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Molecular mechanism of intracellular lipid accumulation: Suppressive effect of Pycnogenol^{R} in liver cells

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Submission date: July 18, 2013; Acceptance date: September 25, 2013; Publication date: September 30, 2013

ABSTRACT:

Cells are physiologically ready to accumulate lipids such as triacylglycerides in the cytoplasm. Five classes of perilipin (PLIN) family proteins are known to be involved in the process of intracellular lipid accumulation. PLIN2 is expressed ubiquitously including adipocytes, hepatocytes and macrophages. Over-expression of PLIN2 is demonstrated in the lesions of fatty liver diseases and atherosclerosis. Suppression of PLIN2 expression prevents from developing these pathological conditions in animal models, suggesting that PLIN2 could be a therapeutic target molecule for excessive intracellular lipid accumulation which leads to various metabolic derangements. The PLIN2 gene promoter has two important *cis*-acting elements in close proximity:AP-1 element which mediates inflammatory signals and PPRE which mediates free fatty acid effect. In NMuLi mouse liver cells, FFA such as oleic acid requires both functional AP-1 and PPRE simultaneously to stimulate the promoter activity, indicating the presence of intimate interaction of inflammatory and metabolic signals on this gene. Pycnogenol^{R}, French maritime pine bark extracts, suppressed the oleic acid-induced PLIN2 expression and lipid accumulation in NMuLi cells. We found that Pycnogenol^{R} did not suppress the PLIN2 promoter activity or AP-1 binding to DNA. Instead, Pycnogenol^{R} facilitates the PLIN2 mRNA degradation, leading to suppression of lipid accumulation. This effect seems to be independent of antioxidant effect of Pycnogenol^{R}. We raise the idea that PLIN2 is a putative target molecule for prevention of pathological condition induced by

excessive lipid accumulation, and this class of natural compounds could be putative therapeutic modalities.

Key words: Pycnogenol^{R}, lipid droplet, perilipin, fatty liver disease

INTRODUCTION:

Most types of cells accumulate lipids in the cytoplasm in a form of lipid droplets, because lipids are indispensable for living cells or organisms. Intracellular lipid droplets are energy storage in adipocytes. Lipid droplets provide materials for production of steroid hormones and eicosanoids as well. Lipids are components of cell membranes, transferred to whole body in a form of lipoprotein, and excreted as milk [1]. To date, five structurally related proteins are known to be involved in lipid droplet formation in the cells [2, 3]. These are called perilipin family proteins: perilipin (PLIN)1 (perilipin), PLIN2 (ADRP or adipophilin), PLIN3 (Tip47), PLIN4 (S3-12) and PLIN5 (OXPAT or MLDP) [4]. PLIN2 was identified adipocyte precursor cells during the process of adipocyte maturation, thus originally named adipose differentiation-related protein (ADRP) [5]. Among these 5 classes of proteins, PLIN2 is ubiquitously expressed, not only in adipocytes but also other cell types including hepatocytes, macrophages and so on [6].

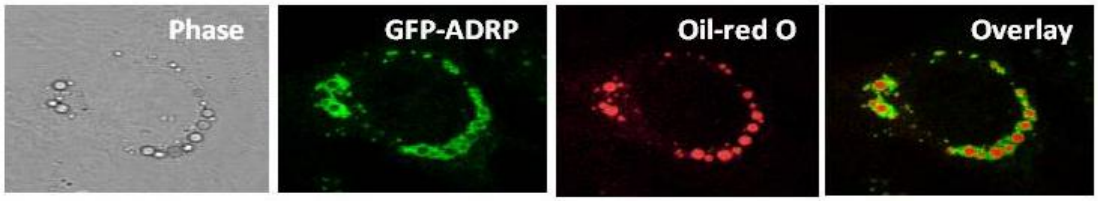

Fig.1 PLIN2 is a lipid droplet-associated protein. GFP-labeled PLIN2 (ADRP) was forcedly expressed in Swiss 3T3 cells. PLIN2 (ADRP) promotes intracellular lipid accumulation. A representative single cell is shown. Oil-red O staining shows the lipid droplets in the cytoplasm. GFP-labeled PLIN2 locates on the surface of lipid droplets. (Modified from ref. 8, with permission).

Physiological function of PLIN2 has not been fully elucidated as yet. PLIN2 stimulates uptake of long chain fatty acids [7]. We and others showed that forced expression of PLIN2 promotes lipid accumulation and lipid droplet formation in fibroblasts [8] (Fig.1), hepatic stellate cells [9] and macrophages [10]. PLIN2 acts on lipid packaging in concert with PLIN3 and PLIN4 [11]. We found that, in addition to PLIN2, PLIN3 is also specifically related to triacylglyceride (TAG) accumulation in macrophages [12, 13]. It has been proposed that fatty

acids, which are taken-up or de novo synthesized in the cells, are transported to endoplasmic reticulum (ER) lipid bilayer, and TAGs are synthesized and packaged [14]. PLIN2 is likely to be co-translated on the ER membrane in parallel with TAG synthesis. Thus, PLIN2 plays an important role for TAG accumulation and lipid droplet formation. Whatever the precise mechanism is, organisms are always ready for energy storage as a form of TAG during excess of nutrients. Actually, PLIN2 protein is rapidly degraded through proteasomal pathway when lipids are not available [15, 16]. Organisms might have acquired "depository genes" like PLIN2 during the process of evolution in order to survive the starvation, which could maximize the function of "thrifty genes" [17].

