Oleuropein, a Phenolic Compound in Extra Virgin Olive Oil, Increases Uncoupling Protein 1 Content in Brown Adipose Tissue and Enhances Noradrenaline and Adrenaline Secretions in Rats

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Summary The effects of oleuropein, a phenolic compound in extra virgin olive oil (EV-olive oil), on triglyceride metabolism were investigated by measuring the degree of thermogenesis in interscapular brown adipose tissue (IBAT), and noradrenaline and adrenaline secretions in rats. In Experiment 1, rats were given a high-fat diet (control diet) with the oleuropein supplementation of 1.2 or 4 mg/kg of diet (0.1, 0.2 or 0.4% oleuropein diet, respectively). After 28 d of feeding, body weight, perirenal adipose tissue, epididymal fat pad, and plasma triglyceride, free fatty acid and total cholesterol concentrations were reduced by the 0.1, 0.2 or 0.4% oleuropein diet and were significantly lowest in rats fed the 0.4% oleuropein diet, as compared with those of rats fed with the control diet. The content of uncoupling protein 1 (UCP1) in IBAT and urinary noradrenaline and adrenaline excretions were significantly higher in rats fed the 0.1 or 0.2% oleuropein diet, as compared with those of rats fed with the control diet, although there were no significant differences in rats fed the 0.4% oleuropein diet. In Experiment 2, the effects of oleuropein on noradrenaline and adrenaline secretion were evaluated. The intravenous administration of oleuropein and oleuropein aglycone significantly increased plasma noradrenaline and adrenaline concentrations. Furthermore, oleuropein aglycone induced the secretions of noradrenaline and adrenaline about ten fold more potently than oleuropein. These results suggest that the phenolic compound oleuropein in EV-olive oil enhances thermogenesis by increasing the UCP1 content in IBAT and noradrenaline and adrenaline secretions in rats.

Key Words oleuropein, extra virgin olive oil, uncoupling protein 1, noradrenaline, adrenaline

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Olive oil appears to be an example of a functional food with various components, such as monounsaturated fatty acids that may contribute to its health benefits, and is also a good source of phytochemicals, including polyphenolic compounds (1–10). Olive oil is a source of at least 30 phenolic compounds (6–8), and particularly, extra virgin olive oil (EV-olive oil) contains considerable amounts of phenolic compounds, e.g., hydroxytyrosol and oleuropein, which are responsible for its peculiar taste and high stability (1). There is accumulating evidence that olive oil phenolics are powerful antioxidants, both in vivo and in vitro, and that they exert other potent biological activities that could partially account for the observed beneficial effects of the Mediterranean diet (1, 11, 12). However, there have been few reports on the nutritional effects of phenolic compounds in olive oil on triglyceride catabolism. Furthermore, the components of olive oil that are effective in enhancing triglyceride catabolism have not been clarified yet. In a recent study, olive oil feeding induced the highest uncoupling protein (UCP) 1, UCP2, and UCP3 mRNA expression levels in IBAT (13). It was suggested that olive oil induces the up-regulation of UCP mRNA, which is probably not mediated by systemic metabolic changes and is related to local effects on interscapular brown adipose tissue and skeletal muscle (13). However, there is as yet no information available on the effects of olive oil components on UCP content and mRNA expression level. A previous study in our laboratory showed, in vivo and in situ, that phenols except hydroxytyrosol in EV-olive oil enhance thermogenesis by increasing the UCP1 content in IBAT and enhancing noradrenaline and adrenaline secretions in rats (14). Furthermore, it was suggested that the oleuropein fraction (mainly containing oleuropein and oleuropein aglycone) in the phenolic fraction from EV-olive oil enhances noradrenaline and adrenaline secretions (14). Oleuropein is the pungent principle of olives
and is found in EV-olive oil and in its aglycone form. Therefore, the present study was carried out to further determine in detail whether oleuropein enhances triglyceride catabolism and thermogenesis. Triglyceride metabolism is known to be stimulated by catecholamines (noradrenaline and adrenaline) released through the stimulation of the activities of the sympathetic nervous system and subsequent thermogenesis (15–18). Noradrenaline secretion, in response to sympathetic nervous system stimulation, likely plays a major role in the regulation of thermogenesis in brown adipose tissue (BAT) (19, 20). Sympathetic nervous system stimulation has been reported to regulate thermogenesis by increasing the content of UCP, particularly subtype 1 (UCP1) but not UCP2 or 3, in BAT (15, 21–23). In this study, we performed in vivo (Experiment 1) and in situ experiments (Experiment 2) on rats to determine whether oleuropein stimulates triglyceride catabolism by measuring UCP1 content in interscapular BAT (IBAT) and noradrenaline and adrenaline secretions.

