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QUANTITATIVE RELATIONSHIP OF LYSOSOMAL GLYCOGEN ACCUMULATION TO LYSOSOMAL α-GLUCOSIDASE INHIBITION IN CASTANOSPERMINE-TREATED RATS

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Abstract—To quantitatively examine the relationship between lysosomal acid α-glucosidase (LAAG, α-D-glucoside glucohydrolase, EC 3.2.1.20) inhibition and glycogen accumulation, rats were treated with castanospermine (CS), and liver lysosomal/mitochondrial fractions were analyzed for glycogen content and LAAG activity. Liver lysosomal glycogen accumulation positively correlated ($r = 0.90$) with the amount of LAAG inhibition when inhibition was about 50% or greater. Glycogen did not accumulate when LAAG inhibition was less than 50%. The route of CS administration had little effect on the amount of LAAG inhibition observed. In rats killed 17 hr after CS administration, the doses estimated to cause 50% LAAG inhibition were 0.77, 0.11, and 0.22 mg/kg for i.p., i.v., and oral administration respectively. After 89% inhibition of LAAG activity with a single oral dose of 10 mg CS/kg, LAAG activity returned to 50% of normal value in about 2.5 days. Accumulated glycogen disappeared as LAAG activity recovered. Surprisingly, twelve daily CS doses of 1 mg/kg had only a small cumulative effect on LAAG inhibition and did not cause more glycogen accumulation than a single dose.

Lysosomes normally contain a small fraction of the total cellular glycogen [1–3] which is hydrolyzed to glucose by lysosomal acid α-glucosidase (LAAG, α-D-glucoside glucohydrolase, EC 3.2.1.20) and recycled to the cytosol. However, lysosomal glycogen may accumulate when LAAG is deficient [4, 5] or when rats or cell cultures are treated with certain α-glucosidase inhibitors [6–10]. A genetic deficiency of LAAG activity is the primary defect in type II glycogenosis [4, 5]. In the severe, infantile form of type II glycogenosis known as Pompe's disease, LAAG activity is often undetectable and lysosomal glycogen occurs in massive amounts [2, 11]. In the less severe, adult form of the disease, LAAG activity may be present at 10–20% of normal activity [11, 12], and glycogen accumulation appears to be much less pronounced.

Fibroblasts isolated from patients with varying disease severity contain little lysosomal glycogen when the cells have 10% or more of normal LAAG activity [2]. In HepG2 cells, treatment with the α-glucosidase inhibitor, BAY m 1099, causes lysosomal glycogen accumulation and reduces LAAG activity to 10% of control [6]. These observations suggest that lysosomal glycogen accumulation may not be expected *in vivo* after treatment with LAAG inhibitors until LAAG activity is inhibited to about 10% of its normal activity. However, two other LAAG inhibitors, acarbose and castanospermine (CS, [(1*S*,6*S*,7*R*,8*R*,8a*R*)-1,6,7,8-tetrahydroxyindolizidine)], cause liver lysosomal glycogen accumulation at doses that appear to inhibit LAAG only about 50% or less [8–10]. No clear dose-dependent effect on LAAG inhibition or on lysosomal glycogen accumulation has been described using either inhibitor. The lack of a quantitative relationship between LAAG inhibition and lysosomal glycogen accumulation has prompted some authors to propose additional mechanisms to explain lysosomal glycogen accumulation [9].

Because CS behaves as an irreversible inhibitor of rat liver LAAG activity [13], recovery of LAAG activity will not occur during cell fractionation and enzyme assay. Therefore, the amount of LAAG inhibition observed *in vitro* should reflect the amount of LAAG inhibition *in vivo*. In this study, we have used CS as a tool to examine the quantitative relationship between liver LAAG inhibition and lysosomal glycogen accumulation in rats. In a similar manner, we have demonstrated previously that CS can be useful to quantitatively evaluate the relationship between intestinal sucrase activity and rate of glucose absorption [14].

