



# Changes in lipolysis and hormone-sensitive lipase expression caused by procyanidins in 3T3-L1 adipocytes

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**OBJECTIVE:** To find out whether lipid stores are influenced by phenolic compounds in wine.

**DESIGN:** Differentiated 3T3-L1 cells were treated with catechin, epicatechin or procyanidin extracts with different degrees of polymerization at 150  $\mu$ M for different periods of time (0.5–24 h).

**SUBJECTS:** Cell line 3T3-L1.

**MEASUREMENTS:** Cellular viability, glycerol-3-phosphate dehydrogenase activity, glycerol release in the medium, HSL mRNA levels, triacylglycerols and protein.

**RESULTS:** Catechin, epicatechin and procyanidin extracts were not toxic for the 3T3-L1 cells in the conditions assayed. Glycerol-3-phosphate dehydrogenase activity was markedly decreased by 150  $\mu$ M procyanidin extracts. The release of glycerol into the medium was increased in 150  $\mu$ M procyanidin extract-treated cells and reached a plateau after 15 h exposure. Procyanidins caused a time-dependent reduction in the HSL mRNA levels.

**CONCLUSIONS:** These results suggest that procyanidins from grape and wine affect lipid metabolism whilst their monomers (catechin and epicatechin) do not. This effect is more pronounced when the degree of polymerization is higher. Procyanidin extracts cause a time-dependent reduction in the HSL mRNA levels, inhibit triacylglycerol synthesis and also favour triacylglycerol hydrolysis until the HSL mRNA had reached very low levels.

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**Keywords:** procyanidins; lipolysis; 3T3-L1 cells; hormone-sensitive lipase (HSL)

## Introduction

Flavonoids, a group of polyphenolic natural products found in vegetables, fruits and beverages such as tea and wine, have a broad pharmacological activity.<sup>1,2</sup> The molecular mechanisms underlying the biological effects of flavonoids are not completely understood, although it is known that they can bind to enzymes, hormone carriers and DNA, chelate transition metal ions and scavenge free radicals.<sup>3–7</sup>

The main classes of flavonoids in grapes and wines are anthocyanins, flavonols, catechins (flavan-3-ols) and procyanidins (oligomers of catechins).<sup>8</sup> There is a lot of information about the antioxidant effects of phenolic compounds in wine and their protective effects on the cardiovascular system, and it would be interesting to know whether they have any effect because there is some controversy about the relation between wine, alcohol and obesity.<sup>9</sup>

In adipose tissue, HSL (hormone-sensitive lipase) functions as the rate-limiting enzyme in lipolysis by mediating the hydrolysis of triacylglycerol and diacyl-

glycerol after the phosphorylation of the enzyme by protein kinase A. This phosphorylation is activated by a variety of hormonal stimuli.<sup>10</sup> Many therapeutic agents cause direct lipolysis or anti-lipolysis in adipocytes,<sup>11,12</sup> and short-term hormonal regulation of HSL activity is well characterized, whereas little is known about how HSL gene expression is controlled.<sup>13</sup>

Some flavonoids are potent inhibitors of protein kinases (PKA, PKC, MLCK, CDPK),<sup>14</sup> and a number of procyanidin preparations from a variety of plants are among the most potent inhibitors of eukaryote protein kinases yet found.<sup>15</sup> While there are many studies of the flavonol quercetin and its glycosides, less is known about procyanidins that are found in large quantities in grapes and wine.<sup>8</sup> Quercetin has inhibitory effects on phosphodiesterase (PDE) activity which are similar to, or greater than, 3-isobutyl-2-methylxanthine.<sup>16</sup> It increases lipolysis by acting synergistically with adrenaline. Some authors<sup>17</sup> have suggested that flavonoids affect lipolysis not by inhibiting phosphodiesterase but by influencing the  $\beta$ -adrenergic receptor. The fact that these compounds modify intracellular cAMP levels<sup>14</sup> suggests that they act on lipid metabolism in adipocytes, because the key enzyme which regulates lipolysis (hormone-sensitive lipase) is clearly modulated, in the short term, by phosphorylation/dephosphorylation mechanisms which are dependent on cAMP-protein kinase.<sup>10</sup>

