Prevention of diet-induced obesity by apple polyphenols in Wistar rats through regulation of adipocyte gene expression and DNA methylation patterns.

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Abbreviations: AP, apple polyphenols; DIO, diet-induced obesity**;** HFS, high-fat-sucrose**;** IPGTT, intraperitoneal glucose tolerance test; RQ, respiratory quotient; WAT, white adipose tissue**;** HOMA, homeostatic model assessment

Key words: high-fat-sucrose diet; adiposity; lipolysis; nutrigenomics; epigenetics.

Abstract

This study was conducted to determine the mechanisms implicated in the beneficial effects of apple polyphenols (AP) against diet-induced obesity (DIO) in Wistar rats, described in a previous study from our group. Supplementation of high-fat-sucrose (HFS) diet with AP prevented adiposity increase by inhibition of adipocyte hypertrophy. Rats supplemented with AP exhibited improved glucose tolerance while adipocytes isolated from these rats showed an enhanced lipolytic response to isoproterenol. AP intake led to reduced *Lep*, *Plin* and *Srebf1* mRNA levels and increased *Aqp7*, *Aebp1* and *Ppargc1*a mRNA levels in epididymal adipocytes. In addition, we found different methylation patterns of *Aqp7*, *Lep*, *Ppargc1a* and *Srebf1* promoters in adipocytes from apple-supplemented rats compared to HFS-fed rats. The administration of AP protects against body weight gain and fat deposition and improves glucose tolerance in rats. We propose that AP exerts the anti-obesity effects through the regulation of genes involved in adipogenesis, lipolysis and fatty acid oxidation, in a process that could be mediated in part by epigenetic mechanisms.

Apples are a rich source of polyphenols, which consist of a complex mixture of chlorogenic acid, phloridzin, quercetin, catechin, epicatechin, procyanidin and rutin, among others compounds [1]. These phenolic substances have been reported to display antihyperglycemic, antihyperlipidemic and anti-inflammatory properties [2]. Furthermore, previous studies have demonstrated the effect of apple polyphenols (AP) on fat deposition, by decreasing the weight of visceral adipose tissue in rats fed a control [3] or high fat-diet [4]. In the same way, AP were able to decrease triglyceride absorption by inhibiting pancreatic lipase activity in mice and humans [5]. Our group have recently published a study where supplementation of HFS diet with AP during 8 weeks (700mg/Kg body weight) prevented body weight and adiposity gain (in all fat pads) promoted by this obesogenic diet, without changes in food intake [6]. AP was also found to protect from HFS diet-induced hyperglycemia, hyperleptinemia and insulin-resistance.

Since the mechanisms implicated in the effects of AP against DIO have not been well elucidated yet, the purpose of this work was to evaluate changes in cellularity, mRNA expression and DNA methylation induced by the ingestion of AP on rat adipocytes, in order to achieve a deeper understanding of the molecular changes in response to AP supplementation.

Our results from histological studies demonstrated that supplementation of HFS with AP markedly reversed the enlargement of adipocyte volume induced by HFS diet intake (Table 1) in the epididymal fat pad, reducing it by almost 28%, without changes in the subcutaneous one. AP supplementation reversed the increase on the population of

large epididymal adipocytes, especially with diameters higher than 130 μm (Supporting information: Fig. S1).

AP supplementation reversed the increase in blood glucose levels observed in HFS-fed rats after the intraperitoneal glucose tolerance test (IPGTT) (Fig. S3A) with a significant decrease of 14% in the total area under the curve (Fig. S3B).

We further explored the lipolytic capacity of epididymal adipocytes from animals supplemented with AP (Fig. S4). HFS intake impaired the catecholamine-induced lipolytic activity of adipocytes, with an inhibition of 54%. Interestingly, supplementation with AP tended to restore the lipolytic response of fat cells to isoproterenol stimulation (HFS 0.42±0.32; HFS+AP 0.77±0.32 μmoles glycerol/100mg lipids/90 min; p=0.065).

