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Research report

Increased ATP content/production in the hypothalamus may be a signal for energy-sensing of satiety: studies of the anorectic mechanism of a plant steroidal glycoside

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Abstract

A steroidal glycoside with anorectic activity in animals, termed P57AS3 (P57), was isolated from *Hoodia gordonii* and found to have homologies to the steroidal core of cardiac glycosides. Intracerebroventricular (i.c.v.) injections of the purified P57AS3 demonstrated that the compound has a likely central (CNS) mechanism of action. There is no evidence of P57AS3 binding to or altering activity of known receptors or proteins, including Na/K-ATPase, the putative target of cardiac glycosides. The studies demonstrated that the compound increases the content of ATP by 50–150% in hypothalamic neurons. In addition, third ventricle (i.c.v.) administration of P57, which reduces subsequent 24-h food intake by 40–60%, also increases ATP content in hypothalamic slice punches removed at 24 h following the i.c.v. injections. In related studies, in pair fed rats fed a low calorie diet for 4 days, the content of ATP in the hypothalami of control i.c.v. injected animals fell by 30–50%, which was blocked by i.c.v. injections of P57AS3. With growing evidence of metabolic or nutrient-sensing by the hypothalamus, ATP may be a common currency of energy sensing, which in turn may trigger the appropriate neural, endocrine and appetitive responses as similar to other fundamental hypothalamic homeostatic centers for temperature and osmolarity.

Keywords: Hypothalamus; Appetite regulation; ATP; Energy sensing; Plant steroidal glycoside

1. Introduction

There has been a resurgence of interest in traditional and folkloric medicines as possible therapeutic agents as well as clues or leads to novel therapeutic mechanisms. An example of such an anecdotal or folkloric agent is the sap from a group of South African cactus-like plants of the species *Hoodia* (including subspecies *Hoodia gordonii* or *Hoodia lugardi*), a member of the large milkweed family. Based on a limited number of reports from and subsequent interviews with both native and foreign South Africans, the sap apparently assuaged both the feelings and sensations (e.g., 'pangs') of hunger that occurred during long treks in the dry bush [2]. Several active components, in particular that described in this report, were isolated by South African scientists of the Council of Scientific and Industrial Research (CSIR) and have been disclosed in recent patents. Dried extracts of the plant sap, which contain not only the putative active agent but multiple other components, have been administered long term to several species, including the diabetic obese Zucker rat. In those experiments, anorectic activity and reversal of diabetes are maintained for the duration of dosing, up to 8 weeks; other studies demonstrate that the food inhibition and weight loss are independent of diet nutrient content and also occur in animals who overfeed on a highly palatable diet (Pytopharm and Pfizer, unpublished). Animal safety studies do not demonstrate deleterious effects independent of the weight loss itself. In addition, Phytopharm has recently disclosed short-term studies in humans, during which the plant extract was well tolerated.

The putative active component in these sap extracts is a trirhabinoside, 14-OH, 12-tigloyl pregnane steroidal glycoside ($M_W = 1008$). The core steroid, particularly regarding the 14-OH substitution, is somewhat similar to other cardenolides

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[8,16,44]. Based on this similarity, and because initial characterization failed to identify binding or activity with a full range of neurotransmitter or neuropeptide receptors, ion channels, or Na/K-ATPase (the target of cardiac glycosides) we undertook further studies with the purified steroidal glycoside (P57AS3) to identify its possible mode(s) of action.

2. Materials and methods

2.1. Chemicals

A plant steroidal glycoside (P57AS3, $M_W = 1008$) and related congeners were prepared by the S.A. CSIR and supplied by Phytopharm and Pfizer (Groton, CT). The compounds reported here include P57AS3, the aglycone termed P57ASA and a-19-substituted compound termed P57NS5. 86-rubidium (^{86}Rb) was purchased from Amersham (United Kingdom). The Enliten™ ATP Assay kit was purchased from Promega (Madison, WI).

2.2. Animals

Male Sprague–Dawley rats weighing an average of 170 ± 5 g were purchased from Charles River Laboratories (Wilmington, MA). Rats were housed individually in metabolic cages in a temperature-controlled environment (22 °C) with 12-h light/12-h dark cycle. All rats had ad libitum access to pelleted rat chow and tap water except when noted otherwise. Animals were weighed daily or as appropriate and daily food intakes were measured using standard metabolic cage techniques. For in vivo experiments, 4–6 rats were studied per group or dose; including repeated experiments approximately 36–72 animals were used for each separate study. For neuronal cultures, hypothalami were obtained from day 17 fetal rats; approximately one dissociated hypothalamus was required per plated well. The Animal Care Committee at the Rhode Island Hospital approved the study protocols used in these experiments.

2.3. Intracerebroventricular (i.c.v.) (III ventricle) injections

In several experiments to determine subsequent effects on food intake, hypothalamic Na/K-ATPase activity or ATP content, P57AS3 steroidal glycoside or congeners were administered by i.c.v. injection to rats. Animals were anesthetized with sodium pentobarbital (6 mg/100 g BW). Under stereotaxic fixation (David Kopf), with aseptic technique, a 25-μl Hamilton microsyringe (Reno, NV) needle tip was inserted according to the published references: 2 mm lateral to the midline at the bregma; − 4.3 mm to the bregma; 5.5 mm deep to the dura; − 1.3 mm with the shaft inclined medially at 13° to the vertical. The P57 was dissolved in DMSO from a 20 mM stock concentration, and 4 μl (or vehicle) was microinjected into the third (III) ventricle. All injections were calculated and expressed in absolute amount, i.e., nanomoles. After rats recovered from anesthesia, they were returned to individual cages and food intake measurements continued for 24-h intervals until the end of the experiment.

At the end of each experiment, the animals were administered a lethal anesthetic overdose prior to dissection and subsequent ex vivo assay of brain tissue.

2.4. Animal experiments

Food intake was controlled in the caloric deprivation study by supplying daily to each rat a pre-weighed, fixed amount of powdered chow (10 g).