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Our work analyses the effects of catechin, epicatechin and procyanidin grape extracts, with a different degree of polymerization, on triacylglycerol synthesis and hydrolysis and HSL gene expression in adipocytes. We have addressed this question using the adipocyte cell line 3T3-L1, which is a cell culture model suitable for investigating adipocyte differentiation and for determining factors that regulate the physiology of the mature adipocyte.<sup>18</sup>

## Materials and methods

### Cell culture

The 3T3-L1 preadipocyte cell line was propagated and induced to differentiate in Dulbecco's modified Eagle's medium (DMEM). Proliferating preadipocytes were maintained at low density in a culture medium (growth medium) that consisted of DMEM supplemented with 10% calf serum, 2 mM glutamine, 100 U/ml penicillin and 100/1.5 µg/ml streptomycin/fungizone. Differentiation was induced by refeeding confluent preadipocytes (day 1) with DMEM supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100/1.5 µg/ml streptomycin/fungizone, 5 µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine and 0.25 µM dexamethasone (D1 medium). Cells were re-fed 48 h later (day 3) with D2 medium (DMEM supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100/1.5 µg/ml streptomycin/fungizone and 5 µg/ml insulin). After a further 48 h incubation (day 5), the cells were re-fed with D3 medium (DMEM supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100/1.5 µg/ml streptomycin/fungizone). After 72 h incubation (day 8), the cells were re-fed with fresh medium (D3) and then exposed to the phenolic compounds.

### Cell treatments with phenolic compounds and cytotoxicity assay

Catechin, epicatechin (purchased from Fluka) and grape seed procyanidin extracts (purchased from Les Dérivés Résiniques et Terpéniques, Dax, France) were dissolved in ethanol. Procyanidin extract A had a mean molecular weight of 1246 and essentially contained monomers (31%) and oligomeric procyanidins, 19% of which were dimers and 50% of which were larger structures (3–10 units). According to the manufacturer, procyanidin extract B had a mean molecular weight of 1399 and contained essentially monomers (21.3%) and oligomeric procyanidins, 17.4% of which were dimers, 41.3% were trimers and 20% were larger structures (4–13 units).

Cells were exposed to these compounds at different concentrations (50–300 µM) and for different periods of time (0.5–24 h) and cellular viability was assessed

by the neutral red assay,<sup>19</sup> which depends on the inability of dead and damaged cells to take up neutral red (NR) dye. If dye-treated cells are lysed, the colour is released and can be measured photometrically. For the NR assay, the cells were grown in 12-well multiplates. Cells incubated with or without ethanol were used as control cells.

### Measurement of lipolytic activity

The lipolytic response of differentiated adipocytes to the compounds assayed was determined by measuring the amount of glycerol released into the incubation medium. Aliquots (200 µl) of medium from multiplates were assayed for glycerol content by the Garland and Randle method.<sup>20</sup>

### Glycerol-3-phosphate dehydrogenase assays

Cells grown on six-well multiplates were rinsed twice with phosphate-buffered saline (PBS), scraped into 750 µl of 50 mM Tris-HCl, 1 mM EDTA, 1 mM β-mercaptoethanol and sonicated. The resulting extract was frozen at –80°C. The glycerol-3-phosphate dehydrogenase activity was determined using the Wise and Green method.<sup>21</sup> The assay buffer contained 100 mM TEA (pH 7.5), 2.6 mM EDTA, 0.1 mM β-mercaptoethanol, 0.120 mM NADH and 0.2 mM dihydroxyacetone phosphate. Reactions were started by adding cellular extract, and the change in absorbance at 340 nm was monitored at room temperature with a spectrophotometer (Hitachi U-2000).

### Northern blot analysis

Total RNA was isolated from treated 3T3-L1 cells by the adapted phenol method.<sup>22</sup> The total RNA (10 µg) was size-fractionated by 1% agarose–gel electrophoresis in 2.2 M formaldehyde. The gels were blotted onto a nitro-cellulose membrane (BA-S 85, Schleicher & Schull). The membrane was exposed to UV light to bind the blotted RNA covalently, and then hybridized with the isolated <sup>32</sup>P-labelled cDNA for HSL<sup>23</sup> or for rat β-actin using a multiprime labelling kit (Multiprime DNA labelling systems, Amersham UK). The <sup>32</sup>P-labelled cDNA that bound to the corresponding mRNA was exposed to X-ray film, and the level of the specific mRNA was estimated by a densitometer (Bio Image Intelligent quantifier, Bio Image Systems Corporation, USA).

