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Extract of grains of paradise and its active principle 6-paradol trigger thermogenesis of brown adipose tissue in rats

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ABSTRACT

Grains of paradise (GP) is a species of the ginger family, *Zingiberaceae*, extracts of which have a pungent, peppery taste due to an aromatic ketone, 6-paradol. The aim of this study was to explore the thermogenic effects of GP extracts and of 6-paradol. Efferent discharges from sympathetic nerves entering the interscapular brown adipose tissue were recorded. Intragastric injection of a GP extract or 6-paradol enhanced the efferent discharges of the sympathetic nerves in a dose-dependent manner. The enhanced nerve discharges were sustained for as long as 3 h. The rats did not become desensitized to the stimulatory effects these compounds on sympathetic nerve activity. The tissue temperature of brown adipose tissue showed significant increase in rats injected with 6-paradol. These results demonstrate that GP extracts and 6-paradol activate thermogenesis in brown adipose tissue, and may open up new avenues for the regulation of weight loss and weight maintenance.

1. Introduction

Obesity develops when energy intake exceeds energy expenditure. Therefore, reducing energy intake and/or increasing energy expenditure can reduce body weight and prevent body weight gain. A number of food ingredients have been proposed as tools for weight loss and weight maintenance, since they may increase energy expenditure (Diepvens et al., 2007; Kovacs and Mela, 2006; Westerterp-Plantenga et al., 2006). In particular, pungent spices have attracted considerable interest, since they may potential affect thermogenesis and fat oxidation (Diepvens et al., 2007; Kovacs and Mela, 2006; Westerterp-Plantenga et al., 2006). Capsaicin, the principle pungent ingredient of red pepper, for instance, has been shown to reduce adiposity in humans and in rodents by increasing energy and lipid metabolism (Kawada et al., 1986a, 1986b; Kobayashi et al., 1998; Watanabe et al., 1988). In addition, the pungent ingredients of mustard (allyl isothiocyanate), cinnamon (cinnamaldehyde), pepper (piperine) and ginger (gingerols and shogaols) have also been shown to be thermogenic in humans and in other animals (Kawada et al., 1988; Kovacs and Mela, 2006; Westerterp-Plantenga et al., 2006). It is thus reasonable to assume that pungent compounds enhance energy expenditure, and would be helpful in achieving body weight loss and preventing weight gain.

The sympathetic nervous system (SNS) is thought to play an important role in increasing energy expenditure after the ingestion of pungent compounds (Doucet and Tremblay, 1997; Matsumoto et al., 2000; Watanabe et al., 1988). The sympathetically-mediated increase in energy expenditure is achieved directly by stimulating the nerves and indirectly by boosting catecholamine secretion from the adrenal medulla. In fact, it has been shown that pungent compounds stimulate SNS activity and enhance catecholamine secretion from the adrenal medulla (Kawada et al., 1988; Ohnuki et al., 2001; Watanabe et al., 1987, 1988). Under enhanced energy-expenditure conditions, the SNS is not activated uniformly in all tissues, but rather selectively in specific tissues (Brito et al., 2008; Rahmouni et al., 2009). One of the major target tissues for sympathetically-mediated energy expenditure is brown adipose tissue (BAT), the tissue involved in metabolic heat production (Cannon and Nedergaard, 2004; Silva, 2006; Wijers et al., 2009). When norepinephrine (NE) released from sympathetic nerve endings acts on β-adrenoceptors in BAT, the consumption of oxygen is dramatically increased as a result of the increased mitochondrial oxidation of fatty acids (Cannon and Nedergaard, 2004; Silva, 2006; Wijers et al., 2009). In the mitochondria of BAT, substrate oxidation is poorly coupled to ATP synthesis because of the presence of a protein called uncoupling protein (UCP)-1, thereby leading to energy dissipation, *i.e.*, heat production (Cannon and Nedergaard, 2004; Silva, 2006; Wijers et al., 2009).

