Piperine’s mitigation of obesity and diabetes can be explained by its up-regulation of the metabolic rate of resting muscle

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We identify a target for treating obesity and type 2 diabetes, the consumption of calories by an increase in the metabolic rate of resting skeletal muscle. The metabolic rate of skeletal muscle can be increased by shifting myosin heads from the super-relaxed state (SRX), with a low ATPase activity, to a disordered relaxed state (DRX), with a higher ATPase activity. The shift of myosin heads was detected by a change in fluorescent intensity of a probe attached to the myosin regulatory light chain in skinned skeletal fibers, allowing us to perform a high-throughput screen of 2,128 compounds. The screen identified one compound, which destabilized the super-relaxed state, piperine (the main alkaloid component of black pepper). Destabilization of the SRX by piperine was confirmed by single-nucleotide turnover measurements. The effect was only observed in fast twitch skeletal fibers and not in slow twitch fibers or cardiac tissues. Piperine increased ATPase activity of skinned relaxed fibers by 66 ± 15%. The Kd was ~2 μM. Piperine had little effect on the mechanics of either fully active or resting muscle fibers. Previous work has shown that piperine can mitigate both obesity and type 2 diabetes in rodent models of these conditions. We propose that the increase in resting muscle metabolism contributes to these positive effects. The results described here show that up-regulation of resting muscle metabolism could treat obesity and type 2 diabetes and that piperine would provide a useful lead compound for the development of these therapies.

Significance

We have developed a method for finding pharmaceuticals that would treat obesity and type 2 diabetes by increasing the metabolic rate of resting skeletal muscle. The metabolic rate is increased by shifting the motor protein myosin from a low activity state to a higher activity state. We devised an assay, screened for compounds, and found one molecule, piperine. Piperine increased the metabolic rate of resting muscle fibers. Piperine does not have the properties required to be a pharmaceutical in humans, but it would make a good lead compound for finding compounds that do. Our results provide proof of concept that these metabolic diseases can be treated by future pharmaceuticals that target myosin to increase the metabolism of excess calories.

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spend most of their time in the SRX in both animals and in humans. A simple calculation shows that if all myosin heads were transferred from the SRX to the DRX, human whole-body metabolic rate would increase by ∼2-4 MJ·d⁻¹ (5). Thus, a pharmaceutical that destabilized a large fraction of myosin in the SRX would be an effective therapy for obesity and type 2 diabetes.

A major hurdle to discovering small molecules that destabilize the SRX has been the limitations imposed by the single-nucleotide turnover assay previously used and the requirement of observing it in fibers. We recently overcame these obstacles by finding a fluorescent probe on a subunit of myosin, the regulatory light chain (RLC), which showed an increase in emission intensity and shift to shorter wavelengths upon the transition from the SRX to the DRX (Figs. S1 and S2) (18). We used this signal to carry out a high-throughput screen of 2,128 compounds approved for human consumption by the FDA (see Supporting Information for description). This screen identified one compound, piperine, the main alkaloid component of black pepper.

Results

The Signal Used in the Screen. Our previous work had identified the ratio of fluorescent intensities as a reporter of the population of the SRX (18). We used an RLC mutant labeled with a coumarin maleimide (MDCC) on cysteine 5. The emission of the probe shifted to lower wavelengths upon the transition from rigor or the DRX to the SRX. The ratio obtained at shorter wavelengths, 440 nm, was divided by that obtained at longer wavelengths, 520 nm, to produce a ratio that was intrinsic to the state of myosin and independent of the number of fibers being visualized, intensity of excitation, and photobleaching.

To strengthen the hypothesis that this signal reports the population of the SRX, we measured the population of the SRX by the ratio signal at different temperatures. The population of the SRX measured by the mant-chase protocol and the binding of myosin heads to the core of the thick filament, measured by X-ray diffraction, have both shown a strong temperature dependence (12, 19). As shown in Fig. S3, the population of the SRX measured by the ratio signal also has a strong temperature dependence, increasing by a factor of 2.2 ± 0.2 (SEM, n = 6) on raising the temperature from 15 °C to 30 °C. This change in population is similar to that observed previously using the chase protocol and the binding of myosin heads that were in the slow component by 34 ± 18% over the same temperature range. These observations provide additional support for the hypothesis that the fluorescence ratio reports the population of the SRX.