### Other methods

Protein was determined by the Bradford method<sup>24</sup> using the Bio-Rad protein reagent.

The amount of triacylglycerol deposited in adipocytes was determined enzymatically by glycerol-phosphate oxidase (QCA, Tarragona, Spain).<sup>25</sup>

## Statistics

Results were expressed as the mean  $\pm$  s.e.m. of five experiments. The ANOVA test was used for statistical analysis followed by Scheffe's test.

## Results

A toxicity assay was carried out in order to define the non-toxic concentration of the flavonoids in 3T3-L1 cells. As ethanol was used to dissolve these compounds, two types of cell cultures were used as control: with and without ethanol. The volume of ethanol added to the cells was the same as the amount in which the flavonoids were dissolved. There was no difference between the two types of control cell (results not shown).

The effect of catechin, epicatechin and procyanidins on 3T3-L1 cell viability was assayed using different concentrations (50, 100, 150, 200 and 300  $\mu$ M) for 24 h (Table 1). The NR assay did not indicate a statistically significant decrease in the viability of the adipocytes at any of the concentrations tested. However, cultures incubated with 200 and 300  $\mu$ M of procyanidin extract B showed a 10% and 15% decrease in viability, respectively. Therefore we decided to work with catechin, epicatechin and procyanidin extracts A and B at concentrations of 150  $\mu$ M.

The effect of these compounds on adipocytes was studied by analysing the two main pathways involved in maintaining adipose stores: the biosynthesis and

hydrolysis of triacylglycerols. Table 2 shows the lipolysis index (measured as the release of glycerol to the culture medium) in adipocytes cultivated with 150  $\mu$ M epicatechin, 150  $\mu$ M catechin, or 150  $\mu$ M procyanidin extracts for up to 24 h. It can be seen that catechin and epicatechin had no effect on glycerol release. In contrast, the two procyanidin extracts considerably increased the glycerol release at longer exposures (15–24 h), with a statistically significant difference between the effect of procyanidin extracts A and B. This increase is greater with procyanidin extract B, which contained longer oligomers than extract A.

The flux throughout the triacylglycerol biosynthetic pathway was assessed by measuring the activity of glycerol-3-phosphate dehydrogenase, an enzyme which plays an important role in the synthesis of lipids in the adipocyte.<sup>26</sup> Figure 1 shows the glycerol-3-phosphate dehydrogenase activity in adipocytes cultivated with 150  $\mu$ M epicatechin, 150  $\mu$ M catechin or 150  $\mu$ M procyanidin extracts for up to 24 h. Epicatechin had no effect, neither did its isomer, catechin, but the procyanidin extracts reduced this enzyme activity to 70% (procyanidin extract A) and 40% (procyanidin extract B) of the control values. Again, the procyanidin extract with longer oligomers (procyanidin extract B) had a greater effect.

To analyse the lipolytic response further, we studied the gene expression of the hormone-sensitive lipase, which catalyses the rate-limiting step in adipocyte lipolysis.<sup>10</sup> This study was carried out with procyanidin extract B because it has the greatest effect on both the pathways involved in fat storage. Figure 2 shows the results of the analysis. In Figure 2(b) we