MATERIALS AND METHODS

Materials. Castanospermine was isolated from seeds of the Australian "black bean" tree, *Castanospermum australe*, as previously described [15]. *p*-Nitrophenyl derivatives of phosphate, sulfate, α-D-glucopyranoside, β-D-galactopyranoside, β-D-glucuronide, and *N*-acetyl-β-glucosaminide were obtained commercially (Sigma, St. Louis, MO). Male, 175–250 g Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN) were used in all experiments. When the time between the CS dose and sacrifice was 2 hr, rats were fasted for 17 hr before the dose; otherwise, all rats were dosed with CS and then fasted for 17 hr prior to being killed.

Determination of lysosomal enzyme activities. A

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lysosomal/mitochondrial pellet was prepared from 3.0 to 3.2 g of liver from each rat by a differential centrifugation method in which the lysosomal/mitochondrial pellet was washed three times to remove cytosolic glycogen [1]. Soluble lysosomal enzymes and glycogen were hypoosmotically released by resuspending the lysosomal/mitochondrial pellet in water to 2 mL total volume, yielding a lysate containing 5–10 mg protein/mL. Preliminary experiments indicated that the following amounts of lysate were suitable for determining enzyme activity: 2 μL for acid phosphatase, 10 μL for β-glucuronidase and *N*-acetyl-β-glucosaminidase, and 100 μL for LAAG, β-galactosidase, and aryl sulfatase. Assays were performed at 37° in a total volume of 0.3 mL containing the lysate and 5 mM concentration of the *p*-nitrophenyl derivative of phosphate, sulfate or the appropriate glycoside in 67 mM sodium acetate, pH 5.0 [16], for each enzyme except LAAG. LAAG activity was assayed at pH 4.2 in 67 mM sodium acetate plus 16.7 mM KCl [13]. Acid phosphatase reactions were incubated for 10 min, β-galactosidase reactions for 30 min, and the other enzyme reactions for 60 min. In each case, 2% or less of the substrate was hydrolyzed during the incubation period and the reaction rate was directly proportional to the amount of lysosomal/mitochondrial lysate added. The acid phosphatase reaction was terminated by addition of 1 mL of 0.1 M Na_2CO_3. All other enzyme reactions were terminated by heating at 90° for 2 min followed by centrifugation and transfer of 100 μL of the supernatant fraction into 1.2 mL of 83 mM Na_2CO_3. The amount of *p*-nitrophenol released was determined by its absorption at 410 nm. A unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of *p*-nitrophenol/min. Protein was determined by a modified Lowry method using a commercially available kit (P5656, Sigma, St. Louis, MO).

Determination of lysosomal glycogen content. Glycogen content in the lysosomal/mitochondrial lysate was determined by a modified procedure of van der Ploeg *et al.* [2]. To 100 μL of the lysate was added 100 μL of 100 mM sodium acetate, pH 4.5, containing 45 μg *Aspergillus niger* amyloglucosidase (Sigma, St. Louis, MO). The reaction was incubated at 37° for 1 hr and stopped by heating at 90° for 2 min. After centrifugation, glucose was determined in the supernatant fraction using a commercial kit (Seradyn Diagnostics, Indianapolis, IN). Rabbit liver glycogen, used as a standard, gave the theoretical yield of glucose.

Effect of CS treatment on lysosomal enzyme activities and glycogen content. To compare lysosome yields from control and CS-treated rats, lysosomal/mitochondrial pellets were isolated from rat livers 17 hr after an oral dose by gastrogavage of CS (0 or 10 mg/kg body weight in 10 mL water/kg body weight). In this experiment, lysates from three pellets were pooled to obtain sufficient lysate and assayed for protein and lysosomal glycogen content and LAAG, aryl sulfatase, acid phosphatase, β-glucuronidase, β-galactosidase, and *N*-acetyl-β-glucosaminidase activities. To establish dose–response curves for CS inhibition of LAAG activity under various conditions, CS was administered to rats i.p. or i.v. in 0.9% NaCl (2.0 mL/kg body weight) or orally in water (10 mL/kg body weight). The doses of CS included 0.001, 0.01, 0.1, 1, 10, 100, and 1000 mg/kg body weight and were administered 2 or 17 hr before the rats were killed; not all doses were used in every experiment. A lysosomal/mitochondrial pellet was isolated from each rat and the lysate assayed for LAAG and acid phosphatase activities and protein concentration. Next, LAAG activity and glycogen and protein content were measured in lysates from rats killed 17 hr after an oral dose of CS (0.1, 1, 10, 100, or 1000 mg/kg body weight) and in rats killed at various times (2 hr to 7 days plus 17 hr) after an oral dose of 10 mg CS/kg body weight. In a multiple dose experiment, rats received a daily oral dose of 0.1 or 1 mg CS/kg body weight for 12 days. Rats were killed 17 hr after the final CS dose, and LAAG activity and protein and lysosomal glycogen content were measured.