2.5. Hypothalamic slice punch, ^{86}Rb uptake

Net potassium flux across cellular membranes may be measured using an isotope of rubidium, ^{86}Rb, which has similar chemical properties to potassium, in the same atomic element group. ^{86}Rb uptake thus has been used for a whole-cell assay of Na/K-ATPase activity, as that enzyme is the primary determinant of cellular sodium–potassium exchange. The whole brain of rats was removed from the skull and inserted into a Precision Brain Slicer (RMB 3000C, Braintree Scientific). As scaled from the rear, a brain slice was removed between the 8 and 10 scale (about 2 mm). The medial-basal hypothalamus was punched using a round mouth #18 1-mm diameter needle with attached syringe. The tissue included most of arcuate nucleus of the hypothalamus and a small part of nucleus premamillaris ventralis. The tissue was pushed into 0.5 ml DMEM medium in a 24-well cell culture plate. Following pre-incubation at 37 °C, in air/5% CO_2 for 30 min, concentrations of P57 were added for an additional 30 min. Finally, ^{86}Rb, 1 μCi, was added to each well. at room temperature and allowed to equilibrate between the intracellular and extracellular compartments for 20 min. The tissue was washed and then transferred to tubes with a protein dialysis solution in order to lyse the cells and dissolve protein. An aliquot of cellular lysate was added to 5 ml scintillation fluid and the photoemissions determined in a β-counter (Beckman). Results were expressed as CPM per mg wet weight tissue.

To measure the effect of the P57 on hypothalamic ATP in diet-controlled hypocaloric rats, hypothalamic slice punches were prepared as described above 24 h following P57 i.c.v. injection. Following extraction, the content of ATP in these slice punches was measured by the Enliten™ ATP Assay system (see below). In some studies, in order to parallel the 86-rubidium conditions, the punches were maintained in vitro as described above for a 20-min incubation period at a final P57AS3 concentration of 100–500 nM.

2.6. Hypothalamic neuronal culture

Primary rat hypothalamic cells were prepared from fetal rats (day 17) using previously described methods [29].

Briefly, the hypothalamus was removed and dispersed enzymatically for 2 h with neutral protease in culture medium at a concentration of 100 U/dl. The dispersed cells were then plated at a low density of 10^6 cells/ml in 24-well culture dishes pre-coated with 20 μg/ml poly-D-lysine (Sigma). The cells were maintained in bicarbonate buffered Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), at 37 °C, 5% CO_2 and 95% humidity [29].

For the described experiments, hypothalamic neurons were pre-cultured for 11 days to allow maturation of a relatively pure neuron culture. Before testing the effect of compounds, neurons were washed once with a buffered salt solution 0.01 M Tris·HCl–0.15 M NaCl (pH 7.4). In initial determination of the IC50 for ^{86}Rb uptake, ouabain was added at concentrations of 10, 100, 1000, 10,000, and 100,000 nM. For subsequent experiments, ouabain was added to a final concentration of 1000 nM (the approximate IC50). For testing the effect of P57, neurons were pre-incubated at selected concentrations in 0.4 ml DMEM for 10 min, and then ouabain was added to a final concentration of 1000 nM for an additional 20 min at 37 °C. Finally, ^{86}Rb (Amersham) 1.0 μCi was then added to the wells, which were kept at room temperature for another 20 min. Medium was removed and neurons were triple washed with ice-cold NaCl solution to remove free ^{86}Rb. The cells were then lysed in 100 μl 0.05% Triton-100 in PBS and the lysate harvested with an additional 0.5 ml PBS. The lysate was added to 5 ml scintillation fluid and cellular ^{86}Rb photon emissions counted for a minimum of 5 min.

2.7. Measurement of ATP content

The Enliten™ ATP Assay system with bioluminescence detection kit (Promega) was used for ATP measurement. The principle of the assay is that luciferase from *Photinus pyralis* catalyses D-luciferin in the presence of ATP and oxygen to oxyluciferin, Pi, AMP, carbon dioxide, and light. As described above, hypothalamic tissues after removal or removal/incubation were transferred to 1.5 ml Eppendorf tubes with 50 μl TCA (5%) and immediately homogenated with a plastic homogenizer. The suspension was spun at 2000 rpm and the supernatant stored at −80 °C until assay. ATP was measured according to the kit protocol. Briefly, samples were neutralized to pH 7.4 with 10 μl 4 M Tris and 10 μl added to a new tube with 90 μl ATP-free water. The luciferase reagent was added 1 s before a 5-s measurement in the luminometer, as described by the supplier. Light photons were measured by a luminometer and were compared with an ATP standard curve to calculate ATP concentration [23]. ATP content is expressed as mol per mg protein for tissue samples, mol per 5 mg tissue for section punches, and as moles per neuron culture well; expressed in appropriate quantitative units for each experiment.

2.8. Statistics

In the text, table, and figures, all data are presented as means ± S.E.M. Most described experiments were repeated three times, using similar, but not always, identical conditions and with similar overall results. For figures and tables, the results of a single representative experiment are shown. Food intake and body weight data used for graphical presentation and statistical analysis are expressed as per experimental time periods or for the 24 h following i.c.v. injection. Food intake, body weight, ATP content and ^{86}RB uptake data were analyzed by ANOVA statistic program using one and two factor analysis of variance of repeated measures. P values for all F-tests are $P < 0.01$. Tukey's test was used for post-hoc comparisons among individual means.

3. Results

3.1. Effect of i.c.v. P57AS3 and other steroidal glycosides on food intake during 24-h periods

To determine whether the anorectic activity may occur within the brain, specifically within the hypothalamus, P57AS3 was injected into the third ventricle under stereotactic guidance at doses of 0.4–40 nmol, dissolved in 4 μl DMSO (Fig. 1). DMSO alone was injected as the control.

As shown in Fig. 1a for one representative study, in multiple experiments the P57AS3 reduced food intake over the 24 h following i.c.v. injection by 50–60%. The effect increased with dose, with 0.4 nmol having a significant but relatively minor effect. The duration of effect was approximately 24–48 h, depending on dose. The animals appeared to groom and behave normally following recovering from anesthesia. A 20-nmol injection administered once i.p. did not significantly reduce food intake (data not shown).