MATERIALS AND METHODS

Animal care. Male Sprague-Dawley rats (Japan SLC, Inc., Shizuoka, Japan) were housed individually in stainless steel wire-bottom cages in a room maintained at 22–24°C and with a relative humidity of about 50%. The room was illuminated from 07:00 h to 19:00 h. Tap water was freely available. Four-week-old rats were purchased for Experiment 1 and 7-wk-old rats were purchased for Experiment 2; these rats were given a commercial diet (CE-2, CLEA Japan, Inc., Tokyo, Japan) for 3 d before starting the experiments. This study was approved by the Institutional Animal Care and Use Committee of Kobe Women’s University, Faculty of Home Economics.

Chemicals. The rats were anesthetized using α-chloralose and urethane (24), which were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Tokyo Chemical Industry, Co., Ltd. (Tokyo, Japan), respectively. Oleuropein (No. 0204, Extrasynthese Genay, France) was a commercially available chemical with a purity of approximately 80%. Oleuropein aglycone was prepared by the enzymatic removal of glucose (37°C, 36 h) with β-glucosidase (Wako306-50941, from almond, 2,000 U/54.4 mg; Wako Pure Chemical Industries, Ltd.), i.e., the reaction was performed by mixing 200 mL of oleuropein solution (10 mg/mL) and 10 mL of β-glucosidase solution (20 mg/mL) and shaking at 100 strokes/min at 37°C for 36 h. Both oleuropein and β-glucosidase solutions were prepared by dissolving in 0.1 mol/L acetate buffer (pH 4.2). The removal of the glucose molecule in oleuropein was confirmed by TLC analysis and the purity of oleuropein aglycone obtained was approximately 100%. The structures of oleuropein and oleuropein aglycone are shown in Fig. 1.

Experiment 1 (in vivo). The experimental diet used in Experiment 1 was a high-fat diet containing 30% shortening (control diet) with oleuropein supplementation (1. 2 or 4 mg/kg of diet: 0.1, 0.2 or 0.4% oleuropein diet, respectively), as shown in Table 1. Rats weighing 80–90 g were separated into four groups of 6 to 7 rats and were each given the control diet or 0.1, 0.2 or 0.4% oleuropein diet for 28 d. Each group of rats was offered the diets in appropriate amounts such that the three groups consumed the same amount of metabolizable energy during the experimental period, and the food consumption in all the three diet groups was approximately equivalent to the maximal diet that rats can consume under these conditions. At the end of the experimental period, the rats were transferred into individual metabolic cages, where urine and feces were separately collected for 1 d. Previously, we confirmed that the daily urinary excretions of noradrenaline and adrenaline are not affected by the stress of placing the animals in a metabolic cage. Each urinary sample was collected in a bottle containing 1 mL of 6 mol/L HCl. After the collection, urinary total noradrenaline and adrenaline excretions were determined by the method of Davidson and Fitzpatrick (25). Urinary creatinine excretion was measured by the method of Clark and Thompson (26). After being fed for 28 d [on day 29], the rats were anesthetized by intraperitoneally injecting of α-chloralose and urethane (75 and 750 mg/kg of body weight, respectively). Blood samples were collected from the abdominal aorta, and plasma was separated by centrifugation (3,000×g for 15 min). After collecting blood samples, the liver, kidney, perirenal adipose tissue and epidydimal fat pad were immediately excised and weighed. All samples were stored at −40°C until analysis. Plasma triglycerides and free fatty acid concentrations were determined enzymatically using commercial kits (triglycerides, Triglyceride G-test Wako; free fatty acids, NEFA C-Test Wako, Wako Pure Chemical
Table 1. Compositions of experimental diet (Experiment 1).

