of allantoin-induced β-endorphin release. The plasma glucose lowering action of allantoin was markedly reduced in bilateral adrenalectomized STZ-diabetic rats. Moreover, the increment in plasma β-endorphin is not observed in adrenalectomized diabetic rats receiving allantoin in effective doses. Thus, the adrenal gland may be responsible for the allantoin-induced secretion of β-endorphin. Such allantoin-induced actions in STZ-diabetic rats are also dose-dependently reduced by opioid receptor blockers. Therefore, β-endorphin released from the adrenal gland may be related to the plasma glucose lowering action of allantoin in STZ-diabetic rats.

The actions of β-endorphin are partly mediated by opioid μ-receptors that are believed to express in specialized neurons for pain transmission in both spinal and supraspinal sites (*18*, *21*). Previous studies indicate that β-endorphins enhance glucose uptake in the soleus muscle and stimulate glycogen synthesis in hepatocytes isolated from STZ-diabetic rats. Both actions of β-endorphins are blocked by naloxone and naloxonazine (*22*), which imply that opioid μ-receptors located in peripheral tissues can be activated to decrease plasma glucose by improving glucose utilization (*12*, *22*). Consistent with previous studies, the action of allantoin in STZ-diabetic rats is inhibited by blocking opioid μ-receptors using naloxone or naloxonazine. Two subtypes of the μ-receptors ($\mu_1$ and $\mu_2$) have been postulated, with the $\mu_1$ subtype as naloxonazine sensitive (*23*, *24*). Thus, the plasma glucose lowering effect of allantoin in STZ-diabetic rats may be mediated by peripheral opioid $\mu_1$-receptors, which warrants further investigation.

In diabetes, elevated blood glucose is a consequence of increased hepatic glucose output in concert with reduced peripheral glucose utilization (*25*). Glucose transport, which depends on insulin-stimulated translocation of glucose carriers to the cell membrane, is the rate-limiting step in carbohydrate metabolism of skeletal muscle, a major site for glucose disposal (*26*). Some glucose transporters are mediated in glucose transport across the cell membrane, whereas the subtype, GLUT4, is predominantly in the skeletal muscle (*27*). Decreasing expression of GLUT4 mRNA and protein in diabetes has been reported (*28*).

It has been previously demonstrated that endogenous β-endorphin activates opioid receptors to play a role as a positive regulator in glucose utilization under insulin-deficient state (*22*). In the present study, the plasma glucose lowering activity of allantoin is reduced but not abolished by opioid μ-receptor inhibitors and bilateral adrenalectomy in STZ-diabetic rats. Therefore, an allantoin-induced blood glucose lowering effect may be achieved through a β-endorphin-independent pathway. Further investigation shows a concentration-dependent increase of glucose uptake in isolated soleus muscle from STZ-diabetic rats (**Figure 2**), which indicates that allantoin may increase glucose uptake directly.

Gene expression of GLUT4 is then examined to elucidate the mechanism of allantoin in regulating plasma glucose in an insulin-deficient state. In STZ-diabetic rats, allantoin effectively decreases plasma glucose after 3 days of repeated treatment, which is accompanied by a marked elevation of GLUT4 expression in the soleus muscle. The increment in skeletal muscle glucose transport is associated with increased GLUT4 gene expression (*28*). Moreover, allantoin increases the expression of GLUT4 in the soleus muscle of diabetic rats with bilateral adrenalectomy, which further implies that allantoin may increase glucose uptake directly.

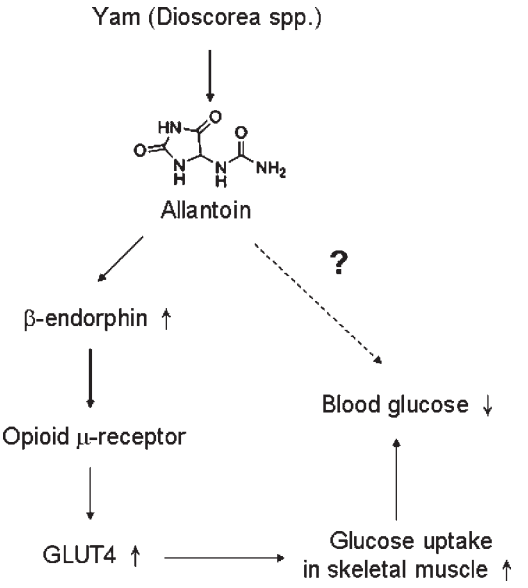


**Figure 4.** Scheme of the blood glucose regulation induced by allantoin, which was obtained mainly from botanical extracts of yam (*Dioscorea* spp.). Allantoin administration increased the secretion of β-endorphin from the adrenal glands of rats. The released β-endorphin can activate opioid μ-receptors to increase GLUT4 expression to increase uptake of glucose into the skeletal muscle. Pretreatment of opioid μ-receptor antagonist partially blocked the effect of allantoin on blood glucose, implying that other mechanisms might be involved and warrant further investigations.

Allantoin is nature-identical, safe, and nontoxic (*1*). The present study characterizes that allantoin aids in the regulation of blood glucose homeostasis (**Figure 4**). Although allantoin is degraded in the gastrointestinal tract (*14*) and easily lost after oral administration (*15*), development of a new method or substance that can protect this may help the application of allantoin in the future. Thus, the suitable intake of allantoin-containing natural products, such as tea or yam, with others that can decrease the degradation of allantoin may be beneficial in the control of blood glucose. This is the first study to highlight this point.

In conclusion, allantoin may increase the secretion of β-endorphin from the adrenal glands of STZ-diabetic rats. Such plasma glucose lowering action is related to the released β-endorphin that can activate opioid μ-receptors in peripheral tissues to increase GLUT4 gene expression via an insulin-independent mechanism. Allantoin may also increase glucose uptake directly in skeletal muscle through increased GLUT4 expression. Thus, allantoin may improve glucose utilization in skeletal muscle through β-endorphin dependent- and independent-pathways that decrease plasma glucose in STZ-diabetic rats.

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