Statistics. The two-sample *t*-test was used to examine differences in glycogen content and enzyme activities between lysosomes recovered from CS-treated rats and control rats and the effect of multiple doses of CS vs a single dose on lysosomal glycogen content and LAAG activity. The slopes of the linear portion of the log dose–response curves of LAAG activity in CS-treated rats were determined by linear regression analysis and compared using the Z-test [17]. The ED_{50}, defined as the dose of CS (expressed as mg/kg body weight) causing 50% inhibition of LAAG activity, was determined from a linear regression analysis of the linear portion of the log dose–response curve. Dose–response relationships between CS and LAAG activity and between CS and lysosomal glycogen content were determined using the linear trend test [17]. A plot of lysosomal glycogen content vs LAAG activity was analyzed by linear regression.

RESULTS

Preliminary studies indicated that an oral dose of 10 mg CS/kg resulted in lysosomal glycogen accumulation and inhibition of LAAG activity in treated rats. To determine if our method of lysosome isolation resulted in approximately equal yields of lysosomes from control and CS-treated rats, we measured the activities of several lysosomal enzymes from rats killed 17 hr after an oral dose of 10 mg CS/kg body weight (Fig. 1). Compared to controls, lysosomal glycogen content increased 4-fold and LAAG activity decreased 5-fold in CS-treated rats, while the activities of aryl sulfatase, acid phosphatase, β-glucuronidase, β-galactosidase, and *N*-acetyl-β-glucosaminidase were not affected significantly. Acid phosphatase activity was initially selected for normalization of lysosomal data, but in later studies we found that acid phosphatase activity was often increased in CS-treated rats, particularly at the doses which affected LAAG and glycogen. Therefore, we used the protein content of the lysosomal/mitochondrial lysate to normalize lysosomal data.

To establish dose–response curves for CS inhibition of LAAG activity, rats were given various doses of CS by i.p., i.v., or oral administration and killed 2

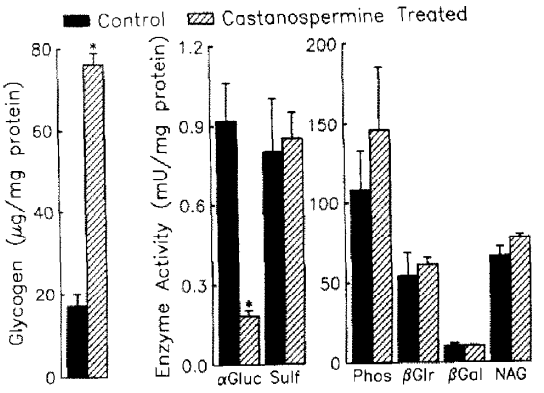

Fig. 1. Liver lysosomal glycogen content and enzyme activities in rats administered a single oral dose of water or 10 mg castanospermine/kg body weight 17 hr before being killed. Bars represent the mean ± SE for each parameter. N = 3 mitochondrial/lysosomal preparations. αGluc = acid α-glucosidase, Sulf = aryl sulfatase, Phos = acid phosphatase, βGlr = β-glucuronidase, βGal = β-galactosidase and NAG = *N*-acetyl-β-glucosidaminidase activity. Key: (*) indicates a significant difference ($P < 0.05$) from the control group.

