The Metabolic Effects of Pokeweed Mitogen in Mice

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Lectins are a family of proteins that stimulate cellular responses after binding to carbohydrate chains on plasma membranes. In the study described here, a mixture of lectins—pokeweed mitogen (PKW)—was shown to have insulinomimetic effects in mice. After receiving PKW (15 mg/kg intraperitoneally [IP]), serum glucose declined from 154 ± 3 to 23 ± 10 mg/dL by 24 hours later. Anorexia developed, and by 3 days, there was a significant decline in body weight. Carcass weights were 10% lower, and epididymal fat pad weights were 45% lower. When given for 16 days, PKW 3 mg/kg every other day caused a sustained 10% weight loss. Severe combined immune deficiency (SCID) mice were sensitive to PKW, showing that B and T lymphocytes were not required for the effects to develop. Cytokine antagonists attenuated the hypoglycemia and anorexia, but only by 50%. Further study showed that PKW has insulin-like effects in vitro. Glucose uptake was stimulated when murine C2C12 myotubes were exposed to an enriched fraction of PKW. These results demonstrated that PKW has both insulin-like activity and weight-reducing effects when administered to mice. The development of therapy for adult-onset diabetes or obesity based on lectins from pokeweed may be possible.

ADULT-ONSET TYPE II DIABETES mellitus is a major health problem in the United States.[1] Because it is exacerbated by obesity, treatment of this disease is often two-pronged, using diet therapy to promote weight loss and maintain normoglycemia,[2] and drugs when diet therapy is not effective.[3] In this report, we describe a type of lectin that did both of these things—decreased circulating glucose levels and body weight.

Lectins are a general class of proteins that bind to carbohydrates.[4] Included in this group of proteins are wheat germ agglutinin (WGA),[5] concanavalin A,[6] and pokeweed mitogen (PKW).

There are five different forms of PKW, from the plant *Phytolacca americana*,[7,8] all of which bind to *N*-acetylglucosamine.[7,9] One of these lectins stimulates B lymphocytes,[10-12] while the others stimulate T lymphocytes.[10-12] One of the forms of PKW that stimulate T cells is similar in structure to WGA.[13,14]

Since WGA has insulin-like activity,[15,16] binds the same ligand as PKW,[5] and has structural homology to at least one of the PKW lectins,[13,14] we determined whether PKW may mimic this hormone, as well, when administered to animals. Mice that received PKW developed hypoglycemia, a decline in food intake, and weight loss. The appearance of interleukin-6 (IL-6) in the circulation after PKW treatment probably contributed to these changes, but the use of a murine muscle cell line showed that PKW also had direct insulinomimetic effects.

MATERIALS AND METHODS

Animals

Male BALB/c, C57BL/6, CB6 F1 (C57BL/6 × BALB/c), and *ob/ob* C57BL/6 mice were from Jackson Laboratories (Bar Harbor, ME), and male BALB/c SCID (severe combined immune deficiency) mice were from Taconic Farms (Germantown, NY). The mice were housed with a 12-hour light/dark cycle and fed standard laboratory chow (Purina Mills, St Louis, MO) and water ad libitum for at least 2 days before use. Except for the group that was fasted for 18 hours after receiving PKW, all mice had access to food at all times. The different strains of mice each responded similarly to PKW administration, as did C3H/hej mice, which are resistant to lipopolysaccharide (LPS) (data not shown).

CB6F1 mice were made diabetic by injecting streptozotocin (200 mg/kg intraperitoneally [IP] in phosphate-buffered saline [PBS]) one time and measuring blood glucose levels 4 days later. At this time, mice given streptozotocin had serum glucose concentrations of 420 ± 10 mg/dL, twice normal values.

Blood was collected by orbital sinus puncture to determine the diabetic state prior to receiving PKW and to measure tumor necrosis factor-α (TNFα) and IL-6 concentrations 1 and 3 hours, respectively, after PKW treatment, as well as in *ob/ob* mice before and after receiving PKW in the corresponding studies. For other measurements, blood obtained by cardiac puncture and tissue samples were collected after death by cervical dislocation. The carcass weight was represented as the body with all viscera removed (with only the skin, brain, muscle, and bone remaining), and the fat content was represented as the weight of the right epididymal fat pad.

Reagents

PKW, streptozotocin, penicillin-streptomycin solution, PBS, bovine serum albumin (BSA), bovine insulin, horse serum, and Triton X-100 were purchased from Sigma Chemical (St Louis, MO), and chloroform from J.T. Baker (Phillipsburg, NJ). Serum glucose levels were measured using a commercial kit (Sigma), and insulin concentrations were determined using a radioimmunoassay kit (ICN, Costa Mesa, CA) with porcine insulin as the standard. The BCA protein assay was purchased from Pierce (Rockford, IL). IL-6 and TNFα levels were measured using enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, MN, and Biosource International, Camarillo, CA, respectively). The TNFα antagonist (TNFbp) was a kind gift from Amgen Boulder (Boulder, CO). TNFbp consists of the extracellular domains of two type 1 (p55) TNFα receptors linked to polyethylene glycol.[17] The anti-IL-6 antibody (20F3) was grown in nude mice from hybridoma cells and purified using standard antibody isolation procedures (Pierce, Rockford, IL). 2-Deoxy-D-[1-^{3}H]glucose was purchased from Amersham Life Science (Elk Grove, IL). For the study that used C2C12 myotubes, Dulbecco's modified Eagle's medium containing L-glutamine (DMEM), RPMI medium containing L-glutamine, and fetal bovine serum were purchased from JRH Biosciences (Lenexa, KS). Antibodies used for B- and T-lymphocyte isolation were obtained from Bectin-Dickinson (Mountain View, CA). For T- and B-lymphocyte isolation, Ficoll was from Sigma, magnetic beads were from Dynal (Lake Success, NY),