**Table 1** 3T3-L1 cell viability after 24 h exposure to catechin, epicatechin or procyanidin extracts

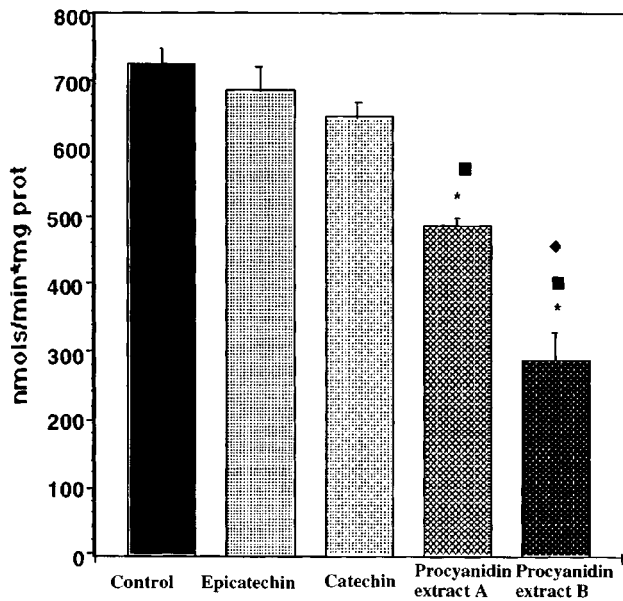
Concentration	Catechin	Epicatechin	Procyanidin extract (A)	Procyanidin extract (B)
50 $\mu$ M	96.7 $\pm$ 3.1	101.4 $\pm$ 8.3	101.9 $\pm$ 3.6	104.2 $\pm$ 2.2
100 $\mu$ M	98.9 $\pm$ 3.6	109.6 $\pm$ 7.2	109.7 $\pm$ 2.2	109.3 $\pm$ 1.2
150 $\mu$ M	93.4 $\pm$ 4.4	95.0 $\pm$ 4.5	112.1 $\pm$ 3.9	100.0 $\pm$ 2.7
200 $\mu$ M	109.8 $\pm$ 5.6	99.2 $\pm$ 9.1	118.1 $\pm$ 5.9	90.6 $\pm$ 2.6
300 $\mu$ M	107.4 $\pm$ 5.8	97.0 $\pm$ 5.2	96.7 $\pm$ 1.4	85.9 $\pm$ 3.2

Differentiated 3T3-L1 cells were exposed to five different concentrations of catechin, epicatechin, procyanidin extracts A and procyanidin extracts B for 24 h. At the end of this period, a neutral red assay was made for toxicity. Cells with no compounds, and ethanol-treated cells were used as a control. Results are expressed as a percentage of the control and are the mean  $\pm$  s.e.m. of five separate experiments.

**Table 2** Influence of catechin, epicatechin and procyanidin extracts on glycerol release into the culture medium

Hours	Control	Epicatechin	Catechin	Procyanidin extract A	Procyanidin extract B
0.5	0.74 $\pm$ 0.09	0.98 $\pm$ 0.06	1.02 $\pm$ 0.05	0.96 $\pm$ 0.07	0.97 $\pm$ 0.08
3	0.80 $\pm$ 0.07	1.03 $\pm$ 0.11	1.23 $\pm$ 0.10	1.13 $\pm$ 0.04	1.15 $\pm$ 0.03
6	1.37 $\pm$ 0.19	1.23 $\pm$ 0.15	1.28 $\pm$ 0.16	1.42 $\pm$ 0.12	1.51 $\pm$ 0.07
15	2.38 $\pm$ 0.17	2.22 $\pm$ 0.33	2.38 $\pm$ 0.27	4.12 $\pm$ 0.25 <sup>ab</sup>	6.75 $\pm$ 0.55 <sup>abc</sup>
18	3.1 $\pm$ 0.40	2.61 $\pm$ 0.40	2.18 $\pm$ 0.30	6.05 $\pm$ 0.15 <sup>ab</sup>	8.13 $\pm$ 0.85 <sup>abc</sup>
21	2.14 $\pm$ 0.55	2.37 $\pm$ 0.17	2.31 $\pm$ 0.42	5.06 $\pm$ 0.87 <sup>ab</sup>	6.45 $\pm$ 0.97 <sup>ab</sup>
24	2.81 $\pm$ 0.23	3.22 $\pm$ 0.19	3.01 $\pm$ 0.14	6.52 $\pm$ 0.32 <sup>ab</sup>	7.72 $\pm$ 0.67 <sup>abc</sup>

Differentiated 3T3-L1 cells were exposed to 150  $\mu$ M epicatechin, 150  $\mu$ M catechin, 150  $\mu$ M procyanidin extract A or 150  $\mu$ M procyanidin extract B for different periods of time: 0.5–24 h. The glycerol content of the medium ( $\mu$ mol/mg protein) was assayed at the times indicated in the table. Cells with no addition to the medium were used as controls. Values are means  $\pm$  s.e.m. of five separate experiments. <sup>a</sup> $P < 0.05$ , significant difference vs control cells; <sup>b</sup> $P < 0.05$ , significant different vs catechin or epicatechin treated cells; <sup>c</sup> $P < 0.05$ , significant difference vs procyanidin extract A-treated cells.