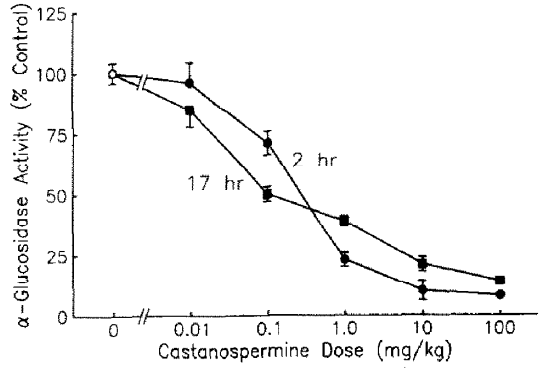

Fig. 2. Dose–response curves for castanospermine inhibition of lysosomal acid α-glucosidase activity. Rats were administered a single oral dose of castanospermine 2 (●) or 17 (■) hr before being killed. Values were calculated as percent of the control group α-glucosidase activity (1.07 ± 0.04 mUnits/mg protein) and are expressed as mean ± SE. In some cases, the error bars were less than the size of the symbol. N = 36 for the control group (presented as 0 mg castanospermine/kg) which included rats i.p., i.v., or orally dosed with 0.9% NaCl or water 2 or 17 hr before being killed. N = 2–6 for each dose of castanospermine, totalling 14 and 20 for the 2-hr and 17-hr groups respectively.

or 17 hr later. The dose–response curves of LAAG activity after oral administration of CS are presented in Fig. 2. Because LAAG activity did not vary significantly among rats administered 0.9% NaCl or water 2 or 17 hr before being killed, the data from all controls were averaged and are presented as 0 mg CS/kg body weight in Fig. 2. The ED_{50} values and slope of the log dose–response curves for each administration route are presented in Table 1. Each route of CS administration inhibited LAAG activity in a similar dose-dependent manner. The 95% confidence limits of the ED_{50} values overlapped in all cases. The slopes of the 2-hr log dose–response curves did not differ significantly among administration routes. The slopes of the 17 hr i.v. and oral log dose–response curves differed significantly from each other but neither differed from the slope of the i.p. curve. For each route of CS administration, the slope of the 2-hr log dose–response curve was significantly steeper than the slope of the 17-hr curve. Because the administration route appeared to have little effect on the potency of CS inhibition of LAAG activity, we used the oral route for all further studies.

The relationship between CS-inhibited LAAG activity and lysosomal glycogen content was examined in rats killed 17 hr after an oral dose of 0.1 to 1000 mg CS/kg body weight (Fig. 3). A dose of 0.1 mg CS/kg inhibited LAAG activity by 49% but did not cause a detectable increase in glycogen content. At higher doses, LAAG activity decreased and lysosomal glycogen content increased dose dependently. Lysosomal glycogen content was positively correlated ($r = 0.90$) with percent LAAG inhibition at doses of 0.1 to 1000 mg CS/kg.

LAAG activity and lysosomal glycogen content were examined in rats killed at various times after an oral dose of 10 mg CS/kg body weight (Fig. 4). LAAG activity and glycogen content did not vary significantly among control rats killed throughout the study; therefore, the data were averaged and are presented as 0 days after CS in Fig. 4. LAAG activity decreased 89% in rats killed 2 hr after CS and then gradually increased, reaching 67% of initial activity in animals killed 7 days plus 17 hr after CS. Lysosomal glycogen content increased 10-fold in rats killed 17 hr after CS and thereafter gradually returned to control levels. Recovery of LAAG activity to about 50% of initial levels coincided with the return of glycogen content toward control levels. Plotting these data as log activity versus time allowed an estimation of 2.5 days for the half-recovery time of LAAG activity.

To determine if multiple doses of CS would have a cumulative effect on LAAG activity and lysosomal glycogen content, rats were given twelve daily oral doses of 0.1 or 1.0 mg CS/kg and killed 17 hr after the final dose (Table 2). For comparison the appropriate data are also presented from an earlier study (Fig. 3) in which rats were killed 17 hr after a single oral dose of CS. After one or twelve doses of CS, 0.1 and 1.0 mg/kg significantly inhibited LAAG activity, but only 1.0 mg/kg caused glycogen accumulation. Twelve doses of 1 mg/kg appeared to inhibit LAAG only slightly more than one dose did. A cumulative effect on lysosomal glycogen content was clearly not observed in rats administered twelve doses of CS.