<table>
<thead>
<tr>
<th>High fat diet (g/kg)</th>
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<tbody>
<tr>
<td>Casein¹</td>
</tr>
<tr>
<td>Shortening²</td>
</tr>
<tr>
<td>Vitamins³</td>
</tr>
<tr>
<td>Minerals⁴</td>
</tr>
<tr>
<td>Cellulose⁵</td>
</tr>
<tr>
<td>Sucrose⁶</td>
</tr>
<tr>
<td>Oleuropein⁷</td>
</tr>
<tr>
<td>Energy density (MJ/kg)⁸</td>
</tr>
</tbody>
</table>

¹ Oriental Yeast Co., Ltd., Tokyo, Japan.
² Crisco®; partially hydrogenated vegetable shortening from Procter and Gamble Co., Cincinnati, OH.
³ Purchased from Oriental Yeast Co., Ltd. The vitamin mixture (mg/kg of diet) contained retinyl acetate 17, cholecalciferol 0.0425, all-rac-a-tocopherol acetate 85, menadione 88.4, thiamin-HCl 20.4, riboflavin 68, pyridoxine-HCl 13.6, vitamin B₁₂ 0.0085, vitamin C 510, d-biotin 0.34, folic acid 3.4, Ca-pantothenate 85, p-aminobenzoic acid 85, nicotinic acid 102, inositol 102, choline chloride 3,400, and cellulose powder 12,419.809.
⁴ Purchased from Oriental Yeast Co., Ltd. The mineral mixture (mg/kg of diet) contained CaHPO₄·H₂O 7,280, KH₂PO₄ 12,860, NaH₂PO₄·H₂O 4,675, NaCl 2,330, Ca lactate 17,545, Fe-citrate 1,590, MgSO₄·7H₂O 60, MnSO₄·H₂O 60, CuSO₄·5H₂O 15, and KI 5.
⁵ Added at 1, 2 or 4 g/kg of diet as oleuropein (0.1, 0.2 or 0.4% oleuropein diet, respectively) to the high-fat diet (control diet).
⁶ Energy values were as follows: starch, soluble carbohydrates and protein (16.70 MJ/kg); fat (37.70 MJ/kg).

TABLE 1 Effects of Oleuropein in Extra Virgin Olive Oil

| Energy values (MJ/kg) | 21.21 |

Energy values were as follows: starch, soluble carbohydrates and protein (16.70 MJ/kg); fat (37.70 MJ/kg).

Industries, Ltd.) Plasma total cholesterol concentrations were measured by the method of Pearson et al. (27). Plasma leptin concentrations were determined using commercial kits (Rat Leptin ELISA Kit Wako, Wako Pure Chemical Industries, Ltd.)

The experimental IBAT UCP1 content analysis by Western blotting was performed as reported previously (28). IBAT was immediately removed from the rats and weighed, and IBAT mitochondria were isolated as previously reported elsewhere (29). Western blotting was performed as reported previously using commercial kits (Rat Leptin ELISA Kit Wako, Wako Pure Chemical Industries, Ltd.) Plasma total cholesterol concentrations were measured by the method of Pearson et al. (27).

The relationships between the plasma noradrenaline and adrenaline concentrations and the administrations of oleuropein and oleuropein aglycone for dose-response measurements in Experiment 2 were determined by regression analysis. Differences with p<0.05 were considered significant.

RESULTS

Experiment 1

After 28 days of dietary treatment, the body weight, body weight gain, energy efficiency and epidydimal fat pad weight of rats fed the 0.4% oleuropein diet were significantly lower than those of rats fed the control diet, and those of rats fed the 0.1 and 0.2% oleuropein diets were lower, although there were no significant differences. The perirenal adipose tissue weights of rats fed the 0.2 and 0.4% oleuropein diets were significantly lower than those of rats fed the control diet, and those of rats fed the 0.1% oleuropein diet were lower, although there were no significant differences. There were no significant differences in liver weight, kidney weight and urinary creatinine level among rats fed the experimental diets (Table 2). The plasma triglyceride and leptin concentrations of rats fed the 0.2 and 0.4% oleuropein diets were significantly lower than those of rats fed the control diet, and those of rats fed the 0.1% oleuropein diet were lower, although there were no significant differences. The plasma free fatty acid and total cholesterol concentrations of rats fed the 0.1, 0.2 and 0.4% oleuropein diets were significantly lower than those of rats fed the control diet (Table 2). The IBAT UCP1 contents of rats fed the 0.1 and 0.2% oleuropein diets were significantly higher than those of rats fed the control diet, whereas there were no significant differ-
Weight gain (g) 184.0
Energy efficiency (g gain/MJ consumed) 19.23