PLIN2 could be a putatve therapeutic target molecule: Despite the physiological importance of lipid storage in cells, excessive or inappropriate lipid accumulation causes not only obesity but also various metabolic derangements. Increased expression of PLIN2 has been proved in these pathological conditions. In atherosclerotic lesions, high expression of PLIN2 mRNA was demonstrated [18, 19]. Up-regulation of PLIN2 expression was observed in human fatty liver and rodent model of liver steatosis induced by high fat diet [20]. Adenovirus-mediated PLIN2 transgenic mice developed fatty liver and increased TAG content in the liver (Sonoda N et al, unpublished observation). It was shown that increased expression of PLIN2 protein in skeletal muscle negatively correlates with insulin sensitivity [21], although the opposite finding was also reported [22].

On the other hand, a number of reports demonstrated that reduced or deficient expression of PLIN2 decreases intracellular lipid accumulation, leading to amelioration of these pathological conditions in animal models. Genetic ablation of the PLIN2 gene reduced the number of lipid droplets in foam cells in atherosclerotic lesions in *ApoE*$^{-/-}$ mice and protected the mice against atherosclerosis [23]. *PLIN2*$^{-/-}$ mice showed reduced hepatic TAG content and were protected against fatty liver formation [24, 25]. Antisense oligonucleotide-mediated reduction of PLIN2 in the liver also ameliorated hepatic steatosis, hypertriglyceridemia and insulin resistance in *Lep*$^{ob/ob}$ as well as diet-induced obese mice [26].

Taken together, all these results indicate that PLIN2 plays a significant role not only in physiological but also in pathological accumulation of intracellular lipids. And these findings implicate that PLIN2 could be a possible therapeutic target molecule. Thus, the reduction of PLIN2 expression could be effective for treatment or prevention of conditions induced by excessive intracellular lipid accumulation such as fatty liver disease or atherosclerosis. Based on this presumption, we have been searching for putative synthetic or natural compounds which suppress the PLIN2 expression, and been finding out the mechanism of the action.

Regulatory mechanism of the PLIN2 gene by free fatty acids: Inflammo-metabolic connection: It is necessary to disclose the regulatory mechanisms of PLIN2 gene expression in order to achieve our purposes. Long-chain, but not short-chain, free fatty acids such as oleic acid effectively stimulate the expression of PLIN2 mRNA and its protein in NMuLi mouse liver cells (Fig.5A) [27]. We have characterized a 2.8kb region of the mouse PLIN2 gene promoter and identified several important *cis*-acting elements for regulation of the gene [15, 27].

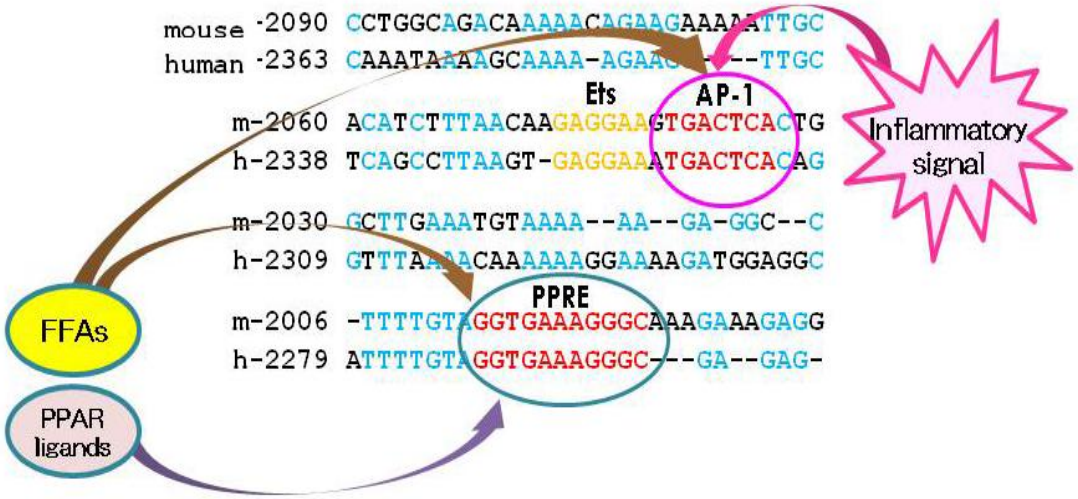

Fig.2 Inflammo-metabolic connection. Two important *cis*-acting elements, Ets/AP-1 composite element and PPAR-response element (PPRE), are located in close proximity, both in mouse and human PLIN2 gene promoter. AP-1 mediates inflammatory signals. PPRE mediates the effects of specific chemical PPAR ligands, while the effect of long-chain free fatty acids is mediated both by AP-1 and PPRE. This surmise is proved in Fig.3 and Fig.4.