DISCUSSION

Liver lysosomal glycogen accumulation correlated with the amount of LAAG inhibition when inhibition was greater than 50%; however, glycogen did not

Table 1. ED_{50} and slope of dose–response curves for castanospermine inhibition of lysosomal acid α-glucosidase activity

Pretreatment*	N (range)†	ED_{50}‡	Slope§
2 hr, i.p.	8 (0.1–10)	0.54 (0.16, 2.1)	−0.35 ± 0.07‖
2 hr, i.v.	12 (0.1–10)	0.53 (0.25, 0.97)	−0.33 ± 0.05‖
2 hr, oral	12 (0.01–10)	0.42 (0.18, 0.95)	−0.27 ± 0.03‖
17 hr, i.p.	22 (0.001–1000)	0.77 (0.24, 2.5)	−0.15 ± 0.02¶**
17 hr, i.v.	26 (0.001–1000)	0.11 (0.02, 0.64)	−0.11 ± 0.02**
17 hr, oral	18 (0.01–10)	0.22 (0.11, 0.42)	−0.18 ± 0.02¶

* Pretreatment is the time interval between castanospermine administration and sacrifice. Rats were treated with i.p., i.v., or oral castanospermine at doses that included 0, 0.001, 0.01, 0.1, 1, 10, 100, and 1000 mg/kg body weight.

† N is the total number of rats (range of doses) included in the linear regression analysis for each pretreatment–route combination.

‡ ED_{50} is expressed in mg CS/kg body weight and includes 95% confidence limits in parentheses.

§ Slope is expressed as mean ± SE from the linear regression analysis of the linear portion of the log dose–response curve.

‖ Significantly different ($P < 0.05$) from the corresponding 17-hr group.

¶** Groups not followed by a common symbol were significantly different ($P < 0.05$).

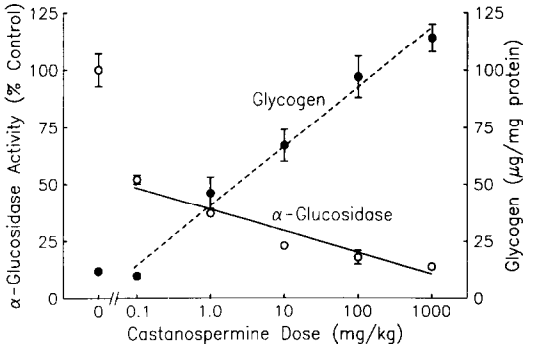

Fig. 3. Relationship between lysosomal acid α-glucosidase activity and glycogen content in rats administered a single oral dose of water or castanospermine 17 hr before being killed. Values are the means ± SE of the lysosomal glycogen content (●) and acid α-glucosidase activity (○) in control and castanospermine-treated rats. In several cases, the error bars were less than the size of the symbol. Linear regression lines were fit to the data from the castanospermine-treated rats. Acid α-glucosidase activities were calculated as percent of the control group activity (1.47 ± 0.10 mUnits/mg protein). N = 3 for the control group and for each dose of castanospermine.

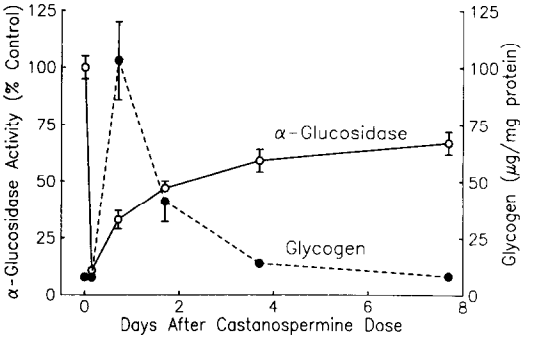

Fig. 4. Time course of lysosomal acid α-glucosidase inhibition and glycogen accumulation in rats killed 2 hr, 17 hr, 1 day plus 17 hr, 3 days plus 17 hr or 7 days plus 17 hr after a single oral dose of 0 or 10 mg castanospermine/kg body weight. Values are the means ± SE of lysosomal glycogen (●) and acid α-glucosidase activity (○) in control and castanospermine-treated rats. In several cases the error bars were less than the size of the symbol. The data were averaged for control rats killed throughout the study and are presented as 0 days after castanospermine. Acid α-glucosidase activities were calculated as percent of the control group activity (1.50 ± 0.07 mUnits/mg protein). N = 15 for the control group. N = 3–9 for each time point, totalling 24 for all castanospermine-treated groups.

accumulate when LAAG inhibition was less than 50%. CS treatment did not simultaneously reduce the activities of other lysosomal enzymes, indicating that the decrease of LAAG activity was not the result of a decreased recovery of lysosomes.