Table 2. Effects of oleuropein supplementation on body , liver, kidney , perirenal adipose tissue and epididymal fat pad weights, and plasma concentrations of triglyceride, free fatty acids and total cholesterol in rats fed high-fat diet for 28 d.1

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>0.1% oleuropein diet</th>
<th>0.2% oleuropein diet</th>
<th>0.4% oleuropein diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>121.1±2.7a</td>
<td>122.2±1.3a</td>
<td>121.9±4.0a</td>
<td>119.2±3.2a</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>303.3±2.3a</td>
<td>296.3±4.6ab</td>
<td>298.5±4.1ab</td>
<td>287.1±3.2b</td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>184.0±2.6a</td>
<td>171.5±10.3b</td>
<td>173.9±3.8b</td>
<td>168.6±4.1b</td>
</tr>
<tr>
<td>Energy intake (MJ/28 d)</td>
<td>9.120</td>
<td>9.120</td>
<td>9.120</td>
<td>9.120</td>
</tr>
<tr>
<td>Energy efficiency (g gain/MJ consumed)</td>
<td>19.23±0.30a</td>
<td>17.93±0.41ab</td>
<td>18.18±0.40ab</td>
<td>17.63±0.43b</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>13.25±0.41a</td>
<td>12.70±0.36a</td>
<td>12.73±0.38a</td>
<td>12.38±0.36a</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>2.43±0.05a</td>
<td>2.19±0.04a</td>
<td>2.23±0.09a</td>
<td>2.23±0.05a</td>
</tr>
<tr>
<td>Urinary creatinine (mmol/d)</td>
<td>7.50±0.16a</td>
<td>7.145±0.29a</td>
<td>6.81±0.16a</td>
<td>6.98±0.62a</td>
</tr>
<tr>
<td>Epididymal fat pad weight (g)</td>
<td>6.64±0.43b</td>
<td>6.03±0.26b</td>
<td>5.95±0.46b</td>
<td>5.16±0.25b</td>
</tr>
<tr>
<td>Triglyceride (mmol/L plasma)</td>
<td>20.71±5.3a</td>
<td>9.86±0.8ab</td>
<td>7.50±0.7b</td>
<td>9.3±1.2b</td>
</tr>
<tr>
<td>Free fatty acids (μmol/L plasma)</td>
<td>44.1±7.1a</td>
<td>26.4±1.9b</td>
<td>26.0±1.4b</td>
<td>23.2±1.3b</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L plasma)</td>
<td>2.43±0.14a</td>
<td>1.76±0.12b</td>
<td>1.77±0.11b</td>
<td>1.47±0.06b</td>
</tr>
<tr>
<td>Leptin (nmol/L plasma)</td>
<td>1.543.9±123.7a</td>
<td>1.171.2±124.8ab</td>
<td>1.154.2±159.2ab</td>
<td>876.2±76.9b</td>
</tr>
</tbody>
</table>

1 Values are expressed as means±SE, n=6 or 7. Within a row, values with different superscripts are significantly different, p<0.05.

Dose-response relationships with respect to plasma noradrenaline and adrenaline concentrations in rats following oleuropein administration are shown in Fig. 4. Both plasma noradrenaline and adrenaline concentrations were significantly increased in rats that received 30 mmol/L (16.2 mg) or 50 mmol/L (27 mg) oleuropein compared with those that received the vehicle alone, whereas with the administration of 10 mmol/L (5.4 mg) or 20 mmol/L (10.8 mg) oleuropein, such an increase was observed but there were no significant differences. The observed increase was dose-dependent, and there was a significant positive correlation between the noradrenaline and adrenaline concentrations and the dose of oleuropein [noradrenaline, p=0.001 (r=0.549); adrenaline, p<0.0001 (r=0.581)].