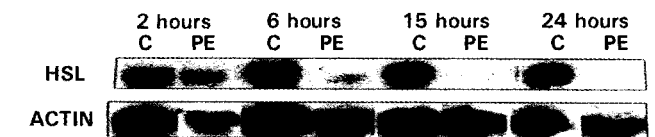
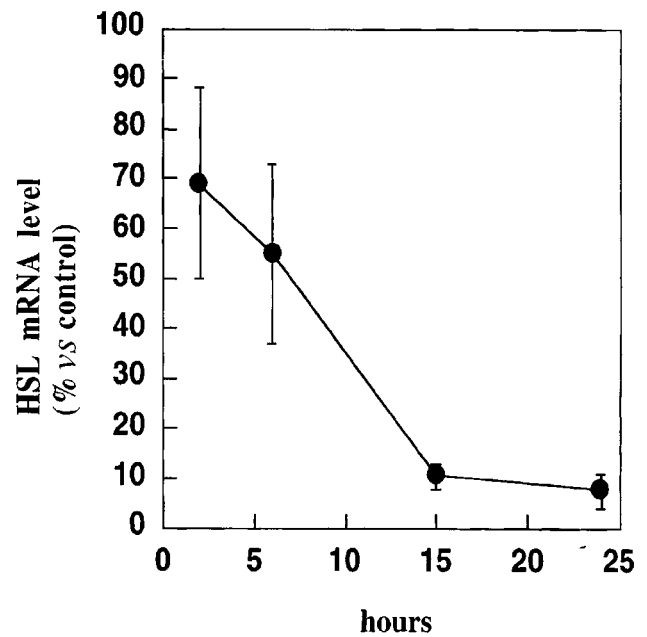
**Figure 1** Glycerol-3P-dehydrogenase activity of 3T3-L1 cells treated with catechin, epicatechin or procyanidin extracts.

Glycerol-3P-dehydrogenase activity of 3T3-L1 cells treated with 150  $\mu$ M catechin, 150  $\mu$ M epicatechin, 150  $\mu$ M procyanidin extract A or 150  $\mu$ M procyanidin extract B. Cells with no addition were used as controls. After 24 h exposure, cells were scraped in a buffer solution and sonicated. Glycerol-3-phosphate dehydrogenase (nmols/min mg protein) activity was assayed in this homogenate. Values shown are means  $\pm$  s.e.m. of five separate experiments. \* $P < 0.05$  vs control;  $\blacksquare P < 0.05$  vs catechin and epicatechin;  $\blacklozenge P < 0.05$  vs procyanidin extract A.

can see that procyanidin extract B causes a time-dependent reduction in the mRNA expression of this enzyme, which has been quantified and illustrated in Figure 2(a). After 2 h exposure, the expression in cells treated with procyanidin extract B was 70% of the controls, and after 15 h it fell to only 10%.

## Discussion

There is some controversy about the relation between obesity, wine consumption and the effects of ethanol.<sup>9</sup> Most studies analyse the impact of ethanol as an energetic fuel on caloric intake and weight gain.<sup>27,128</sup> Alcohol has been shown to suppress fat oxidation by favouring a positive fat balance,<sup>29</sup> but the results are controversial because they are affected by a considerable diversity of human habits and characteristics. Few reports (either epidemiological or clinical) have specifically evaluated the influence of wine consumption on the regulation of body weight.<sup>30,31</sup> Tebib *et al*<sup>32</sup> supplemented the rats' diet with amounts of monomers or polymers of grape seed tannins which were equivalent to a human intake of 0.5 l red wine. They found that those rats which consumed polymers gained less weight than the other animals. There are only a few studies on the interaction of flavonoids with adipose tissue<sup>16,17</sup> and, as far as we know, none on the effects of procyanidins in wine on adipocytes.