Two other P57 congeners were also tested i.c.v. at doses of approximately 40 nmol in DMSO. One, the aglycone P57ASA, was similar except for the absence of the triglycoside chain off the A-ring [16]. The other, termed P57NS5, has a 5-carbon substitution at the C-19 position of the D-ring. As shown in Fig. 1b, neither compound altered subsequent 24-h food intake. Finally, ouabain was also injected at approximately 8 nmol in 4 μl DMSO. Ouabain resulted in motor hyperactivity, a previously reported phenomenon [24], but had no consistent effect on food intake (results not shown).

3.2. Effects of P57AS3 on ^{86}Rb uptake in hypothalamic cultures and explants in vitro

The P57s have a 4-ring core and 14-OH substitution that suggest it is biochemically and phylogenetically related to other plant cardenolides, without the digitalis-specific D-ring lactone [9] [17] (Figs. 2 and 3). For this reason, we speculated that the P57s may interact with a cardiac glycoside binding site, e.g., on the Na/K + ATPase complex,

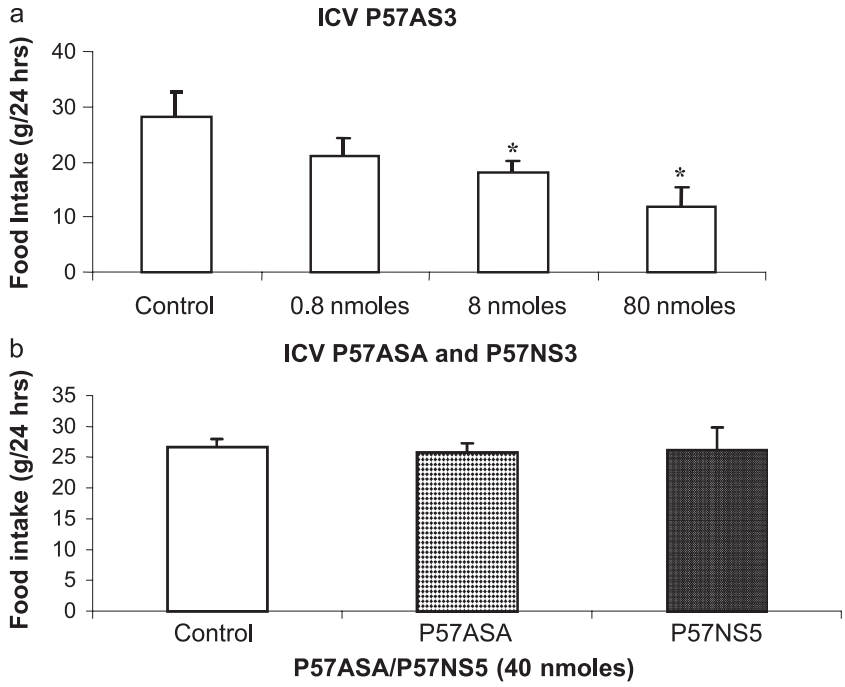


Fig. 1. Effect of i.c.v. administration of P57s on rat food intake. The compounds or DMSO vehicle were administered by microsyringe in 4 μl volumes and food intake measured for the subsequent 24-h period. $N=6$ animals per group, total of 72 animals for three sets of experiments; $^{*}P<0.05$ vs. vehicle control (Tukey's post-hoc). (a) P57AS3 (overall ANOVA, $F=20.3$, $P<0.0001$); (b) P57ASA (aglycone) and P57NS5. $N=6$ animals per group, total of 36 animals for three sets of experiments.

which in turn might result in anorexia [25,41]. Initial competitive binding studies in membrane concentrates from brain, kidney and gut, failed to demonstrate inhibition of ouabain binding or Na/K-ATPase activity (P. Cornelius, unpublished data). However, in whole cell hypothalamic culture, we found that P57AS3 inhibited the effect of ouabain on 86-rubidium uptake. Ouabain dose-dependently inhibits ^{86}Rb uptake into such cultures (Fig. 2a), an effect consistent with its inhibitory effect on Na/K exchange mediated by Na/K-ATPase.

As shown in Fig. 2b, over a wide concentration range, P57AS3 alone had no effect on ^{86}Rb uptake. However, as in a representative experiment in Fig. 2b, the compound significantly blocked the inhibition of ^{86}Rb uptake that occurs with ouabain alone. This activity was observed at the two higher concentrations studied, 500 and 5000 nM. The P57ASA and P57NS5 (Fig. 2c) compounds had no effect.

A similar effect of P57AS3 on ^{86}Rb uptake was also observed in incubated hypothalamic slice punches (Fig. 3). As expected, ouabain inhibited ^{86}Rb cellular uptake whereas P57AS3 alone at concentrations up to 5000 nM had no effect. However, as in the cultures, the compound almost completely blocked the inhibitory effect of ouabain.

3.3. Absence of evidence of P57 binding to the ouabain receptor

Despite previous evidence cited above that the P57AS3 does not bind to or inhibit activity of membrane extracts enriched with Na/K-ATPase, we sought to confirm that P57 did not bind to ouabain receptors using a whole cell assay system. As shown in Fig. 4, tritiated ^{3H}ouabain binds to (subsequently extracted) cells in hypothalamic culture and is specifically inhibited by higher concentrations of unlabeled ouabain. This binding was unaffected and in some experiments appeared to actually increase during exposure to P57AS3. The other P57s (aglycone and NS5) were not tested.

3.4. Effect of P57AS3 on ATP content in hypothalamic culture or slice punch explants

Although there are several possible explanations for the apparent blockade by P57AS3 of the inhibitory effect of ouabain on putative Na/K-ATPase activity, we presumed that this effect was not related to interference with ouabain binding nor to a direct toxic effect of the compound, for which there has been no in vivo or in vitro evidence. The Na/K-ATPase exchanger or 'pump' is directly fueled by ATP and the activity of this exchanger is known to be modified by availability of ATP substrate [4]. Accordingly, we measured ATP content in hypothalamic culture following incubation with the steroidal glycoside (Fig. 5). Ouabain alone had no effect or slightly reduced ATP content. 5000 nM P57AS3, either alone or in combination with ouabain, significantly increased ATP content by up to 2-fold following 30-min incubations in vitro.