The dose-response relationships with respect to plasma noradrenaline and adrenaline concentrations in rats following oleuropein aglycone administration are shown in Fig. 5. The plasma noradrenaline concentration was significantly increased in rats that received 3 mmol/L (1.14 mg) or 5 mmol/L (1.9 mg) oleuropein aglycone compared with those that received the vehicle alone, whereas with the administration of 10 mmol/L (0.38 mg) or 20 mmol/L (0.76 mg) oleuropein aglycone, such an increase was observed but there were no significant differences. The plasma adrenaline concentration was significantly increased in rats that received 2 mmol/L (0.76 mg), 3 mmol/L (1.14 mg) or 5 mmol/L (1.9 mg) oleuropein aglycone compared with those that received the vehicle alone, whereas with the administration of 1 mmol/L (0.38 mg) oleuropein aglycone, such an increase was observed but there were no significant differences. The observed increase was dose-
dependent, and there was a significant positive correlation between the noradrenaline and adrenaline concentrations and the dose of oleuropein aglycone [noradrenaline, $p<0.001$ ($r=0.595$); adrenaline, $p<0.0001$ ($r=0.661$)]. Although the oleuropein aglycone doses (1, 2, 3, or 5 mmol/L) were one-tenth the oleuropein doses (10, 20, 30 or 50 mmol/L), the noradrenaline and adrenaline concentrations were similarly increased by the oleuropein and oleuropein aglycone administrations, as shown in Figs. 4 and 5. Both plasma noradrenaline and adrenaline concentrations were dose-dependently increased in rats that received 1 mmol/L (0.38 mg) to 3 mmol/L (1.14 mg) oleuropein aglycone and then reached a plateau. Oleuropein aglycone stimulated the secretory activities of noradrenaline and adrenaline about tenfold more potently than oleuropein.

**DISCUSSION**

In Experiment 1, the effects of oleuropein supplementation on thermogenesis in IBAT (UCP1) and on noradrenaline and adrenaline secretions in rats fed a high-
fat diet containing 30% shortening were investigated in vivo. We conducted Experiment 1 as a model for the analysis of the effects of oleuropein on thermogenesis in rats fed a high-fat diet. Our data indicate that oleuropein enhances urinary noradrenaline and adrenaline excretions, and decreases body fat accumulation, as well as the weights of perirenal adipose tissue and epididymal fat pad, by increasing triglyceride catabolism by the elevation of thermogenesis in IBAT via an increase in UCP1 content. In particular, thermogenesis appears to be facilitated by oleuropein in the experimental diet (high-fat diet), and the total intake of 430 or 860 mg of oleuropein during 28 d (from the total diet intake of 430 g) enhances thermogenesis in rats fed the 0.1 or 0.2% oleuropein diet.

Visioli et al. (33) demonstrated that, after olive oil ingestion, 4-hydroxyphenylethanol and hydroxytyrosol are dose-dependently absorbed in humans and are excreted in the urine, mostly as glucuronide conjugates. Edgecombe et al. (34) demonstrated that oleuropein, a
The ingestion of olive oil phenolics, including olive oil phenolics, is absorbed poorly in the small intestine. Oleuropein and ligstroside aglycones are hydrolyzed in the gastrointestinal tract. Oleuropein and ligstroside aglycones are hydrolyzed in the small intestine, and oleuropein (glycosides) and oleuropein and ligstroside aglycones are hydrolyzed in the gastrointestinal tract. Oleuropein and ligstroside aglycones are hydrolyzed in the small intestine, and oleuropein (glycosides) and oleuropein and ligstroside aglycones are hydrolyzed in the gastrointestinal tract.

In the present study, it could be considered that using 60% as the rate of oleuropein absorption as reference (66–73%), the total amounts of oleuropein ingested for 28 d with the 0.1, 0.2 and 0.4% oleuropein diets were about 258 mg (9.2 mg/d), 516 mg (18.4 mg/d) and 1,032 mg (36.9 mg/d), respectively. We considered that excessive oleuropein ingestion in rats fed the 0.4% oleuropein diet for 28 d induces a decrease in the metabolic rate. Similarly, from our preliminary experiment, we confirmed that the oleuropein ingestion of 129 mg per rat with the 0.05% oleuropein diet for 28 d (4.6 mg/d) does not affect thermogenesis (data not shown).