Grains of paradise (GP), a West African spice, is obtained from *Aframomum melegueta*, a species in the ginger family (*Zingiberaceae*). Extracts of GP have a pungent, peppery taste due to the presence of

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aromatic ketones such as 6-paradol (1-(4-hydroxy-3-methoxyphenyl)-decan-3-one) (Lee and Surh, 1998). The aim of the present study was to examine the potential thermogenic effects of GP extracts and their pungent component, 6-paradol. We therefore examined the effects of GP extracts and 6-paradol on the activity of sympathetic nerves entering BAT since thermogenesis in BAT is directly controlled by sympathetic nerves and electrophysiological recordings of their efferent discharges permit reliable assessment of thermogenesis in real time (Shinozaki et al., 2005, 2008). Furthermore, the tissue temperature of BAT was monitored to confirm that the changes in the sympathetic nerve activity were associated with thermogenesis. Our results demonstrate that GP and 6-paradol activate thermogenesis in BAT. Hence, GP and 6-paradol are promising in terms of their potential benefits in weight loss and weight maintenance.

2. Materials and methods

2.1. Animals

Male Wistar rats, 8–10 weeks in age, were obtained from Nihon SLC, Shizuoka, Japan. All rats were housed in plastic cages at 24 ± 1 °C with a 12:12 h light–dark cycle (lights on from 0700 to 1900 h), and were given free access to laboratory chow (LABO MR Stock, Nihon-Nosan, Kanagawa, Japan) and water. They were allowed to adapt to the laboratory housing for at least 1 week prior to use in these studies.

All experimental procedures were approved by the Gifu University Animal Care and Use Committee.

2.2. Neural recording procedure for sympathetic nerves

The electrical activity of the intercostal nerve innervating the BAT was recorded as previously described (Shinozaki et al., 2005). A 12-h-fasted rat was anesthetized with an intraperitoneal injection of a chloralose–urethane solution (50 mg/kg and 500 mg/kg, respectively), and was placed in the prone position on a heating blanket (Homeothermic Blanket Control Unit, Harvard Apparatus, Holliston, MA, USA) to keep body temperature around 37 °C. A small incision was made above the scapula and the interscapular BAT was partially separated from the underlying muscle. The 5 intercostal nerves, which contain sympathetic nerves entering the BAT, were identified and one of the five nerve branches was cut. When isolating the nerve, care was taken to make sure that it was not associated with blood vessels passing through the BAT to the skin. The isolated nerve was placed on a pair of silver/silver-chloride wire electrodes while the nerve branch was kept in mineral oil to prevent dehydration. The original signal of efferent mass discharges was amplified and filtered (low cut at 150 Hz; high cut at 10 kHz). The amplified signal was converted to a digital signal using a Power Lab (ADInstruments, Sydney, Australia) and was then recorded on a computer using recording software (Chart, ADInstruments, Sydney, Australia). The sampling rate was set at 20 kHz. Spikes above a threshold voltage level set just above the background were counted using a Spike Histogram (ADInstruments, Sydney, Australia).

When preparations were complete, the baseline activity was recorded for 30 min, after which the GP extract or 6-paradol was injected through an intragastric cannula. The nerve activity was then continuously monitored for up to 4 h after injection. The rate meter of the Spike Histogram with a reset time of 6 s was used to observe the time-course of the nerve activity. For each experimental condition, the mean spike frequency over a 5 min period of sympathetic nerve activity after application of the stimulus was calculated in Hz.

2.3. Measurements of tissue temperature of brown adipose tissue

The rat was anesthetized with an intraperitoneal injection of a chloralose–urethane solution (50 mg/kg and 500 mg/kg, respectively), and was placed in the prone position on a heating blanket (Homeothermic Blanket Control Unit, Harvard Apparatus, Holliston, MA, USA) to keep body temperature around 37 °C. The 5 intercostal nerves entering the right pad of the interscapular BAT were cut, whereas the nerves to the left pad were left intact. Thus, the right and left pads were used as the denervated and intact BATs respectively. A small thermistor (tissue implantable thermocouple microprobe, Physitemp Instruments Inc., Clifton, NJ, USA) was placed under each pad of the BAT as described previously (Shimizu and Saito, 1991). A similar thermistor was also inserted 4 cm into the rectum. After confirming that the tissue temperatures were stable at around 37 °C, the GP extract or 6-paradol was injected through an intragastric cannula. The tissue temperatures were continuously recoded using a Power Lab (ADInstruments, Sydney, Australia).