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antibodies were from Becton-Dickinson, and ^{3}H-thymidine was from New England Nuclear (Boston, MA).

Treatments

PKW was dissolved in sterile PBS and administered IP or orally, by gavage, in a volume of 200 μL/20 g mouse. TNFbp and anti-IL-6 antibody (20F3) were given 2 hours before PKW in the studies described. The TNFbp dose was 780 μg/mouse in 100 μL vehicle. The quantity of TNFα antagonist was 35-fold greater than a dose that protected against death in the D-galactosamine/LPS model of liver injury.[18] The dose for anti-IL-6 (20F3) was 650 μg/mouse in a 150-μL volume. This quantity of 20F3 attenuated anorexia and the acute-phase protein response in the aseptic turpentine abscess model of inflammation.[19] Control animals received the same vehicle and volume at the appropriate time.

Food Intake

The food consumption per day by each cage of mice was divided by the number of mice in the cage. Because only a single cage was used per treatment, a single average value was derived. The mice were housed three or four per cage.

Chloroform Extraction of PKW

The purpose of the chloroform extraction of PKW was to isolate any nonprotein organic molecules that may have been present as contaminants. For example, triterpenes have been shown to stimulate glucose uptake by sheep red blood cells,[20] and the pokeweed plant contains this type of compound.[21] The PKW solution used to treat mice was mixed thoroughly with an equal volume of chloroform by vortexing at moderate speed for 30 minutes at room temperature. A precipitate appeared in the chloroform layer that was separated by centrifugation. Both the clear chloroform layer and the precipitate were dried under nitrogen at room temperature and then under vacuum to remove the chloroform. These extracts were redissolved in the same volume of the original vehicle (PBS). Mice were treated IP with the extracted pokeweed solution or with the reconstituted extracts (the dried protein precipitate and the dried chloroform residue) in the subsequent study reported herein.

The reconstituted chloroform layer contained no protein, whereas the reconstituted precipitate contained 14% as much protein as the unextracted PKW solution. The protein content of the aqueous layer plus the precipitate was equal to the protein content of the unextracted solution (data not shown).

Human B- and T-Lymphocyte Stimulation by Chloroform-Treated PKW

B and T lymphocytes were purified from heparinized human blood by separating peripheral blood mononuclear cells over a Ficoll gradient, followed by passage over a nylon wool column. Adherent cells were depleted of non–B lymphocyte by using magnetic beads directly conjugated with anti-CD4 and anti-CD8 antibody, or by adding anti-CD16 (0.5 μg/mL final concentration) or anti-CD14 (1.25 μg/mL final concentration) antibody to the cells followed by anti-mouse immunoglobulin G (IgG)- or IgM-coated beads in a secondary step. In both steps, the bead to cell ratio was 3:1. B lymphocytes were plated at 1×10^5 cells/well and T lymphocytes were plated at 2×10^5 cells/well on 96-well plates. The three PKW fractions—unextracted, chloroform-extracted, and chloroform precipitate—were added, and 2 days later, ^{3}H-thymidine 0.5 μCi/well was added. At the end of 8 hours, ^{3}H-thymidine uptake by the cells was measured.

Ex Vivo Exposure of Blood to PKW

Seventy-five micrograms of the PKW mixture in a volume of 50 μL was added to 0.5 mL heparinized blood. This is equal to the contribution of the PKW solution used for acute mouse studies to 2 mL blood, roughly the blood content of a 20-g mouse.[22] The treated blood was incubated for 6 hours at 38°C in 5% CO_2. Blood was removed at intervals, the plasma was separated, and the glucose level was measured.

Glucose Uptake In Vitro

Murine-derived C2C12 myoblast cells were obtained from the American Type Culture Collection (Rockville, MD). Although these cells do not contain GLUT4, they are nevertheless responsive to insulin.[23] The myoblasts were plated in 24-well plates and grown in high-glucose DMEM with penicillin and streptomycin (100,000 U/L and 100 mg/L, respectively) and 10% fetal bovine serum. At confluence, the medium was replaced with high-glucose RPMI containing antibiotics and 10% horse serum. Four days later, myotubes were formed, and the medium was replaced with low-glucose DMEM plus 2% fetal bovine serum containing the treatments. Sixteen hours later, the cells were washed with PBS containing 0.1% BSA. Afterward, 2-deoxy-D-[1-^{3}H]glucose in PBS/BSA was added at a dose of 0.2 μCi/well for 20 minutes. The cells were then washed three times with PBS and solubilized in 1% Triton X-100, and radioactivity and protein concentrations were measured.