High-Throughput Screen. The screens were carried out by observing the change in fluorescence intensity of probes attached to a subunit of myosin, RLC, in skinned rabbit fast skeletal muscle fibers. The wells were loaded using the labeled and chopped fiber preparation as described in Materials and Methods and Supporting Information (Fig. S1). Because of the low homogeneity of the fiber preparation, fiber number varied among wells. Even if fibers were loosely attached to the bottom of the well, it was not possible to exclude movements during the assay. To further complicate matters, the fluorescent excitation intensity was not uniform across the well. These problems have been addressed by the discovery of a fluorescence ratiometric reporter of the myosin state, described above (18). The images were analyzed at two wavelengths by the Fiji macro, and outliers were reexamined by manual analysis. See Supporting Information for a more detailed description of the fiber preparation and the high-throughput screen.

In only one well did the intensity ratio indicate that the SRX of the fibers had been destabilized by the compound present in that well. The compound was piperine, which is the main alkaloid component of black pepper (Fig. 1). Piperine is a well-known compound in ayurvedic medicine and has been shown to interact with a number of targets in biological systems.
The single-nucleotide turnover experiment used to measure the SRX. Single skinned skeletal muscle fibers or strips of cardiac tissue were mounted in a flow cell, incubated in a fluorescent analog of ATP, mantATP, and chased with ATP. The intensity of the fiber fluorescence is plotted as a function of time during the chase phase. Each plot shows samples in the absence (open squares) and presence (solid circles) of 100 μM piperine. Fiber fluorescence decreases in two phases: a fast phase that is largely over in about 20 s, followed by a slow phase with a lifetime of minutes. The slow phase arises from the slow release of nucleotides by myosin in the SRX. (A) Fast twitch skeletal fibers. Data averaged from a number of such experiments showed that populations of the slow fluorescent component were control, 32 ± 3% (SEM, n = 10), piperine, 21 ± 2% (SEM, n = 18), and the lifetimes were control, 191 ± 13 s (SEM, n = 9), piperine, 136 ± 8 s (SEM, n = 8). Both the population and the lifetime show that the SRX has been partially destabilized. (B) Slow twitch skeletal fibers, showing little effect of piperine. Averaged populations of the slow fluorescent component were control, 33 ± 4% (SEM, n = 4), piperine, 33 ± 3% (SEM, n = 8), and the lifetimes were control, 107 ± 10 s (SEM, n = 4), piperine, 98 ± 8 s (SEM, n = 8). (C) Cardiac tissues showing little effect of piperine. Averaged populations of the slow fluorescent component were control, 21 ± 2% (SEM, n = 11), piperine, 21 ± 3% (SEM, n = 13), and the lifetimes were control, 146 ± 24 s (SEM, n = 11), piperine, 178 ± 28 s (SEM, n = 13).

**ATPase Activity.** The data described above show that piperine destabilizes the SRX, shifting a large fraction of its population into the inhibition caused by piperine (Fig. S4A vs. B). The screen and all of the fiber experiments discussed above were performed using rabbit psoas, a fast twitch muscle predominantly composed of myosin type 2B fibers (20–22). To assess a possible fiber-type specificity, the experiment was repeated using fibers isolated from the soleus muscle, which uses a slow twitch type 1 myosin isoform. In contrast to fast twitch fibers, piperine showed little effect on the SRX in the slow twitch fibers (Fig. 2B). The addition of piperine also showed little effect on the SRX in strips of rabbit cardiac tissue (Fig. 2C). Slow twitch skeletal fibers share the same myosin heavy-chain and regulatory light-chain isoforms as cardiac ventricle (20–22), so it is expected that if piperine targets one of these chains, the results would be similar in the two muscles. These observations have important consequences for the development of piperine as a therapeutic compound. An effect of piperine in cardiac tissue would have been very undesirable in a therapeutic agent targeting skeletal muscle. In addition, if the compound only affects fast twitch fibers in humans, as it does in the rabbit fibers, this would limit its power to elevate thermogenesis.