2.4. Blood sampling and measurement of plasma 6-paradol concentration

To examine whether 6-paradol injected into the stomach can be absorbed, plasma concentrations of 6-paradol were measured using high performance liquid chromatography (HPLC). Rats were anesthetized as described previously, and a cardiac cannula was placed into the right jugular vein for blood sampling. Before the intragastric injection of 6-paradol, a blood sample of 0.1 ml was withdrawn at time −10 min, in order to measure the control level. Blood samples were then withdrawn at 15 min, 30 min, 60 min, 120 min and 180 min after the administration of 6-paradol. When a sample was collected, an equal volume of physiological salt solution containing 20 U/ml heparin was added to it. Sera were centrifuged immediately after collection and stored at −80 °C until the concentrations of 6-paradol could be measured.

Aliquots (1 μl) of sample extracts or standards were separated by HPLC on a CAPCELL PAK MF C8 (Shiseido, Tokyo, Japan). A stock solution of 6-paradol (1 μg/ml) was prepared in acetonitrile. Working standard solutions at various concentrations were prepared by dilution of the stock solution with acetonitrile. The mobile phase was aqueous mixed with 35% acetonitrile at a flow rate of 200 μl/min. The monitoring ions were as follows: the precursor ion was 279.2 $[M+H]^+$ and the diagnostic product ion was 137.0 $[M\text{-}C_9H_{16}O]^+$. Data was collected using an Analyst 1.4.1 software package (MDS SCIEX, Downingtown, PA, USA).

2.5. Reagents

Seeds of *A. melegueta* were purchased from a commercial spice in Paris market. A single batch of dry Grains of Paradise seeds were extracted in 5 times 95% ethanol for 24 h at reflux condition with continuous agitation by magnetic stirrer. The resulting ethanolic extract was filtrated and the solvent was removed under vacuum, using a rotary evaporator (yield of the extract 4.2%). 6-paradol was synthesis from vanillin as reported previously (Locksley et al., 1972).

2.6. Statistical analysis

Results are expressed as means ± SE. Statistical significance was determined by the Student's *t*-test. The difference was considered to be significant if $p<0.05$.

3. Results

3.1. Effect of the GP extract on sympathetic nerve activity in BAT

The efferent discharges of sympathetic nerves entering the BAT correlate well with the thermogenic activity of this tissue, and we therefore examined the effect of the GP extract on the nerve activity. A single intragastric injection of the GP extract did not

change the sympathetic nerve activity for up to 30 min after the injection. However, about 30 min after the injection of the GP extract at 30 mg/kg, the number of spikes increased (Fig. 1) and the increased activity persisted at least for 1 h. The mean spike number at 60–65 min after injection (43.8 ± 6.8 spikes/10 s, $n = 5$) was significantly higher than before injection (19.8 ± 3.2 spikes/10 s, $n = 5$). By contrast, the GP extract at 10 mg/kg did not cause any significant change in the nerve discharges (Fig. 1).

3.2. Effect of 6-paradol on sympathetic nerve activity in BAT

The efferent discharges of sympathetic nerves entering the BAT increased as a result of an intragastric injection of 6-paradol (Fig. 2A). The mean spike numbers (spikes/10 s) at 60–65 min after injection of 6-paradol were normalized to the respective basal spike numbers, and are summarized in Fig. 3. The results show that the action of 6-paradol is dose-dependent, with minimum effect at 2 mg/kg.