Statistics

Data are presented as the mean ± SEM (n = 3 to 10 mice per group). Groups were compared using the two-tailed paired or unpaired *t* test or two-way ANOVA, followed by the post hoc Fisher's test, as appropriate. Regarding body weight, except where noted, all groups that were statistically different from the control group at the time of sampling, ie, after PKW administration, also differed when compared with their initial (pretreatment) body weight.

RESULTS

Circulating Glucose, Lactate, and Insulin Concentrations

Glucose concentrations in the vehicle-treated control group were 154 ± 3 mg/dL. Mice with 15 mg/kg PKW showed significant hypoglycemia by 4 hours, declining to 78 ± 11 mg/dL ($P < .05$ *v* controls). These concentrations declined further to 23 ± 10 mg/dL at 24 hours (Fig 1A). Table 1 shows that diabetic mice and *ob/ob* mice also developed hypoglycemia after receiving PKW.

Blood lactate concentrations declined after administration of PKW, although not to as great an extent as glucose. These levels, briefly, were 39 ± 4 mg/dL in PBS-treated animals, 27 ± 4 mg/dL 4 hours after PKW, and 16 ± 2 mg/dL at 24 hours.

The hypoglycemia that followed PKW administration was not secondary to stimulated insulin release. Plasma insulin concentrations decreased after PKW, from 43 ± 10 μU/mL to 24 ± 0 μU/mL 24 hours later ($P < .05$; Fig 1B), while diabetic mice given PKW had a circulating glucose level 45% lower than in the diabetic control group (also $P < .05$; Table 1).

The metabolic effects of PKW were induced by oral administration (by gavage) in 50% of the animals that received it. That is to say, in groups given PKW by gavage, half of the animals had decreased circulating glucose compared with the control group and were considered responders (Table 1), while half appeared unaffected and were considered nonresponders. Because mice given PKW orally were part of two different experiments, the orally treated and control groups were combined and subgroups of responders and nonresponders were included in the comparison (Table 1). Specifically, for nonre-

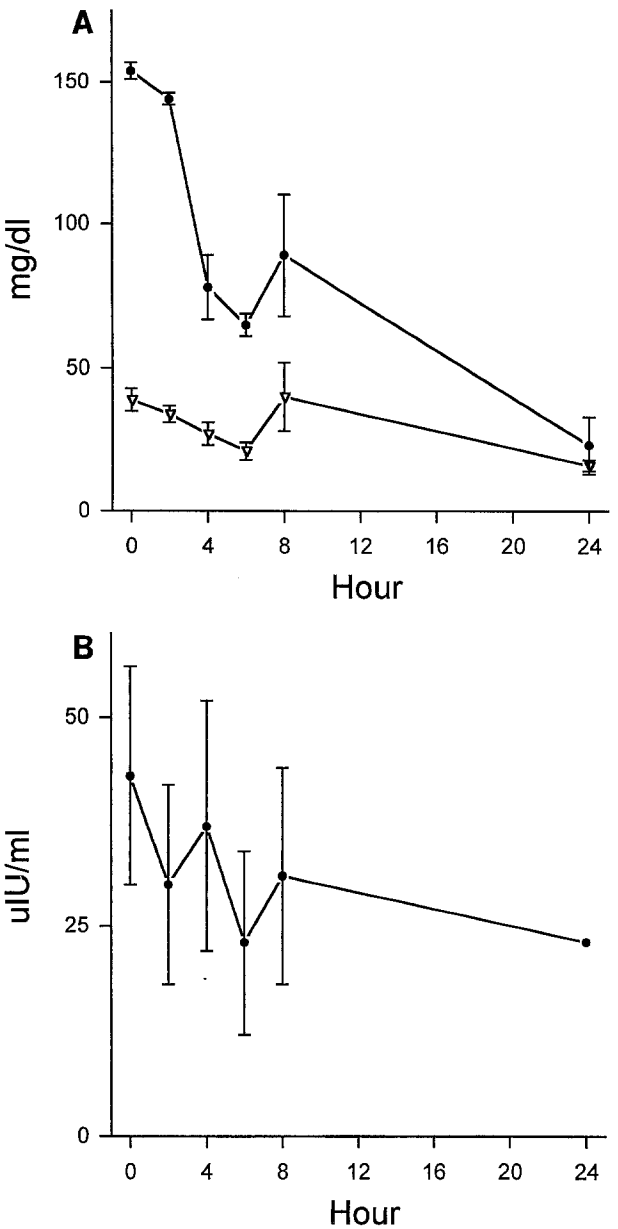

Fig 1. (A) Circulating glucose (●) and lactate (▽) concentrations in mice administered an IP injection of PKW, 15 mg/kg or vehicle. $P <$ .05 for PKW-treated groups *v* vehicle-treated groups from 4 to 24 hours. (B) Circulating insulin concentrations in mice administered an IP injection of PKW, 15 mg/kg or vehicle. $P <$ .05 for PKW-treated groups *v* vehicle-treated groups at 4 h, 6, and 24 hours.

sponders, blood glucose was 210 ± 17 mg/dL, while responders had a blood glucose level of 70 ± 5 mg/dL and significantly lower body weight. Because nonresponders and responders were caged together, food intake could not be compared.