**Fig. 3.** The effect of piperine on the ATPase activity of purified myosin (Fig. 3, columns 5 and 6). This result suggests that the effect of piperine is specific for the myosin heads in the SRX complex and not to myosin itself. Exchange of fibers with RLC-MDCC did not alter the effect of 100 μM piperine on fiber ATPase activity (Fig. S5). Together, the data show that piperine increases the ATPase activity of skinned fibers by destabilizing the SRX. The fraction of myosin heads involved can be determined by blebbistatin-free myosin heads (Fig. 3, columns 3 and 4). Piperine had little effect on the ATPase activity of purified myosin (Fig. 3, columns 5 and 6). The inhibition caused by piperine (Fig. 3, columns 3 and 4). Piperine has little effect on the ATPase activity of purified myosin (Fig. 3, columns 5 and 6). This result suggests that the effect of piperine is specific for the myosin heads in the SRX complex and not to myosin itself. Exchange of fibers with RLC-MDCC did not alter the effect of 100 μM piperine on fiber ATPase activity (Fig. S5). Together, the data show that piperine increases the ATPase activity of skinned fibers by destabilizing the SRX. The fraction of myosin heads involved can be determined by blebbistatin-free myosin heads (Fig. 3, columns 3 and 4). Piperine had little effect on the ATPase activity of purified myosin (Fig. 3, columns 5 and 6).
estimated by comparing the increase in fiber activity, ~0.04 s⁻¹, with that of pure myosin, ~0.06 s⁻¹. This shows that a large fraction of the myosin heads, >50%, would have to be shifted from the SRX to the DRX to produce the observed increase.

Measuring Affinity. Estimating the affinity for the binding of piperine to its target is complicated, because piperine is very hydrophobic and binds to the many hydrophobic surfaces available in the interior of the fiber. Nonspecific binding acts in two ways: it decreases the concentration of free piperine inside the fiber and it provides a larger pool of available binding sites that need to be filled. The full effect of piperine requires about 80 s at a concentration of 100 μM (Fig. 1). The time increased with decreasing piperine and reached more than 1 h at 10 μM. We observed chopp ed fibers in plates, where they were stable for long periods, and we measured ATPase activities after preincubation in relaxing solutions plus piperine, as described in Materials and Methods. In each experiment, data were obtained at a particular concentration and also at a high concentration, 80–100 μM. The value obtained at the lower concentration was normalized by that obtained at the higher concentration, providing a more accurate concentration dependence. In the case of the plate-reader, different concentrations were observed in different wells (Fig. S6). In the case of the ATPase activities each set of fibers was first incubated in a lower concentration of piperine in a relaxing solution, followed by measurement of ATPase activities at that concentration and finally by measurement in 100 μM piperine (Fig. S7).

As the concentration of piperine was lowered, its effect on the ratio of fluorescent intensities and on the fiber ATPase activities decreased. Measurement of the fluorescent intensities in plates produced a Kd of 1.7 ± 0.4 μM (Fig. 4A). Observation of ATPase activities showed a similar Kd of 3 ± 0.8 μM (Fig. 4B). The affinity of piperine for its target is not sufficiently high to be used as a pharmaceutical in humans. However, it would be a good candidate as a lead compound for molecule optimization.

Mechanics. To be an effective therapeutic for metabolic diseases, a compound must increase the metabolic rate of resting fibers without having an effect on the mechanics of active muscle. To explore this possibility, single skinned muscle fibers were mounted on a tensiometer, which could measure tension and shortening velocities (28). Each fiber was observed in the presence or absence of 100 μM piperine, at 25 °C, using a temperature jump protocol to provide better sarcomere stability at 25 °C (Fig. S8). In fully relaxed fibers, piperine had no effect. Addition of 100 μM piperine to fully activated fibers had no significant effect on isometric tension, ratio of piperine to control = 1.05 ± 0.05 (SEM, n = 8) (Fig. S8). Isotonic velocities at 25% of isometric tension were measured (Fig. S9). Piperine had no significant effect on the shortening velocity, ratio of piperine to control = 0.97 ± 0.08 (SEM, n = 10).

Components of Piperine. Once a compound with the desired properties has been identified in a high-throughput screen, a customary next step is to examine similar compounds for further drug development. Piperine consists of two ring systems connected by a short conjugated leash (Fig. 1). The compound is easily broken down into two components: piperidine, the six-membered ring on the left, and piperic acid. The effects of both of these compounds were examined using the single-nucleotide turnover protocols. Neither compound at concentrations up to 200 μM had any effect on either the population or the lifetime of the SRX. Thus, the action of piperine requires that the two ring systems be connected together to produce an effect.