Reductions in body temperature maximally activate the sympathetic nerve activity (Shinozaki et al., 2005). It was thus of interest to compare the degree of nerve activity after treatment with 6-paradol with that observed during a hypothermic condition. The efferent discharges of the sympathetic nerves increased dramatically when the body temperature dropped to 33 °C (Fig. 2B). The mean spike number at 33 °C was 287 ± 39 spikes/10 s ($n = 4$), a value approximately 3-fold higher than that observed after the injection of 10 mg/kg of 6-paradol.

3.3. Temporal pattern of sympathetic nerve activity in BAT before and after injection of 6-paradol

Representative sequential rate histograms of sympathetic nerve discharges before and after intragastric administration of 6-paradol (at 10 mg/kg) are shown in Fig. 4A. The nerve discharges were transiently enhanced immediately after the injection, but thereafter, no obvious change in discharge rate was observed for up to 30 min. At approximately 30 min after the injection of 6-paradol, however, the nerve discharges began to increase, and the enhanced discharges were maintained for up to 3 h. After confirming the return of nerve activity to the basal activity level, i.e., before the injection, the same dose of 6-paradol was re-injected via the intragastric tube. An enhancement in the sympathetic nerve discharges with a time-course similar to the first injection was confirmed once again (Fig. 4B).

3.4. Effect of 6-paradol on tissue temperature of BAT

To confirm the association of enhanced sympathetic nerve activity with substantial heat production, changes in BAT temperature were monitored before and after intragastric injection of 10 mg/kg

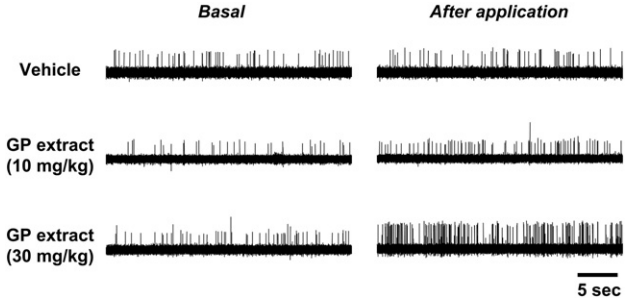

Fig. 1. Effects of the GP extract on sympathetic nerve activity in BAT. Representative recordings of efferent discharges of sympathetic nerves entering the BAT in rats injected with GP extract. After recording the baseline activity for 30 min, the GP extract (10 or 30 mg/kg body weight) or vehicle was injected through an intragastric cannula. Traces on the right ("After application") show nerve activity at around 60 min after the injection. Similar results were obtained from five independent experiments. A significant increase in nerve discharges was observed in rats injected with 30 mg/kg GP extract.

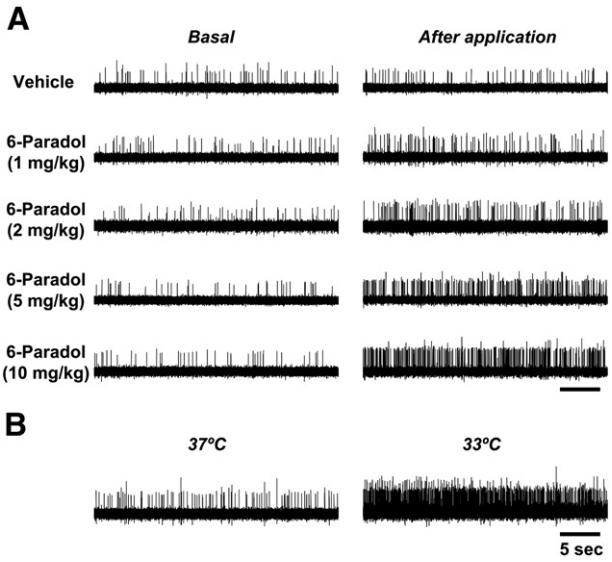

Fig. 2. Effects of 6-paradol on the sympathetic nerve activity in BAT. Representative recordings of efferent discharges of sympathetic nerves entering the BAT in rats injected with 6-paradol [A]. After recording the baseline activity for 30 min, 6-paradol (1–10 mg/kg body weight) or vehicle was injected through an intragastric cannula. Traces on the right ("After application") show nerve activity at around 60 min after the injection. Similar results were obtained from five independent experiments, and the results are summarized in Fig. 3. For comparison, nerve activity observed when the body temperature dropped to 33 °C is shown in panel [B].