Food Intake

The mean food intake declined in all groups of mice that received PKW (Table 1). Fasted mice showed that anorexia did not contribute significantly to the decreased glucose levels in mice given these lectins (Table 1). Stimulated release of leptin was not essential to the anorexia, since *ob/ob* mice were also sensitive to PKW regarding food intake (Table 1).

Body Composition

A single treatment of PKW 15 mg/kg had effects for 3 days. The decline in body weight at this time was due to the loss of both lean tissue and adipose tissue. Lean tissue, represented by carcass weight, was 14.9 ± 0.4 g in the control group and 12.8 ± 0.4 g in the PKW-treated group ($P <$ .05). The right epididymal fat pad, representing adipose tissue, weighed 0.12 ± 0.01 g in the control group and 0.07 ± 0.01 g in the PKW-treated group ($P <$ .05). These tissue comparisons are illustrated in Fig 2.

Long-Term Treatment With PKW

When mice were administered PKW at a dose of 5 mg/kg every other day, body weight and food intake declined dramatically. On day 3, after two treatments, three of the five mice were dead.

In a second part of this study (Fig 3A and B), mice were

Table 1. Circulating Glucose, Food Intake, and Weight Loss Induced by Various Treatments in Normal, SCID, Diabetic, and *ob/ob* Mice

Mice	Glucose (mg/dL)	Food Intake (g/mouse)	Body Weight (g)
Normal			
Vehicle	248 ± 13	3.26	17.5 ± 0.3
PKW	34 ± 7*	0.07	17.0 ± 0.3
PKW + anti-IL-6 antibody	81 ± 22*†	0.22	17.7 ± 0.3
PKW + TNFbp	112 ± 19*†	0.93	17.3 ± 0.4
PKW + anti-IL-6 + TNFbp	120 ± 14*†	0.16	18.5 ± 0.5
Vehicle	191 ± 6	3.55	18.8 ± 0.2
PKW	74 ± 12*	0	16.3 ± 0.6*
Chloroform-extracted PKW	144 ± 19	0.38	16.2 ± 0.3‡
Chloroform extract	265 ± 24*	2.63	17.1 ± 0.7‡
Chloroform precipitate	106 ± 14*	0	16.0 ± 0.2*
Vehicle	205 ± 17	—	—
PKW	96 ± 5*	—	—
Fasting	192 ± 12	—	—
Vehicle	183 ± 6	—	19.4 ± 0.3
Oral PKW	140 ± 26	—	18.1 ± 0.7
Oral nonresponders	210 ± 17	—	18.6 ± 0.7
Oral responders	70 ± 5*	—	16.5 ± 0.2*
SCID			
Vehicle	126 ± 8	2.85	—
PKW	77 ± 8*	0.60	—
Streptozotocin-diabetic			
Vehicle	421 ± 14	—	16.0 ± 0.3
PKW	227 ± 40*	—	13.2 ± 0.8*
ob/ob			
Day 0	424 ± 4	4.15	45.3 ± 1.8
Day 1	55 ± 8*	0	44.5 ± 1.2

NOTE. The PKW dose was 15 mg/kg IP, except for *ob/ob* mice. *ob/ob* mice were given 10 mg/kg IP and used as their own control. The samples were collected 18 hours after PKW administration.

*$P <$ .05 *v* control group in respective study.

†$P <$ .05 *v* group given PKW only.

‡NS *v* initial body weight.

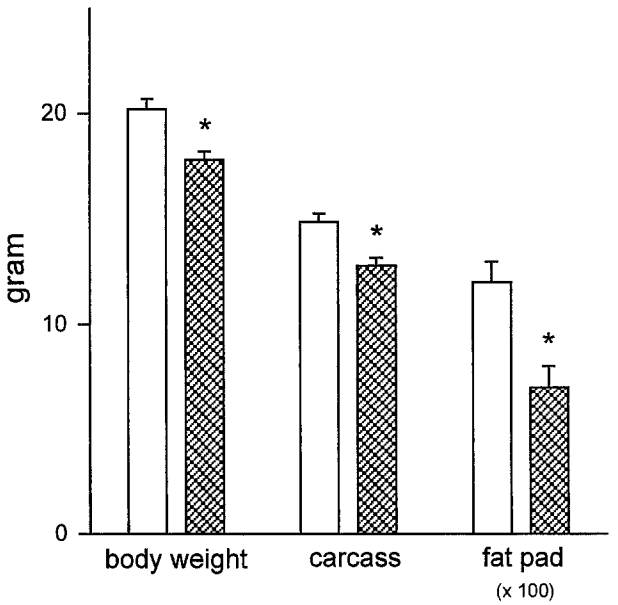

Fig 2. Body weight, carcass weight, and right epididymal fat pad weight of mice 3 days after IP injection of PKW, 15 mg/kg. $P < .05$ for PKW-treated groups (▨) *v* vehicle-treated groups (□).

administered PKW at a lower dose, 3 mg/kg every other day, for 16 days. Food intake recovered 1 day after each treatment. Beyond 3 days, the mean food intake was roughly equal to that of the control group, oscillating above and below it in response to PKW. In the treated group, body weight was 21.0 ± 0.2 g at the start, but declined 15% to 17.9 ± 0.4 g by day 5 ($P < .05$ *v* both day 0 and the control group). The body weight of this group increased after day 5, but remained lower than that of the control group for the additional 10 days of treatment and for 1 week of monitoring after treatment was discontinued. The pattern and extent of weight loss in the experimental group and a pair-fed group were similar (Fig 3A).