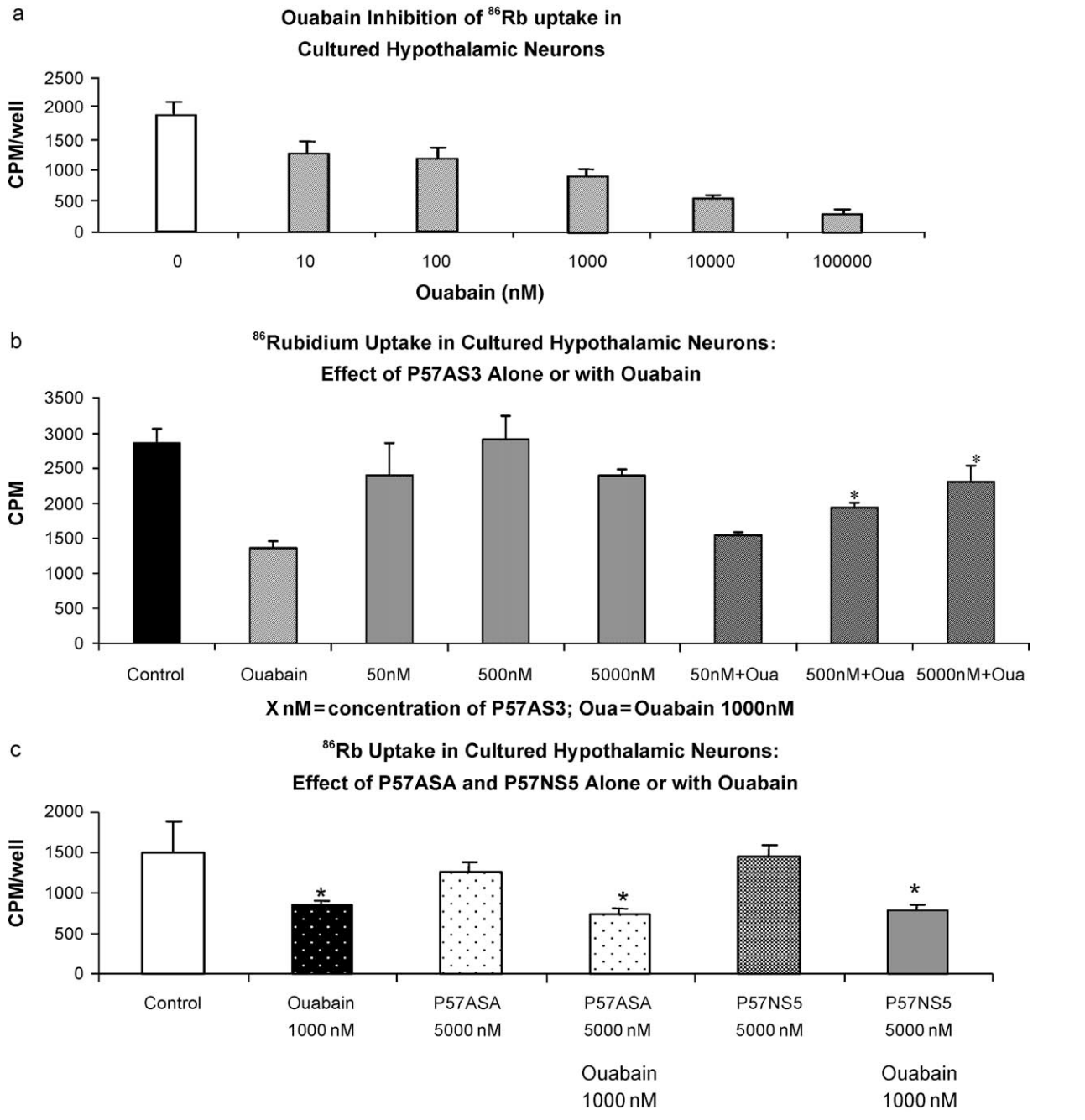


Fig. 2. ^{86}Rb uptake in cultures of fetal rat hypothalamic neurons. Three congeners of P57's were tested for their effect on ouabain inhibition of presumptive Na/K ATPase function as determined by ^{86}Rb uptake. $N=8$ wells per group (pooled experiments), $^{*}P<0.05$ (Tukey's post-hoc). (a) Effect of ouabain alone (overall ANOVA, $F=153.1$, $P<0.0001$); (b) effect of ouabain alone or P57AS3 alone, or the two agents combined on rubidium uptake (overall ANOVA, $F=28.8$, $P<0.0001$); (c) ouabain alone or plus P57ASA or P57NS5 (overall ANOVA, $F=59.0$, $P<0.0001$).

3.5. Effect of P57AS3 on hypothalamic ATP content in rats maintained on a normal or on a hypocaloric diet

To study the possible significance of altered hypothalamic ATP content in P57-treated rats, experiments were first performed to assess the effect of P57AS3 on hypothalamic ATP content and subsequently on animals maintained on a hypocaloric diet (Figs. 6–8). Rats were maintained either on ad libitum or on a 4-day diet restricted to 10 g/day plus ad libitum water. Animals were then sacrificed and regions of the brain and liver were sampled for ATP content.

In rats maintained on a normal diet, P57AS3 i.c.v. injections increased hypothalamic ATP content (Fig. 6, $P<0.05$). In rats maintained for 4 days on a hypocaloric diet, ATP content in the basal hypothalamus (sampled from a larger basal hypothalamic section as shown in Picture 1) was reduced by about 40% ($P<0.001$) (Fig. 7) Liver ATP content was also significantly reduced by about 60%.