Discussion

After identifying piperine in the high-throughput screen as a destabilizer of the SRX, we were pleased to find in the literature that piperine had already been associated with attenuation of weight gain in rodents. Piperine is efficiently absorbed by the gut and is widely distributed in the various tissues (29, 30). However, most of the piperine has been conjugated with glucuronic acid, which may influence its activity. In a typical experiment, rats or mice were fed a high-fat chow for an extended time. All animals gained fat mass, but those also receiving piperine gained less than the controls. The difference in fat mass gain varied between 20 and 70% in different studies (31–35). Changes in lean mass were small, ~10%. One study showed that administration of piperine to mice during caloric restriction had no effect on a series of parameters (36). Thus, the main effect of piperine appears to be to mitigate fat gain during caloric overload. In the studies above there was no change in the amount of food consumed; therefore piperine must affect the amount of fat stored by increasing the amount metabolized. Piperine is approved by the FDA for human consumption, but at doses, 20 mg/d, much lower than used in the experiments in rodents cited above, 20–50 mg·kg⁻¹·d⁻¹. It is approved, not for weight control, but for increasing the bioavailability of other drugs, which it does by inhibiting liver enzymes that metabolize them (37).

Piperine also has a beneficial effect in rodent models of type 2 diabetes (31, 32). Rodents fed high-fat and glucose diets for extended periods have higher blood glucose levels and show insulin resistance. Addition of piperine to their diets lowers levels of blood glucose and insulin. Piperine also improves the rate of glucose removal in a glucose tolerance test (32). All of the above
observations could be explained by an increase in glucose consumption by resting muscle resulting from the increased metabolic rate of myosin produced by piperine.

The mechanisms by which piperine promotes weight loss or improves diabetes were unclear. A number of targets have been identified, but none have been shown to be causative (31, 33, 38–40). Our data show that piperine enhances thermogenesis of resting muscle via a perturbation of the SRX/DRX ratio, thus providing a mechanism. As originally recognized by Ferenczi and coworkers (14) over 30 y ago, in vivo ATP turnover requires almost all myosin in relaxed skeletal frog muscle to be in what we now term the SRX. A transfer of only 20% of the myosin in relaxed muscle from the SRX to the DRX would cause thermogenesis of resting muscle to double (5).

Could the activation of resting muscle metabolism by piperine found here explain the difference in fat gain produced by piperine in rodents fed high-fat chow for an extended period? In the experiments by BrahmaNaidu et al. (31), rats were fed high-fat chow for 42 d, producing one of the largest observed gains in fat mass. All rats gained fat during this time, but those who were also fed piperine gained ~80 g less fat than controls. The oxidation of this amount of fat will yield 40 mol of ATP (41). We first compare this value to the amount of ATP that would be consumed if all of the myosin heads were in the DRX. The amount of myosin is calculated by taking the lean mass of the rats consuming the high-fat chow, 375 g, assuming 50% is muscle and multiplying by the concentration of myosin heads in mammalian muscle, 360 μM (42), to give 72 μmol of myosol. The basal ATPase activity of rat myosin has, to our knowledge, not been measured. To estimate this rate we extrapolated from the values for other myosins using the known variation of basal metabolic rate with body mass (43), producing an estimate of ~0.5 s⁻¹ (see Supporting Information for a more detailed description). Multiplying the total amount of myosin heads times the activity and the duration of the experiment we find that 129 mol of ATP would be consumed if all myosin heads were in the DRX. To estimate the fraction of the myosin heads that would be transferred from the SRX to the DRX, we assume that 50% of the heads are affected and only in fast twitch fibers, taken to be 50% of total fibers, giving an ATP consumption of ~32 mol. This energy is similar to the energy involved in the difference in fat gain observed by BrahmaNaidu et al. (31). Thus, if the piperine concentration used was far above its Kd, ~5 μM, it would explain a rate of the attenuation of fat gain. BrahmaNaidu et al. measured the attenuation of fat gain at different concentrations of piperine, finding only a modest decrease in effect when the piperine dose was lowered from 40 mg·kg⁻¹ to 20 mg·kg⁻¹, suggesting that the doses used were far above the Kd (31). Although it requires a number of assumptions, this calculation suggests that the metabolic changes found here for piperine are of the magnitude to explain its effect on weight gain. The changes amount to an increase in the basic metabolic rate of about 25%. We propose that this is a major factor in the effect of piperine in mitigating weight gain and diabetes.

At saturating levels, piperine destabilizes ~50% of the myosin heads that are in the SRX. This may be because the binding of piperine only provides enough energy to destabilize 50% of the heads in the array. Alternatively, the two myosin heads in the interacting-heads motif are in different configurations, and one of them, the free head, is less stable (15). It could be that piperine acts on that head only.