6-paradol. Fig. 5 shows the representative result of the changes. The temperature of the intact innervated BAT began to increase at around 30 min after the injection. By contrast, the temperature of denervated BAT pad in the same animal did not increase. The net increment was calculated by subtracting the mean temperature observed before injection from that at 60–65 min after the injection. The temperature of the intact BAT was 0.52 ± 0.10 ($n = 5$), significantly higher than the denervated BAT (0.02 ± 0.04, $n = 5$).

3.5. Changes in serum 6-paradol concentration after intragastric injection

To examine whether 6-paradol can be absorbed by the gut, its level in the blood was measured. Although 6-paradol was not detected in the serum at 5 min after intragastric injection (at 10 mg/kg), it reached a detectable level (32 ± 6 ng/ml, $n = 4$) 15 min after the injection. The serum concentration of 6-paradol increased as time elapsed (144 ± 27 and 212 ± 88 ng/ml at 30 and 60 min after the

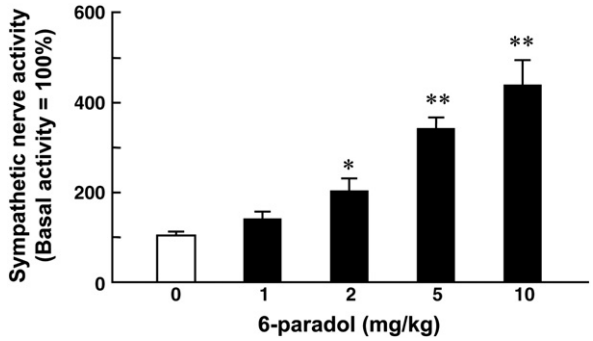

Fig. 3. Dose-dependency of the effects of 6-paradol on sympathetic nerve activity in BAT. The mean spike frequency (spikes/10 s) at 60–65 min after the 6-paradol injection were normalized to the respective basal spike frequency. Values reported are means ± SE ($n = 5$). The action of 6-paradol is dose-dependent, with minimum effect at 2 mg/kg.

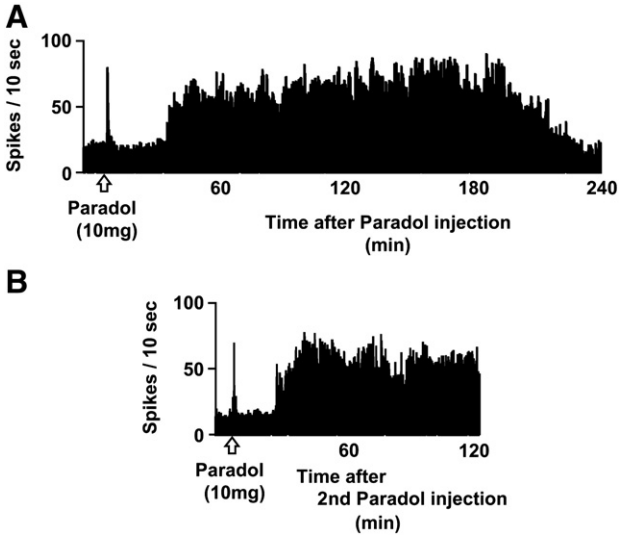

Fig. 4. Temporal pattern of sympathetic nerve activity in BAT before and after 6-paradol injection. Representative sequential rate histograms of sympathetic nerve discharges before and after intragastric administration of 6-paradol (10 mg/kg) [A]. The nerve discharges began to increase at approximately 30 min after 6-paradol injection, and the enhanced discharges were maintained for up to 3 h. After confirming that the nerve activity had returned to the basal level, the same dose of 6-paradol was re-injected [B]. The nerve discharges showed an increase with a time-course similar to the first injection. Similar results were obtained from five independent experiments.

injection, respectively). Concentrations of 6-paradol as high as 100 ng/ml or higher were maintained at least for 3 h.