Bravo (36) reviewed the metabolism of phenolic compounds including olive oil phenolics and suggested that some polyphenolic compounds are metabolized within the gastrointestinal tract. In addition, it was suggested that aglycones and free simple phenolic compounds can be directly absorbed through the small intestinal mucosa conversely and that glycosides must be hydrolyzed to their corresponding aglycones before absorption (36). Therefore, in the present study, it could be considered that the induction of UCP1 in IBAT is caused by the stimulation of noradrenaline and adrenaline secretions via the elevation of oleuropein aglycone concentration in blood due to the accumulation of oleuropein aglycone absorbed in the small intestine after the hydrolysis of oleuropein in the gastrointestinal tract with the daily continuous intake of oleuropein from the 0.1 or 0.2% oleuropein diet for 28 d.

In Experiment 2, to identify the components of EV-olive oil that enhance triglyceride catabolism and thermogenesis, the effects of oleuropein, a phenolic compound in EV-olive oil, on plasma noradrenaline and adrenaline concentrations were investigated in anesthetized rats in situ. Our data indicate that the administration of oleuropein or oleuropein aglycone significantly increases plasma noradrenaline and adrenaline concentrations. Oleuropein is the pungent principle of olives and is found in EV-olive oil and in its aglycone form. The concentrations of oleuropein and oleuropein aglycone have been reported to be 2.04±0.78 and 18.64±3.36 mg/kg in EV-olive oil, respectively (7). On the basis of these data, the doses of oleuropein and oleuropein aglycone in the present study could therefore be considered as being equivalent to physiological levels consumed by the normal dietary intake of EV-olive oil. In the physiological state, it might be considered that another form of oleuropein aglycone exists in the bloodstream, although oleuropein was not observed in rats fed the EV-olive oil. Accordingly, we conducted Experiment 2 (in situ) as a model for the comparison of the effects of oleuropein and oleuropein aglycone on catecholamine secretion by infusion into the femoral vein. The present study indicates that oleuropein aglycone is about 10 times more active than oleuropein in stimulating the secretions of noradrenaline and adrenaline. It could be considered that this stronger secretion-stimulatory activity of oleuropein aglycone is caused by the difference in the degree of pungency between oleuropein and oleuropein aglycone. Both oleuropein and oleuropein aglycone in EV-olive oil increase bitterness and pungency (1, 12). In our preliminary experiment, we confirmed that oleuropein aglycone activates the rat capsaicin receptor TRPV1 (transient receptor potential vanilloid subtype 1) expressed in HEK 293 cells to the same extent as zingerone and that oleuropein shows a lower degree of TRPV1 activation than its aglycone by one order of magnitude or lower (unpublished data). Therefore, it could be considered that the pungency of oleuropein aglycone more strongly stimulates noradrenaline and adrenaline secretions than that of oleuropein, from the results of Experiment 2 in situ. Previously, we reported that in rats, the pungent principles of allyl-containing sulfides in garlic enhance thermogenesis by increasing the UCP1 content in IBAT and also by increasing noradrenaline and adrenaline secretions (37). We suggested that allyl-containing sulfides in garlic enhance thermogenesis via the β-adrenergic stimulation of the sympathetic nervous system by increasing noradrenaline secretion in rats (37). We consider oleuropein and oleuropein aglycone to enhance triglyceride catabolism and thermogenesis by a similar mechanism. In the present study, it could be considered that oleuropein is hydrolyzed in the gastrointestinal tract and absorbed in the small intestine as oleuropein aglycone and that the pungency of oleuropein aglycone enhances thermogenesis via an increase in UCP1 content in IBAT by increasing noradrenaline and adrenaline secretions. Therefore, the present study indicates that a phenolic compound, the pungent principle of oleuropein (both forms of oleuropein and oleuropein aglycone) in EV-olive oil, is responsible for the enhancements of noradrenaline and adrenaline secretions as well as thermogenesis, as indicated by the increased UCP1 content in IBAT.

Acknowledgments
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