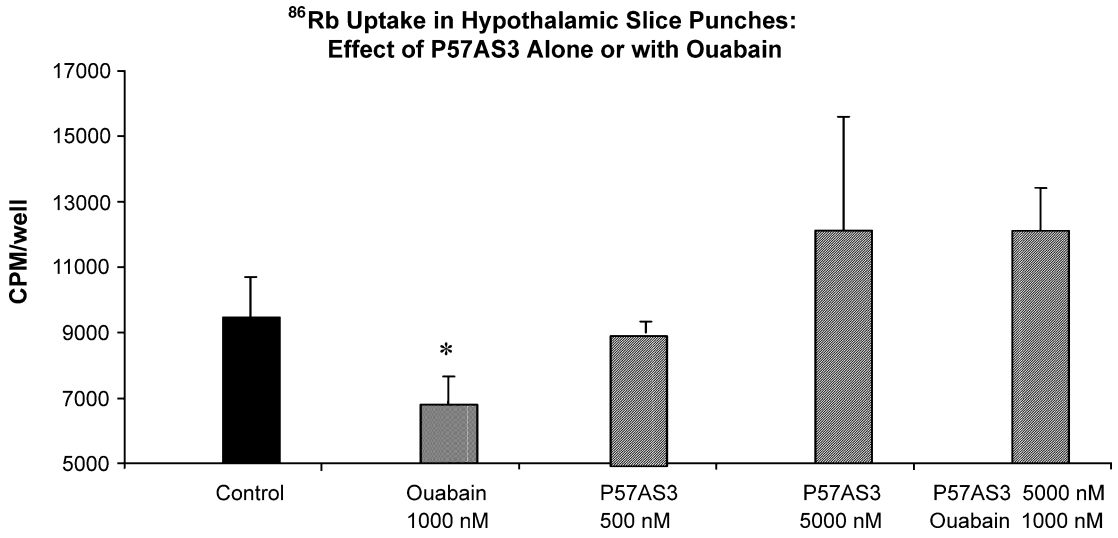


Fig. 3. Effect of P57AS3 on the ouabain effect on ^{86}Rb uptake into rat hypothalamic slice punches. Punches were obtained and incubated as described in the text. $N=6$ samples per group, $^{*}P<0.05$ (Tukey's post-hoc) (overall ANOVA, $F=5.6$, $P<0.003$).

whereas ATP content in the cortex and cerebellum were unaltered vs. ad libitum fed rats.

In subsequent experiments, we sought to determine whether P57AS3 reverses the decline in hypothalamic ATP that was observed in hypocaloric animals. Rats were maintained on 4 days of low-calorie 5 g/day or eucaloric ad lib chow control diets. At the end of the 4th day, the animals were administered by i.c.v. injection 40 nmol P57AS3 or DMSO vehicle. They were then maintained on their previous assigned diets during the postoperative 24 h before sacrifice and harvest of hypothalamic slice punches. ATP content in hypothalamic slice punches was again reduced in hypocaloric/DMSO-treated rats but was similar to ad libitum fed rats in those hypocaloric rats treated with i.c.v. P57AS3 (Fig. 8). In a similar single follow-on experiment in which animals were allowed to feed ad lib following i.c.v. injections, P57AS3 did not significantly inhibit food intake in previously underfed rats compared to either hypocaloric vehicle-injected animals or normal diet vehicle-injected animals: mean (S.D.) 21.0 (4.3), 23.0 (6.9), and 21.8 (6.9) g per 24 h, respectively. Normal diet, P57AS3-injected animals in the same experiment ate 12.4(3.3) g ($P=0.04$).

4. Discussion

As described in this report, these studies led to the key finding that the compound increases the production and/or content of ATP within hypothalamic neurons, either in whole cell cultures of fetal neurons or in hypothalamic slice punches from adult animals. Furthermore, we found a not previously reported reduction in hypothalamic ATP in rats after 4 days of moderate food deprivation (10 g/day)—and the 'reversal' of those ATP reductions in rats treated with intracerebroventricular (VIII) injections of P57AS3. Based on these findings, we hypothesize that a key mechanism of hypothalamic regulation of food intake is altered intracellular concentrations of ATP.

The studies described in this report evolved as we sought to determine the anorectic mechanism of a steroidal glyco-

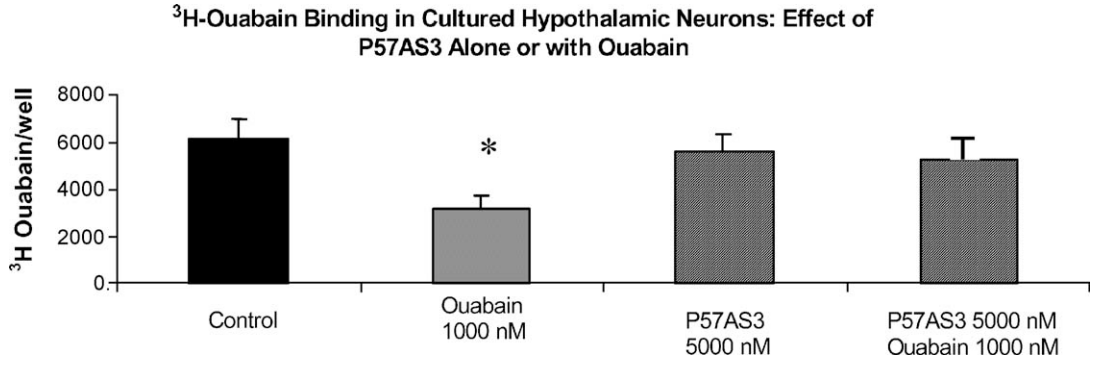


Fig. 4. The effect of P57AS3 on ^{3H}ouabain binding in cultured fetal rat hypothalamic neurons. Cultures were maintained then exposed to ^{3H}ouabain, ouabain and/or P57AS3 as described in the text. $N=6$ wells per group, $^{*}P<0.05$ (Tukey's post-hoc) (overall ANOVA, $F=12.6$, $P<0.0005$).