Immune Mediation of Hypoglycemia

Circulating TNFα did not increase to detectable levels after a 15-mg/kg dose of PKW. However, an elevation in the circulating concentration of IL-6, to 129 ± 6 ng/mL, was found, as compared with undetectable levels in control animals (Table 2). Pretreatment with TNFbp attenuated IL-6 appearance by 68%, ie, to 32% of that in mice not given TNFbp. Blood glucose was significantly higher in mice given PKW + TNFbp, as was food intake. Despite some blocking effect, these indices of PKW action remained lower than in vehicle-treated animals (Table 1).

Mice given anti-IL-6 antibody before PKW did not show a decline in circulating IL-6 concentrations (Table 2). Although IL-6 concentrations appeared unaffected, the anti-IL-6 antibody attenuated hypoglycemia to an extent that was equivalent to the effect of TNFbp, but anorexia was unaffected (Table 1). The cytokine antagonists were not additive in their protective effects. All groups in this specific study lost weight overnight, possibly due to the stress of the two IP injections and blood sampling via the orbital sinus in the span of 3 or 5 hours.

SCID mice, which do not have functional T or B cells and cannot mount an allergic reaction, were not less sensitive to the hypoglycemic effect of PKW (Table 1). These immunodeficient mice also responded with the appearance of circulating IL-6, to 92 ± 35 ng/mL, in the treated animals (Table 2).

Chloroform Extraction of PKW

Data from this study are presented in Table 1. The vehicle-treated group had circulating glucose concentrations of 191 ± 6 mg/dL, while in the PKW-treated group, glucose levels were 74 ± 12 mg/dL ($P < .05$).

The same solution of PKW was mixed with chloroform, and the results are as follows. The aqueous chloroform-extracted PKW solution had only a mild hypoglycemic effect, to 144 ± 19 mg/dl (NS). Food intake declined, but body weight did not.

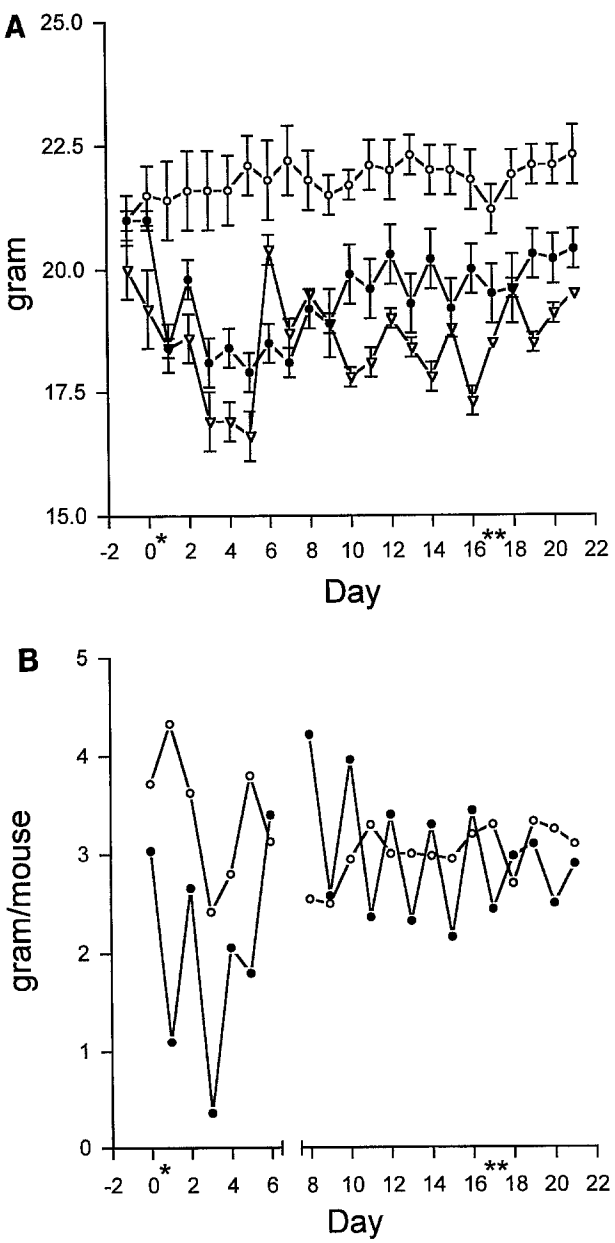

Fig 3. (A) Body weight and (B) food intake of mice treated long-term with PKW 3 mg/kg every other day (●), vehicle (○) or pair-fed (▽). *First treatment; **last treatment.