To examine AP-induced gene expression modifications in isolated epididymal adipocytes, a quantitative RT-PCR primer array was performed (Supporting information: Table S1). Gene expression levels of leptin, perilipin (*Plin*) and sterol regulatory element binding transcription factor 1 (*Srebf1*) were upregulated by HFS whereas this effect was normalized by AP supplementation (Fig. 1). Interestingly, some genes were only regulated by AP supplementation (Fig. 1). Thus, the adipocyte enhancer binding protein 1 (*Aebp1*), aquaporin 7 (*Aqp7*) and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*Ppargc1a*) were significantly overexpressed in the supplemented group compared with the HFS group.

After that, we measured the methylation pattern in the promoters of those genes which expression levels had been modified by AP supplementation (Fig. 2 and Supporting information: Fig. S6). HFS intake resulted in hypomethylation of 3 CpG sites of *Aqp7* promoter (Fig. 2) whereas supplementation with AP reversed these

effects. The opposite pattern was found in 2 CpG sites of leptin promoter, with increased methylation in HFS group and significant decreased methylation in AP group. Notably, AP could also modify the percentage of DNA methylation in one CpG site of *Srebf1* promoter, with a reduction of 52%, and *Ppargc1a* promoter, with an increase of 14%. Moreover, we found a significant correlation between *Lep*_CpG22 methylation degree and leptin gene expression and also between *Aqp7* promoter total methylation and *Aqp7* expression levels (Fig. 2). Additionally, there was a negative correlation between total Aqp7 methylation and *Lep*_CpG22 methylation levels. Epidydimal fat mass was negatively correlated with *Aqp7*_CpG4 methylation while *Aqp7*_CpG4 percentage methylation was inversely correlated with insulin plasmatic levels and leptin mRNA levels.

Our results obtained in the epidydimal depot indicate that AP prevents adipose tissue accumulation mainly through a prevention of hypertrophy rather than hyperplasia. These effects could be mediated through the increased capacity of these adipocytes to respond to catecholamine-induced lipolysis. In this way, other polyphenols have been reported to decrease β-adrenergic mediated lipolysis [7]. However, we cannot rule out other mechanisms that could be implicated, as for example inhibition of dietary fat absorption, since it has been observed that polyphenols induce this effect *in vivo*, probably due to its inhibitory effect on pancreatic lipase activity [5].

In addition, AP supplementation normalizes glucose levels after the IPGTT. In this context, quercetin and phloridzine, polyphenols found in apples, have been reported to decrease intestinal glucose uptake [8,9]. The diminished population of larger-sized adipocytes in rats supplemented with AP could explain this improvement of glucose tolerance, as it has been seen that adipocyte size is related with insulin resistance [10] and that large adipocytes exhibit lower insulin-stimulated glucose oxidation [11].

Evidence from various studies indicates that polyphenols consumption prevents fat accumulation through the activation of β-oxidation [12,13]. Our results show an upregulation by AP of the key transcription factor *Ppargc1a,* which could be related with an increase in fatty acid oxidation. Concomitantly, the increase in *Ppargc1*a mRNA expression could indicate a partial shift from a white to brown adipose tissue phenotype [14].

On the other hand, *Aqp7* is known to modulate adipocyte glycerol permeability thereby controlling triglyceride accumulation and glucose homeostasis. Accordingly, overexpression of *Aqp7* could help to explain the increased glycerol released observed in the *ex vivo* lipolysis experiments and the smaller size of the adipocytes of AP-fed rats, although no changes were found in the mRNA levels of the key lipolytic genes *Lipe* and *Pnpla2*.

Another mechanism that could be implicated in the anti-obesity effects of AP is the inhibiton of adipogenesis, as indicated by the enhanced expression of *Aebp1* [15]. Remarkably, it has been previously described that in preadipocytes, *Aebp1* mRNA levels are modulated by some plant extracts [16]. Although we did not find changes in key transcription factors related with adipogenesis, like *PPARγ* or *CEBPα*, the expression levels of *Srebf1*, which also participates in the control of adipogenesis, were downregulated by AP. In this sense, it has been shown that other polyphenolic extracts inhibited adipocyte differentiation through downregulation of *Srebf1* [17]. Furthermore, decreased mRNA levels of perilipin in adipocytes from AP-supplemented rats could indicate again an inhibition of adipogenesis by AP since higher expression of perilipin levels are related with increased adipogenesis and higher lipid droplets size [18].