A single dose of 1 mg CS/kg inhibited LAAG about 50%, suggesting that CS is about 1000 times more potent than was suggested previously [10]. This difference in potency of CS between the two studies cannot be explained as a difference between i.p. and oral administration because we found that route of CS administration had minimal, if any, effect on LAAG inhibition.

We were surprised that, for all three routes of CS administration (i.v., i.p., and oral), the slopes of the dose–response curve was significantly more shallow in 17-hr pretreated rats than in 2-hr pretreated rats. We know of no single factor that could be responsible for both an increased inhibition at lower doses and a decreased inhibition at higher doses, suggesting that two factors may be responsible. Slow penetration of CS to the lysosome could result in the increased LAAG inhibition observed at lower doses in the 17-hr pretreated rats but would also result in an increased LAAG inhibition at the higher doses. A possible explanation for the observed decrease in

Table 2. Effect of 1 or 12 days of castanospermine treatment on lysosomal acid α-glucosidase activity and glycogen accumulation

Dose* (mg/kg)	N	Treatment (days)	α-Glucosidase (mUnits/mg protein)	Glycogen (μg/mg protein)
0.0	3	1	1.6 ± 0.2†	8.2 ± 0.8
	5	12	1.5 ± 0.1	12 ± 2
0.1	3	1	0.81 ± 0.07‡	12 ± 1
	7	12	0.76 ± 0.03‡	10 ± 1
1.0	3	1	0.76 ± 0.03‡	48 ± 3‡
	6	12	0.55 ± 0.02‡§	46 ± 8‡

* Castanospermine or water was administered orally once/day for 1 or 12 days and the rats were killed 17 hr after the last dose.

† Values are expressed as means ± SE.

‡ Significantly different ($P < 0.05$) from the appropriate 0.0 mg/kg group.

§ Significantly different ($P < 0.05$) from the 1.0 mg/kg group treated for 1 day.

LAAG inhibition at the higher CS doses in the 17-hr pretreated rats is that the synthesis of new LAAG may be stimulated when its activity is inhibited more than 50%. This stimulus may be the accumulated glycogen.

The relationship between LAAG inhibition and lysosomal glycogen accumulation in the dose–response experiment (Fig. 3) and during 8 days following a single oral dose of CS (Fig. 4) indicated that lysosomal glycogen accumulated when LAAG activity was inhibited about 50% or greater and lysosomal glycogen disappeared when LAAG activity was inhibited less than 50%. The time required for 50% recovery of LAAG activity was estimated as about 2.5 days which is somewhat less than the estimated 5- to 7-day half-life for LAAG in fibroblasts [18]. Because LAAG appeared to have a half-recovery time of 2.5 days, daily treatment with a nearly irreversible inhibitor would be expected to have a cumulative effect on LAAG inhibition. Contrary to expectation, little or no cumulative effect on LAAG inhibition and no cumulative effect on glycogen accumulation were observed following 12 days of CS treatment. Possibly multiple CS doses stimulated LAAG synthesis greater than a single dose.

In conclusion, we have demonstrated that lysosomal glycogen accumulated and was quantitatively proportional to the amount of LAAG inhibition when LAAG activity was inhibited about 50% or more. This relationship between LAAG activity and lysosomal glycogen accumulation may be useful for estimating the LAAG inhibition that occurs *in vivo* when studying reversible α-glucosidase inhibitors. Reversible inhibitors can rapidly dissociate from the enzyme during tissue preparation and during enzyme assay, when the lysosome is lysed or the sample volume is increased by buffer and/or substrate addition. Depending upon the extent of the dilution involved, this dissociation allows a partial or total recovery of enzyme activity which could suggest, misleadingly, that the enzyme had not been inhibited. Measurement of lysosomal glycogen content *ex vivo* rather than LAAG activity could circumvent this problem, thus avoiding severely underestimating *in vivo* LAAG inhibition.

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