**Figure 2** Time course of procyanidin extract B inhibition of HSL mRNA levels in 3T3-L1 adipocytes.

Cell cultures were exposed to concentrations of 150  $\mu$ M of procyanidin extracts B for various times. At the times shown, the procyanidin extract (PE) treated cells and their control (C) cells were processed for RNA quantification. Northern blots were hybridized with a HSL cDNA probe or a  $\beta$ -actin cDNA probe. (a) Data from densitometric scanning of autoradiographs are expressed as the percentage of HSL mRNA present in control cells. Values shown are means  $\pm$  s.e.m. of five separate experiments. (b) Representative Northern blot analysis of HSL and  $\beta$ -actin expression during exposure to procyanidin extracts B.

Although little is known about the bioavailability and the plasma transport of flavonoids, some studies have shown that non-negligible concentrations of dietary flavonoids are recovered in rat and human plasma.<sup>33-37</sup>

Several phenolic compounds modify intracellular cAMP levels<sup>14</sup> and are potent inhibitors of protein kinases.<sup>14</sup> This suggests that they can act on the lipid metabolism of adipocytes, because the key enzyme which regulates lipolysis (hormone-sensitive lipase) is clearly modulated, in the short term, by phosphorylation/dephosphorylation mechanisms that are cAMP-protein kinase dependent.<sup>10</sup>

The results of this study indicated that only by incubating 3T3-L1 cells with procyanidin extracts is lipolysis favoured and biosynthesis inhibited, thus degrading fat stores. Monomers (catechin and epicatechin) have no effect on lipolysis, and more glycerol is released with the procyanidin extracts which have more, and bigger, oligomers (extract B). Triacylglycerol synthesis (glycerol-3P-dehydrogenase) was again more inhibited by procyanidin extracts, particularly by extract B, and monomeric forms have no

effect, so there is a clear correlation between the effects of procyanidins on adipocytes and their degree of polymerization. In contrast to catecholamines, which stimulate lipolysis within minutes,<sup>38</sup> procyanidin extracts increase lipolysis only after hours (> 6 h) of incubation, like TNF- $\alpha$ .<sup>39,40</sup>

From these results, and the time required to observe them, it seems that some of the effects may be due to the compounds causing changes in expression of genes, such as the HSL gene. It is well known that hormone-induced changes in the cAMP levels of adipocytes regulate HSL activity,<sup>41</sup> but little is known about how the expression of this activity is regulated. Our results show that, after cells have been incubated with procyanidin extracts, HSL mRNA levels decrease over time with respect to their controls. Other authors have found a similar response when cells are incubated with other compounds.<sup>13,41–44</sup> Doerrler *et al*<sup>43</sup> found similar results when they cultured 3T3-F442A adipocytes with TNF, IFN- $\alpha$  and IFN- $\gamma$  cytokines. Slavin *et al*<sup>44</sup> incubated primary cultures of rat adipocytes with adrenaline or glucagon and there was an increase in medium glycerol but no change in HSL mRNA levels. Plée-Gautier *et al*<sup>13</sup> found that treating adipocytes with cAMP analogues considerably decreased HSL mRNA. The similarity between the Plée-Gautier results<sup>13</sup> and ours reinforces the idea that the procyanidins mediate this effect by modulating cAMP levels. Some authors<sup>13</sup> postulate that long-term cAMP treatment of adipocytes induces a counter-regulatory process that reduces HSL content and, ultimately, limits the depletion of fatty acids from stored triacylglycerol.

Despite the changes induced by procyanidin extracts in the triacylglycerol biosynthesis and hydrolysis pathways in 3T3-L1 cells, we found no variation in the triacylglycerol stores after cells had been exposed to these compounds for 24 h (results not shown). At a first glance, these results seem contradictory, but the levels of glycerol in the medium remained constant between 15 and 24 h of cellular incubation with procyanidins. This suggests that after 15 h there is no more triacylglycerol hydrolysis. The moment at which the lipolysis stops coincides with a considerable reduction in HSL-mRNA (10% of control value).

Therefore, we can conclude that procyanidins from grape and wine affect lipid metabolism, whilst their monomers (catechin and epicatechin) do not. There is a clear positive correlation between the effects of procyanidins on adipocytes and their degree of polymerization. Procyanidins cause a time-dependent reduction in the HSL mRNA levels. They also inhibit triacylglycerol synthesis and favour triacylglycerol hydrolysis until the HSL mRNA levels have reached very low levels. However, there is still much work to do to define the mechanisms involved in these effects, which may be a combination of changes in cAMP levels and/or changes in the gene expression.

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