

Avishek Saha
BSCI360
11/29/11

How can resveratrol, an AMPK activator¹ influence hibernation preparation and duration in yellow-bellied marmots (*Marmota flaviventris*)?

Purpose:

How can the broad-spectrum polyphenic stilbene resveratrol influence body composition, torpor, feeding behavior, and the associated hormonal mechanisms in a hibernating mammal? The aim of this study is to observe the behavior of the yellow-bellied marmot (*Marmota flaviventris*) under the influence of resveratrol. Wild yellow-bellied marmots will be treated with resveratrol in two contexts: either in the spring after arousal from torpor (SR), or one month after the cessation of feeding between late autumn and winter (WR). Leptin, adiponectin, lipid mass and body composition, food intake, and body temperature will be assessed monthly.

Background:

Resveratrol has been studied avidly for over a decade due to its potential role as a calorie-restriction mimetic² as an anti-obesity aid,³ and as a cardioprotective, neuroprotective, anti-diabetic,¹² and anti-cancer treatment.⁴ Hibernation parallels mammalian obesity, but it has not been studied under resveratrol administration.

As in human obesity, in hibernating mammals, leptin and insulin levels rise as fat mass rises during the homeothermic, or fattening phase during the summer months, while adiponectin decreases. In the autumn months, leptin levels drop, slightly before the cessation of feeding and just before the gradual reduction in body mass.⁵ Although resveratrol has not been studied in these mammals, caloric restriction has.⁶ AMPK agonists and leptin treatment have also been tested on hibernating mammals.^{7,8} These three treatments were found to reduce feeding and body mass gain during the fattening months in the summer when hibernating mammals normally feed until they gain enough weight. Resveratrol has actions similar to caloric restriction, AMPK activation, and leptin, which makes it a novel but predictable compound to study in these animals.

Resveratrol was however studied recently in gray mouse lemurs. It was shown that four weeks of resveratrol administration reduced seasonal fattening in these primates.⁹ These findings will parallel our hypotheses. Grey mouse lemurs utilize torpor as an energy saving mechanism seasonally, and daily, however with no clear pattern,¹⁰ whereas hibernating squirrels display predictable seasonal torpor and therefore will be easier to study when tracking the effects of resveratrol administration over a longer period of time.

Yellow-bellied marmots are an ideal organism for this experiment because they are completely aphagic in the winter, as are golden-mantled ground squirrels, another popular study organism for hibernation research, and rely on a circannual rhythm to enter torpor,

whereas other squirrels in the genus *Eutamias* and *Cynomys* will feed intermittently during the winter, relying on cached food as well as endogenous lipid stores.¹¹ The latter group of animals could complicate interpretations of findings since we are aiming to study feeding behavior and how it is influenced by variables such as insulin, leptin, adiponectin, and AMPK which have all been studied in *Marmota flaviventris*. We can predict more accurately the marmots behavior due to its already well-known circannual pattern of feeding, hormonal variations, and body mass fluctuations.

There are six different variables in this study to be tested: food intake, body temperature, body mass and composition, hormonal levels (leptin, adiponectin), and AMPK activation. These will be tested in three separated groups of animals as described previously, SR, WR, and a control group. In SR, we hypothesize prevention of torpor, attenuation of body mass gain, and reduction in feeding. Resveratrol is expected to inhibit hyperinsulinaemia, and leptin resistance, while increasing adiponectin, causing stable feeding patterns, in addition to up-regulating AMPK in SR to prevent hyperphagia and weight gain.

In WR, resveratrol will be administered approximately one month after the initiation of torpor, which should occur shortly after termination of feeding,⁵ which will serve as the signal for administering the treatment. Adiponectin is hypothesized to rise, and body mass hypothesized to decrease. Consistent with a study examining caloric restriction on hibernating squirrels, we expect the body mass loss to come from fat free mass rather than fat mass.⁶ We hypothesize feeding to remain minimal. Although the exact mechanisms that initiate and terminate torpor are not known, if resveratrol interacts with the predicted targets, we could expect the marmots to bypass the need to initiate torpor due to stable hormonal and satiety signals from the hypothalamus. This may help us understand why torpor is an adaptive strategy in squirrels and pinpoint the actual trigger.