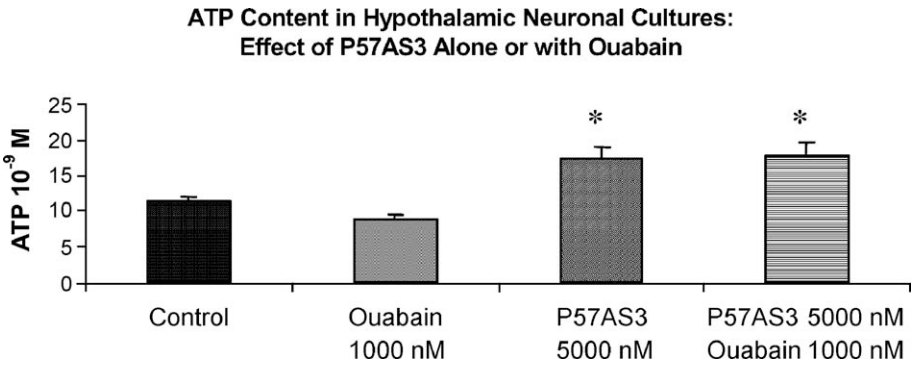


Fig. 5. Effect of P57AS3 on the ATP content in cultured fetal hypothalamic neurons. Cultures were exposed to ouabain, P57AS3 or the two combined as described in the text. $N = 5–6$ samples per group, $^*P < 0.05$ vs. control or ouabain alone (overall ANOVA, $F = 562.5$, $P < 0.0001$).

side purified and derived from a South African plant, *H. gordonii*. The plant species was originally chosen for study based on anecdotal reports of its effect in humans to assuage the pangs of hunger associated with long treks in the bush. The molecule was purified by tracking anorectic activity in single dose bioassays and was shown to have similar maximal activity as a concentrated preparation of whole sap; the mechanism of action or targeted molecule(s) was unknown. Based on the collaboration between Pfizer and Phytopharm and the Council of Scientific and Industrial Research in Pretoria, South Africa, one of us (D.B.M.) had access to the purified extract and several natural analogues that were used in the described studies.

Both dried whole sap and the purified extract were known from previous work to be active using both per os, subcutaneous, and intravenous routes of administration. Our studies using relatively small amounts of material (0.4–40 nmol) injected i.c.v. suggest but do not prove that at least one locus of action is within the CNS. We were unable to do specific pharmacokinetic assays in these studies to directly compare systemic exposure after peripheral vs. i.c.v. administration. However, in our hands, i.p. injections of amounts that were very active when injected i.c.v. had no discernable effect on food intake. That the compound is active centrally, however, by no means excludes that it may also have peripheral effects on appetite regulation, for example on vagal afferent nerves [31], on gastric emptying (vs. those mediated by CNS effects on vagal function), or on potentially anorectic peripheral hormones, such as CCK.

The core structure of these Hoodia-derived P57 compounds has obvious similarities to the cardiac glycosides or cardenolides, both the general planar structure of the steroidal core, the 14-OH group and the glycoside side chain. In addition, the Hoodia compounds have a relatively bulky C-12 substitution, normally hydroxylated on cardenolides [8,44]. Thus, our initial hypothesis was that these compounds may in some way interact with the Na/K ATPase within the brain, and that such effects might in turn alter hypothalamic function by either direct effects on dopamine metabolism [42] glucose sensitive neuron thresholds [1,18,22] or altered consumption of intracellular ATP [30,45]. In addition, there are persistent reports of endogenous ouabain-like cardiac glycosides within the hypothalamus [37,38] for which P57AS3 compounds might conceivably act as an exogenous antagonist.

In multiple Na/K ATPase assays with membrane extracts of brain, gut, and kidney, no evidence was obtained supporting either direct effects or inhibition of ouabain effects on directly measured Na/K ATPase activity (Pfizer, unpublished). In addition, our own studies demonstrated no evidence of interference with tritiated ouabain binding in whole cell preparations of neuronal cultures. Rather, we

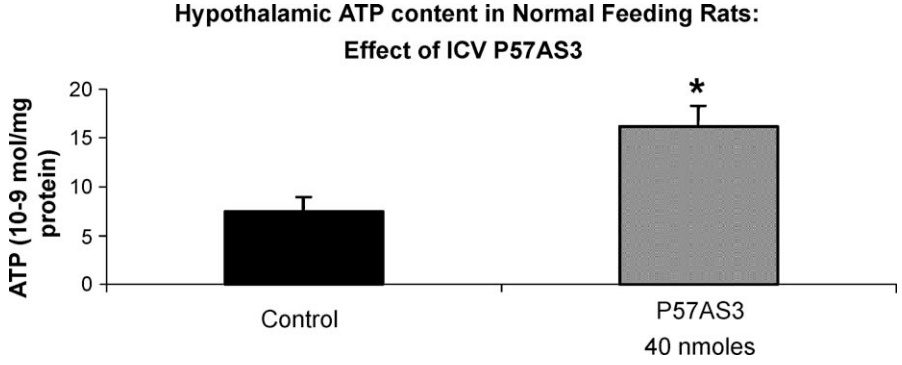
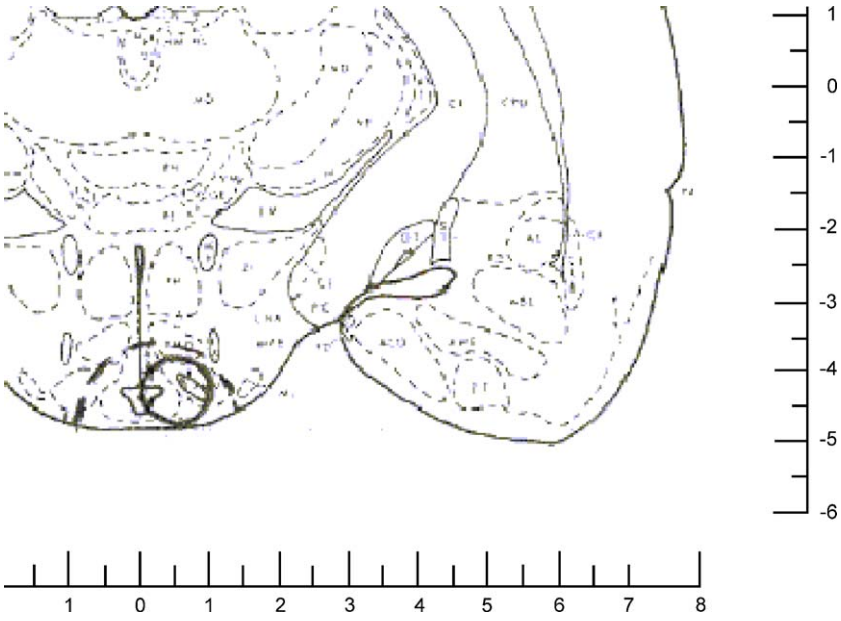


Fig. 6. Effect of i.c.v. P56AS3 (40 nmol) or DMSO vehicle on ATP content in hypothalamic slice punches removed from ad libitum diet-fed rats. Rats were treated i.c.v. and slice punches obtained 24 h later. $N = 6$ rats per group, total of 36 animals for control and P56AS3 groups for three sets of experiments; $^*P < 0.05$.