Table 2. Cytokine Appearance After PKW Administration

Group	TNFα, pg/ml	IL-6, ng/ml
Normal mice		
Vehicle	Not detectable	Not detectable
PKW	Not detectable	129 ± 6*
PKW + anti-IL-6 antibody	—	123 ± 22*
PKW + TNFbp	Not detectable	40 ± 5*
PKW + anti-IL-6 + TNFbp	—	104 ± 5*
SCID mice		
Vehicle	—	Not detectable
PKW	—	92 ± 35*

NOTE. The PKW dose was 15 mg/kg IP. For TNFα, samples were collected 1 hour after PKW; for IL-6, samples were collected 3 hours after PKW.

*$P < .05$ *v* control group in respective study.

The chloroform precipitate induced a significant hypoglycemia (106 ± 14 mg/dL), weight loss, and complete anorexia, despite a lower protein content in the redissolved precipitate, constituting a dose of 2.1 mg protein/kg rather than 15 mg/kg. The chloroform extract, which did not contain any protein, had no effect on body weight or food intake. With this preparation, circulating glucose concentrations increased to 265 ± 24 mg/dL ($P < .05$ *v* controls).

Human B-Lymphocyte and T-Lymphocyte Stimulation by PKW

Unstimulated thymidine uptake by B lymphocytes was 241 ± 4 cpm/well. At 5 μg/mL, the chloroform precipitate increased thymidine uptake by B cells to 2,633 ± 267 cpm/well, 40% greater than unextracted PKW and 265% greater than the chloroform-extracted solution of PKW. Using T lymphocytes, baseline thymidine uptake was 750 ± 46 cpm/well. Extracted PKW caused the greatest increase in cellular thymidine uptake, to 42,213 ± 762 cpm/well. This was 24% greater than the PKW solution and 39% greater than the chloroform precipitate (Table 3).

Ex Vivo Exposure of Blood to PKW

Incubating mouse blood with PKW had no effect on the rate of glucose disappearance in the blood.

Deoxyglucose Uptake by C2C12 Myotubes

When myotubes were exposed to PKW and the two fractions of PKW, the chloroform precipitate stimulated a significant increase in deoxyglucose uptake, while the extracted fraction and the unextracted solution did not. This increase was 39% greater than for untreated myotubes ($P < .05$). In a subsequent study, insulin and the chloroform-precipitate fraction both stimulated a 48% increase in deoxyglucose uptake (both $P < .05$). Data from both studies are shown in Table 4.

Table 3. B-Cell and T-Cell Stimulation by PKW and Chloroform Fractions of PKW

Group	B Lymphocyte	T Lymphocyte
Vehicle	241 ± 4	750 ± 46
PKW	1,876 ± 291	36,696 ± 1,001
Chloroform-extracted PKW	720 ± 10	42,243 ± 767*
Chloroform precipitate	2,633 ± 267*	33,359 ± 1,457

NOTE. Protein administration was 5 μg/mL for all treated groups, with incubations for 3 days. Exposure to ^{3}H-thymidine was for 8 hours, and measurements represent cpm ^{3}H-thymidine uptake per well.

*$P < .05$ *v* other groups.

DISCUSSION

The strategy of treatment for adult-onset diabetes—controlling blood glucose and controlling body weight—includes both dietary modification and drug treatment.[3] However, diet therapy is often ineffective in controlling blood glucose concentrations,[24] and resistance to hypoglycemic drugs can develop.[25] We report here a protein type that may provide both of these means of treating type II diabetes, having hypoglycemic in addition to weight-loss effects. The protein is a lectin found in the pokeweed plant, *P. americana.*

Pokeweed contains a number of compounds in addition to lectins, including triterpenes, multicyclic organic compounds.[21] Triterpenes contained in ginseng stimulate glucose uptake by sheep red blood cells.[20] Although this effect could account for the hypoglycemia that developed after PKW was given to mice, it is unlikely. The decline in glucose was unaffected when PKW was added to blood ex vivo. Further, a nonprotein substance(s) extracted from the PKW preparation by chloroform caused hyperglycermia rather than hypoglycemia. It may be that the triterpenes present in pokeweed have binding properties similar to those of ginseng, but block the glucose uptake that ginseng-derived triterpenes stimulate.