Although the affinity of piperine for its target is reasonably high, sufficient to produce beneficial effects at the high doses used in rodents, it is probably not high enough to be an effective therapy in humans. Piperine has been identified as binding to a number of other molecular targets with a similar affinity, discussed above, which would probably lead to unfavorable side effects if taken at the quantities that would be necessary for effective thermogenesis. Although piperine is probably not an effective therapeutic, it is an excellent lead compound, which could be used to find similar compounds whose properties could be optimized using our in vitro muscle assay systems.

Piperine has many of the qualities that would be required for any compound to be useful as a therapeutic treatment for metabolic diseases in humans: (i) It destabilizes the SRX and can lead to substantial thermogenesis. (ii) It functions only in fast twitch muscle fibers with a marginal effect in cardiac muscle fibers. (iii) It has little effect on fully active muscle fibers. (iv) It is well tolerated at high doses in rodents, with no obvious side effects. If a piperine-like pharmaceutical were developed that destabilized 50% of the myosin heads in the fast muscles of a 70-kg human [assuming 50% fast fibers and a myosin activity in the DRX of 0.09 s⁻¹ (44)], it would increase metabolic rate by 1.2 MJ·d⁻¹. This represents 15% of total daily energy expenditure (TEE) and would consume 32 g fat·d⁻¹ or 12 kg fat·yr⁻¹. How well will an increase of 15% of TEE be tolerated? Overexpression of uncoupling protein 3 or ectopic expression of uncoupling protein 1 in mice raised TEE by 15–25% (45–47). It was also well tolerated and led to decreased adiposity and improved insulin resistance, suggesting that the approach proposed here could be successful.

In summary, our results provide the proof of concept that pharmaceuticals targeting resting muscle thermogenesis can be found and that they will effectively treat the metabolic diseases, obesity and type 2 diabetes, in humans. Muscle is an ideal tissue to target for increasing thermogenesis, as it has a large-reserve metabolic capacity, and the modest increase suggested here can be accommodated. These pharmaceuticals will directly address the fundamental problem in these conditions: the consumption of more fuels than are metabolized. Here we show that high-throughput screens can be performed and that the protocols we used can find molecules that do increase the metabolic rate of resting muscle. Given the immensity of the problem and the need for effective therapies, this approach should be attempted. We suggest that this will open up an area in the field of muscle research and a race to be the first to market with a new class of pharmaceuticals.

Materials and Methods

Fibers and Solutions. White adult New Zealand rabbits were killed according to protocols approved by the University of California, San Francisco Institutional Animal Care and Use Committee #AN108976-02. Psoas and soleus muscle fibers were harvested and stored at ~20 °C in a solution of rigor buffer and glycerol mixed 50/50. The Rigor buffer contained 50 mM 3-(N-morpholino)propanesulfonic acid, 120 mM potassium acetate, 5 mM magnesium chloride, 5 mM EGTA, 4 mM DTT, 5 mM potassium phosphate, pH = 6.8. Relaxing buffers were obtained by addition of 4 mM ATP or 250 μM mANTP to the Rigor buffer. Activating solutions were obtained by addition of 3 mM CaCl₂ to the relaxing buffer. Strips of rabbit cardiac tissues were obtained from the left ventricle, mounted with aluminum clips, and measured as described in ref. 48.

Proteins. The RLC used was from mouse skeletal muscle (MLC2F, National Center for Biotechnology Information identification NP_058034.1) and was expressed in bacteria, labeled, and exchanged into fibers as described in ref. 18. Myosin was made as described by ref. 49. Labeling with MDCC was 60%, and ~50% of the endogenous RLC was replaced by mutant RLC during the exchange (18).

Characterization of Fiber Properties. Single-nucleotide turnovers were measured in flow cells as described previously (12). The ATPase activities of a group of single fibers, 8–12, were measured by direct determination of phosphate using malachite green (50). For a more detailed description of the assay, see Figs. S5 and S7. At concentrations of piperine of 25 μM and below the fibers were preincubated in piperine in relaxing solution for 60–120 min. Single-fiber mechanics were measured using methods and apparatus described previously (28) and in Figs. S8 and S9. All experiments were performed at room temperature, ~22 °C. For more detailed descriptions, see Supporting Information.
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