Picture 1. Diagram of rat brain coronal section at the region obtained between the 8 and 10 scale at the rear of Precision Brain Slicer (about 2 mm)). The circle indicates the punch area that included most of the arcuate nucleus of the hypothalamus and a small part of nucleus premamillaris ventralis. The broken-line half-circle indicates the areas for sampled for ATP content. The diagram was modified from *The Rat Brain in Stereotaxic Coordinates* by George Paxinos and Charles Watson (Academic Press 1982, Australia).

demonstrated in two whole cell systems—hypothalamic neuronal cultures and incubated hypothalamic punch explants from adult rats—a likely functional antagonism of the ouabain inhibition of 86-rubidium uptake. That assay has been a well-characterized system to measure Na/K-ATPase activity, inhibition of which leads to gradual depletion of intracellular potassium (manifested by reduced 86-rubidium uptake) and accumulation of sodium.

However, there are other possible explanations for the apparent preservation of intracellular potassium in the face of inhibitory concentrations of ouabain. The most important of these is the closure of K^+ channels, blocking the efflux of potassium and thus resulting in the accumulation of ^{86}Rb [33,45]. In our experimental conditions, we first added P57s, then vehicle or ouabain, and finally 86-rubidium. Prior closure of the KATP channel (i.e., before equilibration with ^{86}Rb) would be less likely to trap ^{86}Rb than would occur by reversing that sequence. However, at the present time, we cannot exclude that at least some of the apparent antagonism of the ouabain effect is due to this mechanism. Further specific studies of P57AS3 activity at the KATP channel, e.g., in patch clamp models, with channel agonist/antagonists, metabolic blockade or with clamped intracellular ATP, will be required to definitively evaluate for direct rather than indirect (i.e., altered ATP) effects on that channel.

We hypothesized that apparent effects on both the ATP-sensitive K^+ channel and the inhibition of ouabain activity in whole cell but not membrane preparations might be secondary to a common mechanism, specifically increased

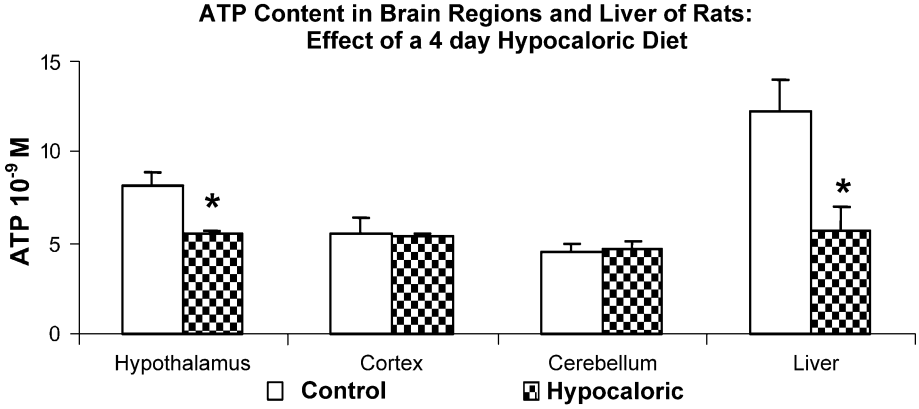


Fig. 7. Effect of a 4-day hypocaloric (5 gm/day) diet in rats on ATP content in brain regions and liver. $N=6$ rats per group, total of 12 animals for control and hypocaloric groups; $^*P<0.05$ (Tukey's post-hoc) vs. control fed animals (overall ANOVA, $F=53.1$, $P<0.0001$).

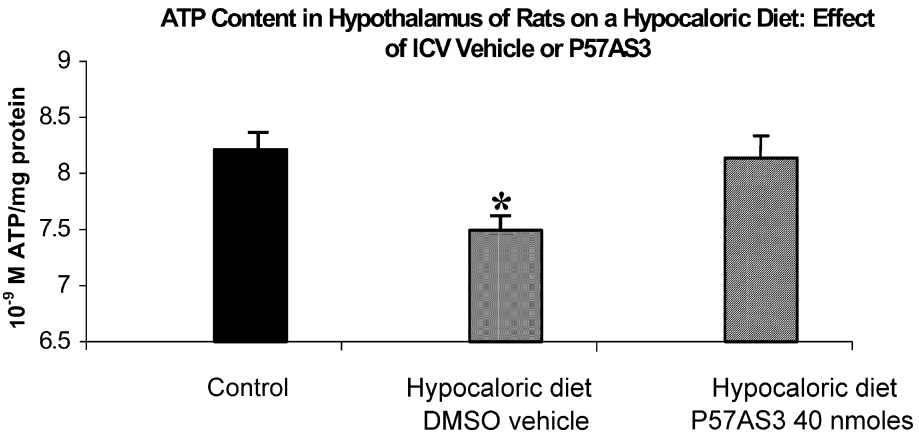


Fig. 8. Effect of i.c.v. vehicle or P57AS3 (40 nmol) on ATP content in hypothalamic slice punches removed from rats maintained on a hypocaloric diet. $N=6$ rats per group, total of 36 animals for two sets of experiments; $^*P<0.05$ vs vehicle-treated or control-fed rats (overall ANOVA, $F=14.3$, $P<0.0007$).