In previous studies, often only chemicals with relatively precise actions were studied, such as insulin,¹³ leptin,⁸ and AMPK activators,⁷ but to date resveratrol has been studied only once in a seasonal model of obesity in a species that displays high inter-population variation in hibernation and torpor strategies.⁹ Since resveratrol has such a wide variety of actions, we can look at a wider array of cellular processes with relatively simple predictions on the physiological changes. This work may spur future research examining the internal genetic and molecular mechanisms in greater detail in order to understand how this natural model of obesity is influenced by mediators such as cytokines and satiety hormones.

Proposed work:

There are a few caveats in the aforementioned hypotheses to understand first before delving into the proposed methods. The variables to be studied once again are body temperature, body mass and composition, food intake, and satiety hormones such as adiponectin and leptin. The latter two hormones will have an influence on the former

three variables, so they must be explored in detail if some predictions are to be generated:

1. *Adiponectin*: Adiponectin is a cytokine that plays an important role in metabolism. In early winter (January), adiponectin levels are low in marmots. By April, levels triple, once the animals commence feeding. In October, when feeding ceases, adiponectin levels are reduced threefold.⁵ Resveratrol has been shown to stimulate adiponectin.¹⁴ In WR resveratrol would be predicted to therefore arouse the mammals from torpor. It could be predicted feeding will begin, but since fat stores are abundant it may not be necessary to feed on exogenous sources of energy. In SR, the predictions are less clear; adiponectin is already elevated and marmots are already feeding. Would we see further elevations in adiponectin, and therefore even more feeding and fattening? This is not likely, since resveratrol has other actions as well, such as reducing leptin, so it is more likely that mechanisms independent of adiponectin will regulate food intake and body mass. In fact, resveratrol's ability to activate AMPK, and the sirtuins (not discussed here) predict a lowering of body mass and food intake.
2. *Leptin*: Concomitant with increased body mass in homeothermic hibernating mammals, there is an elevation in leptin and insulin. Insulin can be ignored in this study because it can be safely assumed that when insulin is high, leptin is high. However, if we were to study the effects of resveratrol on a glucose load, it may be illuminating to study insulin since these mammals are transiently insulin resistant.¹⁵ Leptin levels rise from April to November, and fall sharply afterwards.⁵ Since resveratrol can inhibit leptin¹⁶ we can predict prevention of hyperphagia. It has been found that leptin treatment also prevents hyperphagia,⁸ but perhaps through the same mechanism; exogenous leptin may downregulate endogenous leptin and therefore prevent the subsequent resistance to leptin that these mammals experience by autumn. Similarly, if resveratrol can control the resistance to leptin, and insulin, as has been shown in diabetic mice, it may prevent weight gain, and torpor.
3. *AMPK*: AMPK is a central regulator of metabolism that responds to cellular energy levels.¹⁷ AMPK acts on upstream targets of leptin, such as neuropeptide-Y (NPY) and agouti-related protein AgRP, an appetite stimulator, released from neurons in the arcuate nucleus (ARC). A potent AMPK activator, AICAR, administered to the 3rd ventricle of yellow-bellied marmots inhibited hyperinsulinaemia and reversed torpor in winter marmots.⁷ Since resveratrol also stimulates AMPK^{1,18} and reduces NPY,¹⁹ which stimulates food intake²⁰ it may cause an increase in food intake in WR and arousal from torpor.