4. Discussion

The current study demonstrates that GP extract and its pungent component, 6-paradol, triggers the thermogenic activity of the BAT. Experimental evidence for this conclusion are manifested in the form of (i) enhanced efferent discharges of the sympathetic nerves entering the BAT in rats with the intragastric injection of the GP extract or 6-paradol, and (ii) an increase in tissue temperature in the BAT with enhanced sympathetic nerve activity. The activation of sympathetic nerve activity was sustained for as long as 3 h. In addition, it is noteworthy that 6-paradol had two important characteristics; first, the sympathetic nerves in rats did not become desensitized to the stimulatory effect of the compound, and second, it can be absorbed through the intestine. These characteristics should prove valuable in developing orally active food ingredients for inducing non-shivering thermogenesis and thereby controlling body weight.

Several methods have been established to assess the actual activation of BAT thermogenesis. These include directly measuring tissue temperature, recording the efferent discharges of sympathetic nerves entering BAT (Shinozaki et al., 2005, 2008), determining the norepinephrine turnover rate (Brito et al., 2008; Young et al., 1982), measuring glucose uptake rates (Shimizu et al., 1991; Shimizu and Saito, 1991), and assessing GDP-binding sites in mitochondria isolated from the BAT (Goodbody and Trayhurn, 1981; Sundin, 1981). Of all these methods, we chose the measurement of electrophysiological recordings of sympathetic nerve activity, because it allowed us to assess the effect of drugs on BAT thermogenesis in real time. In addition, changes in tissue temperature in intact innervated BAT and denervated BAT were measured concomitantly in the same animal. Using a combination of these methods, we were able to examine accurately whether or not the GP extract and 6-paradol activated BAT thermogenesis.

We found that a single intragastric injection of the GP extract substantially enhanced sympathetic nerve activity. Similarly, injection of 6-paradol potently activated the sympathetic nerves in the BAT in a dose-dependent manner. An earlier report has identified 6-paradol as the major pungent ingredient of GP extract (Calixto et al., 2005). An HPLC analysis revealed that 40% of the GP extract used in this study was made up of phenolic compounds and that 37% of the phenolics were composed of 6-paradol (unpublished observation). Based on these percentages, 30 mg of GP extract can be assumed to contain about 4.4 mg of 6-paradol. Considering that 30 mg of GP extract activates the sympathetic nerves to a degree comparable to 5 mg of 6-paradol (see Figs. 1 and 2), the action of the extract may have been due to the 6-paradol. We cannot, however, rule out the possible involvement of other chemicals. The maximal levels of sympathetic nerve discharges after treatment with 6-paradol were approximately one third the levels observed in hypothermic rats, which suggests that 6-paradol cannot lead to full activation of BAT function. However, it is important to recognize that heat generation under hypothermic conditions is an urgent need for homothermal animals. Thus, it seems likely that the level of activation by 6-paradol is sufficient for use in helping body weight loss and preventing weight gain.

The intercostal nerves contain sympathetic nerves entering the BAT, but are not exclusively composed of the sympathetic nerves. Therefore, in order to provide a firm conclusion, it is necessary to determine whether the enhanced nerve activity is actually accompanied by heat production in BAT. Direct recordings of tissue temperature revealed that a single intragastric injection of 6-paradol (20 mg/kg) brought about a rise in BAT temperature with a similar time-course as the activation of the sympathetic nerves. Importantly, the rise in temperature was not observed in the denervated pad of the BAT in the same rat. This rules out a possible direct action of 6-paradol on brown adipocytes as well as a possible involvement of humoral mediators. Altogether, it can be concluded that 6-paradol activates BAT thermogenesis through a mediation of the sympathetic nerves in rats.