intracellular ATP [1], ATP being the principle rate-limiting regulator of pump activity [4]. We tested that hypothesis by direct measurement of ATP content in tissue culture, explants of slice punches and slice punches following in vivo treatment with P57AS3. In each of those conditions there were consistent increases in total ATP content. To attempt to physiologically manipulate ATP content, we elected to expand on a previously reported experiment [11], in which reduced hepatic ATP content was induced with a hypocaloric diet. In our study, in addition to liver, we measured ATP content in several brain regions and in dissected hypothalamus that encompassed the region shown in Picture 1. Our experiments confirmed the reduced hepatic ATP content following hypocaloric diet, reported by Ji and Friedman [11]. In addition, we also found reduced content in dissected hypothalamus, but not in cerebellum or cortex. In animals pair fed for 4 days and treated a day prior to sacrifice, ATP content was increased in the hypothalami from P57AS3 vs. those from vehicle-treated animals.

We have also found similar evidence of hypothalamic ATP apparent depletion in another model of relative caloric deficiency, rats treated with supraphysiological doses of triiodothyronine (T3) [27,28]. In that model, the apparent ATP deficit and increased food consumption induced by the presumed hypermetabolic state were partially reversed by i.c.v. P57AS3 to control levels, but not to the intake of P57AS3-treated control animals. Similarly, in animals fed ad lib following hypocaloric food deprivation, P57AS3 was also ineffective relative to eucaloric rats. Both these models suggest that over the short term at least, the absolute availability rather than relative change of ATP may be the 'sensed' energy signal. Finally, it is relatively counterintuitive that an increase in apparent intracellular ATP would be due to a non-specific toxic effect of P57AS3 administration. As described briefly in the Introduction, 30-day toxicological studies in rats have not been associated with significant toxicity.

There is one report of changes in directly measured hypothalamic content of ATP [7] in gonadal steroid-treated rats. We are aware of no studies of brain ATP content in altered nutritional states. Until recently, there were few studies attempting to measure ATP or ATP/ADP ratios in whole brain. MR spectroscopy has been increasingly used for this purpose, to date in predominantly pathological states such as brain trauma, mental illness or recurrent seizures [35,43]. We have been unable to find such results in states of starvation or relative caloric depletion. In light of our findings, such studies would be of interest, particularly regarding hypothalamus vs. other brain regions.

At the present time, we have no mechanistic explanation for the observed changes in ATP following P57AS3 treatment. Based on our in vitro results, the mechanism is likely to be 'local' or intracellular rather than due to a whole organism integrative or hormonal response [12]. We have not identified a specific molecular target or pathway, such as an indirect effect on intracellular substrate oxidation or conversely decreased ATP consumption. However, based on the effects of cardiac glycosides on Na/K ATPase, we speculate that P57AS3 may have a direct effect on another ATPase regulating ATP balance. For example, there is limited evidence that other steroids, e.g., estrogen, may directly regulate the F1/F0 ATP synthase [46,50,51]; Natural product (oligomycin) and semisynthetic inhibitors of the mitochondrial ATP synthase are well characterized [36].

A recent paper by Ji and Friedman [11] suggested that the rate and subsequent termination of refeeding following fasting in rats is directly correlated with feeding-induced hepatic replenishment of ATP. Our findings complement that study and suggest that the hypothalamus may also be a sensing target organ that is vulnerable to ATP fluctuations. Two recent reports also suggest that metabolic activity of hypothalamic neurons directly regulates food intake [48,49]. Loftus and co-workers [26] demonstrated that i.c.v. administration of a fatty acid synthase (FAS) inhibitor, C-75, reduced food intake and also inhibited the expected up-regulation of NPY expression. The result suggested that

FAS inhibition mimicked a caloric replete state. Similar results occur with oleic acid infusions into the hypothalamus [34].

The hypothalamus is also sensitive to insulin and glucose, the latter transducing its effects at least partly through the ATP-sensitive K^+ channel [5,21,22]. These KATP channel functions are analogous, but not necessarily identical to glucose oxidation-dependent regulation of the pancreatic beta cell [39] or cardiac myocyte [14]. In a recently reported knockout of the gene encoding the ATP-sensitive K^+ channel, the KO mice did not respond to hypoglycemia with increased food intake and were also heavier than wild type [33]. Leptin signaling also may be partly mediated through the ATP-dependent K channel [40]; ATP content, as a regulator of that channel, may be one reason for the altered leptin sensitivity described in obesity [15,19] and hypothyroid states [10].

ATP is also a putative neurotransmitter within the hypothalamus, studied regarding the neuroendocrine responses to stress [3,13,47] or regarding regulation of body temperature [6]. We did not characterize the subcellular localization of ATP that was measured in the chemiluminescent assay. While ATP may have direct effects on K^+ channel activity or Na/K-ATPase, many other phosphorylation-dependent transduction pathways may mediate the subsequent integrative response to energy sensing.

Our findings further extend the concept of hypothalamic nutrient and energy sensing and suggest that neurons within the medial basal hypothalamus may be sensitive to altered content of ATP. The sensing of energy homeostasis, as signaled by intracellular ATP, may be directly analogous to other fundamentally simple but tightly regulated hypothalamic homeostatic mechanisms, in particular those controlling osmolarity and body temperature [4,29,32,38,43]. P57AS3 or related molecules may be intriguing probes to further elucidate the amplification of signaling of this energy sensing function. It remains to be determined whether the hypothalamus can be temporarily 'short circuited' by agents that alter energy-sensing—agents which thus might be harnessed to allow energy-sensing remodeling within the hypothalamus [20] or to restore more adaptive energy homeostasis within the whole organism, as a treatment for obesity or insulin resistance [21,23].

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Since submission of this manuscript, Minokoshi et al have reported that intrahypothalamic AMP kinase (AMPK) may regulated food intake. They did not report on or measure hypothalamic ATP. In liver and other tissues, AMPK is regulated in part by concentrations of ATP or the ratio of AMP/ATP and thus may be one effector/or sensor of altered hypothalamic ATP.

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