A crude extract of the lectins from *P. americana* is referred to as PKW. PKW is a mixture of five lectins,[7,8] which are designated Pa-1 to Pa-5. These proteins range in size from 19 to 30 kd and contain approximately 3% carbohydrate.[7] The ligand for all pokeweed lectins is *N*-acetylglucosamine.[7] Despite binding to the same ligand, there is disparity between the bioactivity of these lectins. Only Pa-1 is a polymer,[7] and only Pa-1 stimulates B lymphocytes.[7,8,10] Further, Pa-2 to Pa-5, all of which stimulate T lymphocytes, do so with widely differing potency.[7] The diverse biological effects of the pokeweed lectins were born out by the differing results obtained for the chloroform-extracted PKW solution and the chloroform precipitate. Both PKW fractions contained several proteins when electropho-

Table 4. ^{3}H-Deoxyglucose Uptake by C2C12 Myotubes Incubated With Insulin or PKW

Study	cpm/mg Cellular Protein
I	
Vehicle	44 ± 4
Chloroform precipitate	61 ± 5*
Chloroform-extracted PKW	55 ± 6
PKW	44 ± 4
II	
Vehicle	63 ± 7
Insulin	93 ± 8*
Chloroform precipitate	93 ± 12*

NOTE. Twenty microliters of PBS or the treatment solutions were added to each well, yielding 4 μg/mL precipitate, 26 μg/mL extracted solution, and 30 μg/mL PKW solution used to derive the other treatments. The insulin concentration was 6 μg/mL (1 μmol/L). The myotubes were incubated with the treatments for 18 hours before ^{3}H-deoxyglucose exposure. Incubation with ^{3}H-deoxyglucose was for 20 minutes (n = 8 or 12 wells/group).

*$P < .05$ *v* vehicle.

resed on a nonreducing Western gel (data not shown). For the chloroform-precipitated fraction, the greatest staining on the gel was at 20 kd, roughly the size of Pa-1.[7] The presence of hypoglycemic and weight-reducing effects solely for this fraction, which also induced the greatest stimulation of B lymphocytes, suggests that Pa-1 was the major contributor to the metabolic changes seen in mice administered PKW.

One of these proteins—Pa-4—has been sequenced and was found to have homology to WGA.[13,14] WGA also binds to *N*-acetylglucosamine[5] and also stimulates T lymphocytes.[26] Preliminary studies in which WGA was given IP to mice found that this lectin has only a minor hypoglycemic effect, and although some anorexia is apparent, no weight loss developed after a single treatment with this lectin (personal observation, May 1996). This is similar to what was found in mice given the chloroform-extracted PKW solution, which is high in T-lymphocyte–stimulating lectins such as Pa-2 and Pa-4.

A role for T or B lymphocytes in the changes reported here, ie, hypoglycemia and anorexia, was ruled out by the use of SCID mice. These animals lack functional T and B lymphocytes but responded anyway, including an increase in circulating IL-6. Among potential sources of this cytokine in SCID mice are macrophages, which secrete IL-6[27] and respond to PKW,[28] and fibroblasts, which also secrete IL-6.[29]

The lower blood IL-6 concentrations in mice given the TNFα antagonist before PKW suggests that TNFα synthesis was stimulated by PKW, although secretion was not detected (this antagonist neutralizes both circulating and cell-associated TNFα.[18]) The persistence of an IL-6 response despite blockade of TNFα suggests that it was partly independent of TNFα, and supports other studies showing that PKW induced the direct release of this cytokine.[30]

The positive effect of passive immunization against IL-6 was equal to that of TNFbp treatment. IL-6 is a mediator of the hypoglycemia that follows LPS administration[31] and the anorexia associated with the sterile turpentine abscess.[19] Based on this, it was a likely contributor to the changes induced by PKW. In our earlier study with this model, blockade of another cytokine, IL-1, with anti-IL-1 type 1 receptor antibody (35F5) had effects on hypoglycemia that were equal to but not greater than those of the TNFα antagonist (data not shown). Since IL-6 follows TNFα and IL-1 release in the temporal pattern of cytokine appearance in an inflammatory response,[32] antagonism of either of these cytokines may have attenuated the hypoglycemia through a reduction in IL-6 concentration. Further, the level of IL-6 secretion remaining after TNFbp treatment could still have been large enough to induce the changes found. Nevertheless, all three antagonists were limited in their effectiveness, and when anti-IL-6 and TNFbp were given together, the results were not additive. This showed that IL-6 did contribute to the metabolic changes that developed, but was not the sole cause of the outcome of PKW administration.

The study reported here is the first to show that a decline in blood glucose concentrations follows lectin administration in the healthy animal. The hypoglycemia was not secondary to the decline in food intake. Fasted mice did not develop significant hypoglycemia in the time frame of the short-term studies. Combining fasting with PKW treatment would not be likely to influence the changes that presented, since food intake was completely abolished in several groups of mice given PKW as it was. A further exploration of fasting/PKW interactions would have been, in effect, equivalent to the regimen of the higher long-term dose given in this study, with lethal effects.

Attempts to associate an elevated endogenous insulin secretion with the hypoglycemia that followed PKW treatment were not successful. In fact, serum concentrations of insulin declined after PKW. Mice made diabetic by treatment with streptozotocin also had lower glucose concentrations following PKW treatment, additionally ruling out the involvement of insulin. Since neither a wholly cytokine-mediated effect nor insulin involvement in the decline in circulating glucose could be demonstrated, the possibility of a direct effect of PKW was investigated using a murine muscle cell line.

There are well-documented insulin-like effects of lectins in cell culture.[15,16,33] The elevated deoxyglucose uptake by C2C12 myotubes exposed to the Pa-1–enriched fraction of PKW suggested a direct mechanism for the hypoglycemia in mice given PKW. When deoxyglucose uptake was compared, the chloroform-precipitated fraction and insulin were equivalent at the doses of each that were used.