Methods: First, yellow-bellied marmots of both sexes must be captured; since they do not live in Maryland, some travel or shipment may be required. The capture must occur right after arousal from torpor when leptin and body mass are at their lowest and when these mammals will begin to feed. This should happen around April. They must be kept at room temperature (22±2°C). In August the temperature can be reduced to 15°C to acclimate the animals to the cold, and by October the temperature can be reduced to 5°C and maintained that way until April. The exact timing of temperature

changes are not too critical, since in similar experiments, torpor bout and feeding behavior seem to be independent of atmospheric temperature in yellow-bellied marmots.⁷

After capture and transportation into the laboratory, we will measure the lipid mass and percentage with total body electrical conductance (TOBEC). This can be performed on a monthly basis with anesthetization. Simultaneously, blood samples taken from the anesthetized mammals will be analyzed for leptin content. To determine adiponectin content, we must reverse transcribe RNA from white adipose tissue (WAT) as done previously.⁵ Body temperature can be assessed with an inexpensive temperature probe.

In addition to the above factors, diet must be regulated strictly due to the effect it can have on hibernation. Alternations in fatty acid composition for example can dramatically affect body temperature and food intake.^{21,22} A standard diet such as Purina 5001 may work, although the large amount of soy isoflavones has been a cause for concern.²³ In previous work, it was found that linoleic acid is very important for allowing a healthy hibernation to proceed, so linoleic-acid rich diet will be preferred for this experiment.²⁴ Total food intake will be monitored daily, by averaging the amount of chow eaten as measured from simple arithmetic. Food will be provided *ad libitum*. If total food content per cage is known, we can determine easily from arithmetic how much was eaten, but it would have to be averaged between the number of animals per cage.

In regards to the sample size, since there are three total groups, upwards of 20 marmots are required for the experiment, at least 6 per group, all wild. Ideally, they will be mixed in cages containing marmots in other groups, so that the effects of resveratrol will be independent of social behavior.

The resveratrol treatment will begin one full month after the initial measurements, which are again, to take place as soon as the mammals are received. This is just to ensure that the environment is suitable and that the animals begin to gain weight as they normally would. That next month, most likely to be May, there will be three groups of animals, the SR, WR, and control groups. Only the SR will receive resveratrol. This will continue for the duration of the entire experiment, unless torpor is reached and the mammals stop feeding. When the WR group initiates torpor, as well as ceasing food intake, predicted to be in October or November, one month will be again left for observation only without any changes to the program. After one month of torpor and aphagy in the WR, WR will be treated intracerebroventricularly with resveratrol. Surgery will have to be performed to insert a cannula into the ventricles, connected to a larger catheter as done previously.⁷ The dose will be calculated based on the infusion rate, which will vary depending on the temperature. In the SR, marmots will be given 4g/kg resveratrol mixed with chow. In one month's time, it could be predicted that WR marmots will behave just like SR marmots, only if the initial predictions were correct and resveratrol prevented torpor in SR. The total estimated duration of the study would be from the onset of collection in April, to the very beginning of January. One contraindication is that the SR would not receive ICV resveratrol. ICV resveratrol

administration has been shown to have no effect on body mass in diabetic mice despite mitigating insulin resistance and hyperglycaemia,¹² however intracerebroventricular pumping of insulin and AMPK agonists did influence feeding and body mass.^{7,13} This may be due to the differing actions of these satiety chemicals in the brain over white adipose tissue, but, and this may be one potential flaw of this design.

Impact: I believe this work is inevitable, it is simply a continuation of a train of thought processes that have been brewing for the past decade, and will open up many further avenues of inquiry regarding obesity, hibernation, and lifespan. It would be quite neat for example to compare the lifespans of hibernating mammals treated with resveratrol (unless resveratrol treatment prevented hibernation), with only hibernating mammals, and non-hibernating mammals. We know that hibernating animals do live longer, but resveratrol has not been studied on lifespan of hibernating species.

Furthermore, hibernating mammals do not need an obesogenic diet, or pharmacological intervention to induce obesity; they do it by themselves. This presents a simpler model to work with when studying obesity. In this proposal for example, there are multiple targets that resveratrol could act on that could prevent the transient obesity, and is thus more helpful than studying a single drug with a single action. In terms of animal research, the exact reason for going into torpor may be determined. If resveratrol prevents torpor, we can ask, was storing all that fat even necessary in the first place?

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