AP supplementation reversed the increased leptin expression levels induced by HFS feeding. The role of leptin in the regulation of energy expenditure and food intake has been clearly evidenced [19]. Results of leptin gene expression are in line with the decreased circulating leptin levels and reflect the reduced fat mass observed in AP-supplemented animals.

DNA methylation is involved in the control of gene expression together with other epigenetic processes such as histone modifications and miRNAs [20]. Some studies have evidenced a role of AP on the modulation of DNA methylation in relation with its chemopreventive properties [21]. Our group has reported previously that leptin promoter is modulated by high-fat diet in adipocytes [22]. Now, we describe an increase in leptin CpG (position -292 and -198) metyhlation by HFS diet and a reduction of methylation to normal values by AP in the CpG at the position -198. We have also observed an increase of *Ppargc1a* promoter methylation by AP. Other studies have evidenced an epigenetic control of *Ppargc1a* gene expression [23] in human muscle. On the other hand, we have reported for the first time an epigenetic regulation of *Aqp7* gene promoter both by HFS diet and AP and of *Srebf1* by AP, pointing out these genes as potential candidates for future epigenetic studies.

Aqp7 and Leptin promoter methylation levels were significantly correlated with the corresponding gene expression levels. Methylation of CpG rich sequences in gene promoter regions usually represses gene expression by interfering with binding of proteins required for transcription. Unexpectedly, we found that DNA methylation was positively correlated with gene expression. However, it has been proposed that if DNA methylation occurs on negative regulatory elements in the promoter, the transcriptional activity could be increased [24]. The inverse correlation observed between *Aqp7*_CpG4 percentage methylation and insulin levels as well as leptin expression are especially

relevant because it has been described recently that leptin and insulin directly regulate *Aqp7* expression through the PI3K/Akt/mTOR pathway in cultured adipocytes [25].

Although we have found many beneficial effects induced by AP intake, the dose used is quite high and it is equivalent to a human consumption of 146 mg of AP per kg of body weight, accordingly to the formula proposed by Reagan-Shaw S et al [26]. This fact must be taking into account if human intervention studies are designed.

Taken together, our results demonstrated that AP extract exerts potent anti-obesogenic and anti-diabetic effects mainly through direct activity on adipocytes modulating β-adrenergic stimulated lipolysis as well as adipogenic, lipolytic and oxidative-related genes. Furthermore, some of these genes could be subjected to epigenetic regulation. In conclusion, this study highlights AP extract as a promising functional food ingredient for the management of obesity and its metabolic complications.

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FIGURES CAPTIONS

Figure 1. Effect of apple polyphenols (AP) on mRNA levels in isolated epididymal adipocytes from male Wistar rats fed with high-fat-sucrose (HFS) diet supplemented with or without AP for 56 days. Results were normalized to GAPDH mRNA levels and expressed as fold change respect to controls (control set at unit). Data are means ± SE (n=5-6); ** $p < 0.01$ vs control group. #$p < 0.05$, ## $p < 0.01$ vs HFS group. See supporting information: Table S1.

Figure 3. Effect of apple polyphenols (AP) on the percentage of methylation **(A)** of specific CpG dinucleotides in gene promoters of isolated epididymal adipocytes from male Wistar rats fed with high-fat-sucrose (HFS) diet supplemented with or without AP for 56 days. **(B)** Correlation analyses between percentage of DNA methylation and gene expression levels (R, Pearson's correlation coefficient). Data are means ± SE (n=5-6); * $p < 0.05$, ** $p < 0.01$ vs control group. # $p < 0.05$, ## $p < 0.01$ vs HFS group. See supporting information: Fig. S6.