That the protein Pa-1, from an unpurified fraction of *P. americana,* could induce insulin-like effects in the mouse is indirectly supported by the in vitro demonstration of insulin-like activity of other, highly purified, lectins.[15,16,33] For example, another lectin with confirmed insulinomimetic activity in vitro, from *Momordica charantia,*[33] had a pattern of bioactivity that was identical to PKW in a study that compared their in vivo effects (data not shown).

The agents currently in use for controlling diabetes mellitus are the protein hormone insulin, which is roughly one third to one fifth of the size of the *P. americana* lectins, and relatively small nonprotein molecules such as the biguanides (eg, metformin), sulfonylureas (eg, chlorpropamide), and acarbose. The thiazolidinediones (eg, troglitazone), another type of nonprotein small molecule, are also likely to become choices for management of this disease.[34] Insulin constitutes a replacement therapy, biguanides increase peripheral insulin sensitivity,[35] sulfonylureas stimulate insulin release by the pancreas,[36] and acarbose is an oligosaccharide that inhibits pancreatic α-glucosidase.[37] Among the actions of thiazolidinediones, is sensitization to insulin activity.[34] The nonprotein molecules are orally active and thereby more easily used than current preparations of insulin. If the mechanism of action of Pa-1 includes direct stimulation of the insulin receptor, it is different from the noninsulin therapies for diabetes that are currently used. Why an oral dose of PKW was active, and not denatured and digested in the gastrointestinal tract, was not examined in this study. A study in which rats were fed a diet high in kidney beans, which contain the lectin phytohemagluttinin, also showed effects of this lectin after oral intake.[38] It is possible that the oral activity of PKW is dependent on the presence of food in the gut to provide protection from enzymatic degradation.

The amino acid sequence of Pa-4 has been determined,[13,14] and in several domains it is homologous to WGA, which does not have significant insulin-like activity in vivo (personal observations, May 1996). Because the structure of Pa-1 differs

from that of Pa-4,[7] and it has not yet been sequenced to our knowledge, a structure/function comparison with insulin cannot be presented here.

Further study will elucidate the mechanism of action for the insulinomimetic effect described here. Although the lectin from wheat germ links together two insulin receptors on the plasma membrane and thereby induces some of the effects of insulin,[15,16] WGA has insulin-like bioactivity even when insulin receptors are lacking.[15] This suggests that there are at least two pathways by which glucose uptake is stimulated by lectins.

Because insulin caused a decline in food intake and body weight when it was infused into the brain,[39,40] the anorexia and weight loss may also have been a direct insulin-like effect of PKW rather than a nonspecific cytokine-mediated result. However, these changes are contrary to the results of studies of systemic administration of insulin, where appetite and body weight increased.[41,42] Nevertheless, the half-life of insulin administered intravenously is short, roughly 5 to 15 minutes,[43,44] while that of PKW appears long. The long duration of PKW activity, 3 days when a dose of 15 mg/kg was given, may underlie the differences observed between these lectins and peripheral insulin administration regarding central stimulation. Although weight loss following PKW infusion was not consistently shown in the literature,[45,46] the different findings may have resulted from differing preparations of PKW, with a predominance of different forms of the lectins. For example, Pa-2, Pa-3, and Pa-4 inhibit the activity of Pa-1,[7] and the contribution of these lectins to PKW varies with the season of the year.[7]

The study shown here in which chloroform-extracted PKW induced an 89% decline in food intake without a significant decline in circulating glucose concentrations suggests that anorexia alone was not sufficient to cause the rapid weight loss in mice given PKW. Concomitant hypoglycemia seemed to be required for this to develop. The combination of anorexia and unrelenting hypoglycemia may promote exaggerated mechanisms of adaptation to starvation, ie, elevated mobilization of energy from muscle and fat depots. Indeed, the decline in body weight that we found after PKW was due to a loss of both protein and fat stores. Although we did not measure plasma indicators of elevated fat oxidation such as β-hydroxybutyrate, the measurement of epididymal adipose tissue directly in one study, and the finding that it was significantly lower, suggests that this did occur.

PKW toxicity did not cause the metabolic changes described in this report. Lectins from pokeweed were shown to be nontoxic when given to mice[45-47] and after ingestion by humans.[48-50] A published study suggests that kidney, liver, and lung function were not affected by PKW.[45] Studies of the central nervous[47] and cardiovascular[48] systems in animals and humans exposed to PKW, respectively, also indicate no deleterious effects. The mortality that resulted from the higher long-term dose was probably secondary to starvation. Mice given the lower dose for 16 days did not appear to be distressed and showed no aversive responses to receiving the proteins IP (ie, no biting, defecating, vocalization, diarrhea, piloerection, lethargy, etc.).

In conclusion, a pokeweed lectin has insulin-like activity in mice and induces weight loss in these animals even when given orally. It is possible that such an orally active protein can be used to develop a treatment for diabetes mellitus or obesity, offering a new therapeutic strategy for managing these diseases.

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