It is well known that the action of capsaicin is associated with a strong desensitization (Szallasi and Blumberg, 1999). The possible desensitization property of 6-paradol has been of interest owing to its similarity to capsaicin. In this study, the stimulatory effect of 6-paradol on the sympathetic nerve activity was sustained for about 3 h, and then subsided. After the activity had declined to the baseline level, a second application of 6-paradol produced a response comparable to the first application. This clearly demonstrates that, unlike capsaicin, 6-paradol does not result in desensitization. This property is particularly important since it would allow repeated treatments without a decline in effectiveness. Further study is needed to validate

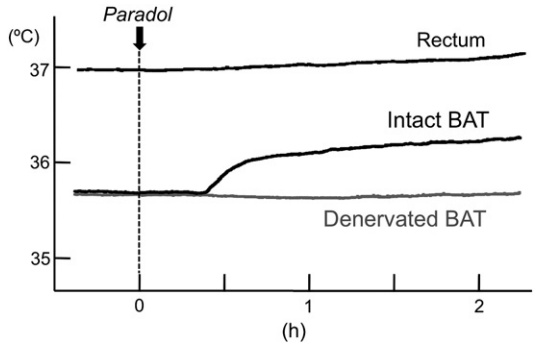

Fig. 5. Effects of 6-paradol injection on temperatures of interscapular BAT and rectum. Representative recordings of temperatures of interscapular BAT and rectum in a rat injected intragastrically with 6-paradol (10 mg/kg) at *time 0* are shown. The 5 intercostal nerves entering the right pad of the interscapular BAT were cut, whereas the nerves to the left pad were left intact. Thus, the right and left pads were used as the denervated and intact BAT, respectively. Similar results were obtained in five independent experiments.

the effects of repeated treatments with GP extract or 6-paradol on body weight gain and BAT hyperplasia.

The transient activation of sympathetic nerves observed just after the intragastric injection of 6-paradol may be due to mucosal stimulation as judged by the immediate onset of the response. This was further supported by evidence that a vehicle injection did not promote the transient activation. On the other hand, the long-lasting enhancement of nerve discharges that began 30 min after the 6-paradol injection can be attributed to its absorption from the gut. In agreement with this idea, the timing of the appearance of 6-paradol in the serum fit well with the onset of nerve activation. It has been demonstrated that capsaicin acts on the hypothalamus to accelerate energy expenditure (Hori et al., 1988; Szolcsanyi et al., 1971). It remains to be seen whether 6-paradol also acts on the hypothalamus.

The present study demonstrated that 6-paradol activates the sympathetic nerves entering BAT. It remains to be determined, however, whether the 6-paradol-induced activation of the sympathetic nerves is restricted to BAT or can be commonly found in other tissues, as well. The sympathetic nervous system generally does not, as a rule, uniformly activate all of the body tissues supplied with sympathetic nerves. Rather, it activates specific tissues selectively in different situations (Diepvens et al., 2007). It therefore seems unlikely that 6-paradol would activate the sympathetic nerves of BAT exclusively. In this regard, Watanabe et al. (1988) demonstrated that capsaicin activates the adrenal sympathetic efferent nerve, in addition to the nerve in BAT. Several pungent compounds are also shown to have similar effects (Kawada et al., 1988; Kovacs and Mela, 2006; Westerterp-Plantenga et al., 2006). Considering the similarities in pungent compounds, 6-paradol is likely to have the ability to activate the adrenal sympathetic nerve.

In summary, the present study demonstrates that treatment with GP extract and its pungent component, 6-paradol have the ability to activate BAT thermogenesis. Furthermore, the present study revealed that 6-paradol can be absorbed through the intestine and the animal does not become desensitized to its effects. These characteristics can be utilized in the development of orally active food ingredients for body weight control. It has been widely believed that BAT contributes little, if anything, to the maintenance of energy homeostasis in adult humans. However, recent studies have revealed that BAT is active not only in newborns but also in adults (Cypess et al., 2009; Saito et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009), indicating the utility of BAT function as a target for anti-obesity drugs. Therefore, 6-paradol should be useful for developing orally active anti-obesity supplements.

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