We, as well as others, identified a peroxisome proliferator-activated receptor (PPAR) response element (PPRE), which responds to specific PPAR ligands as well as long-chain fatty acids, at 2kb up-stream of the transcriptional start site [15, 28]. Additionally, we identified an Ets/AP-1 composite element, which is located closely to the PPRE on its upstream (Fig.2). It is well known that AP-1 proteins are representative mediators of inflammatory stimulation. Sequence of the region encompassing these elements is well conserved between mouse and human genes (Fig.2). Although we demonstrated that transcription factors PU.1 and AP-1 conjointly bind to the Ets/AP-1 composite element and exert the maximal promoter activation in macrophages [15], only AP-1 recognizes the element in NMuLi liver cells because this cell

line does not express PU.1 or other Ets family transcription factors [27].

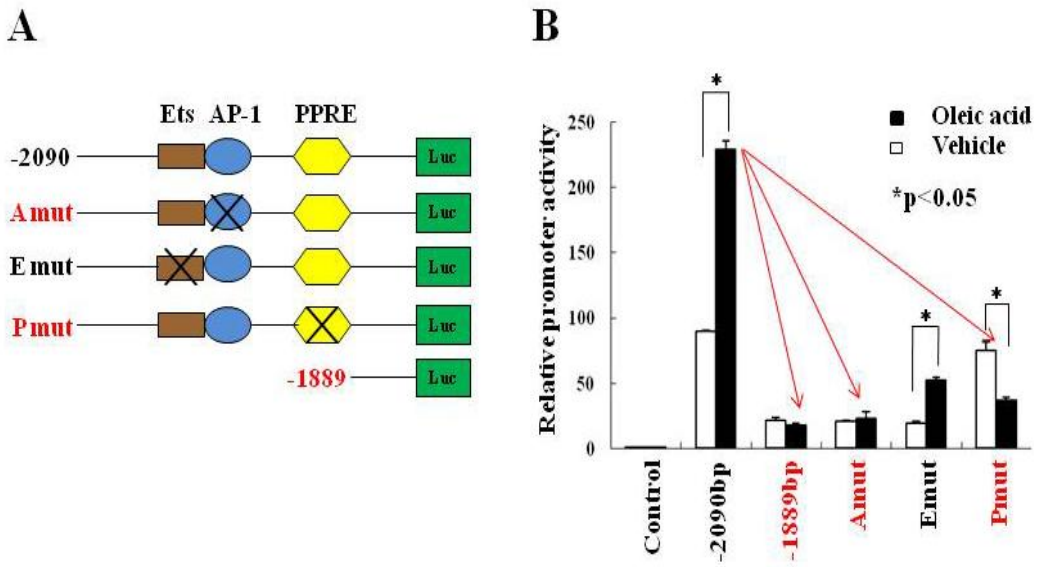

Fig. 3 Oleic acid requires both AP-1 and PPRE to stimulate the PLIN2 promoter activity. A) Schematic presentation of the PLIN2 promoter-driven luciferase constructs. X denotes the loss of function mutation artificially introduced. B) Oleic acid-induced increase in the promoter activity was diminished by deletion of all three functional elements, PPRE mutation or AP-1 mutation. Ets mutation showed decreased basal promoter activity but was still responsive to oleic acid; (Modified from ref. 27, with permission).

In order to characterize the function of these elements, we constructed a series of promoter-luciferase constructs (Fig.3A) and assessed promoter activity. Oleic acid stimulated the activity of wild type promoter (-2090bp), while -1889bp promoter, which lacks all three elements, and PPRE-mutated promoter (Pmut) failed to respond to oleic acid as expected (Fig.3B). Interestingly, stimulatory effect of oleic acid also diminished in the AP-1 mutation (Amut) (Fig.3B). This mode of action of oleic acid is totally different from that of specific PPAR ligands. Thus, synthetic ligands for PPARγ, δ, and α did stimulate the promoter activity of AP-1 mutation, while oleic acid did not (Fig.4). These results indicated that metabolic factors such as free fatty acids, activate PLIN2 expression through or in conjunction with an inflammatory mediator AP-1. We named this interaction as “inflammo-metabolic connection”, and PLIN2 is a target molecule of this connection. In addition to the function as a ligand for PPARs, long-chain fatty acid activates membrane receptors such as GPR40 and GPR120 (29, 30). It was also reported that oleic acid activates AP-1 activity through ERK1/2 activation (31).

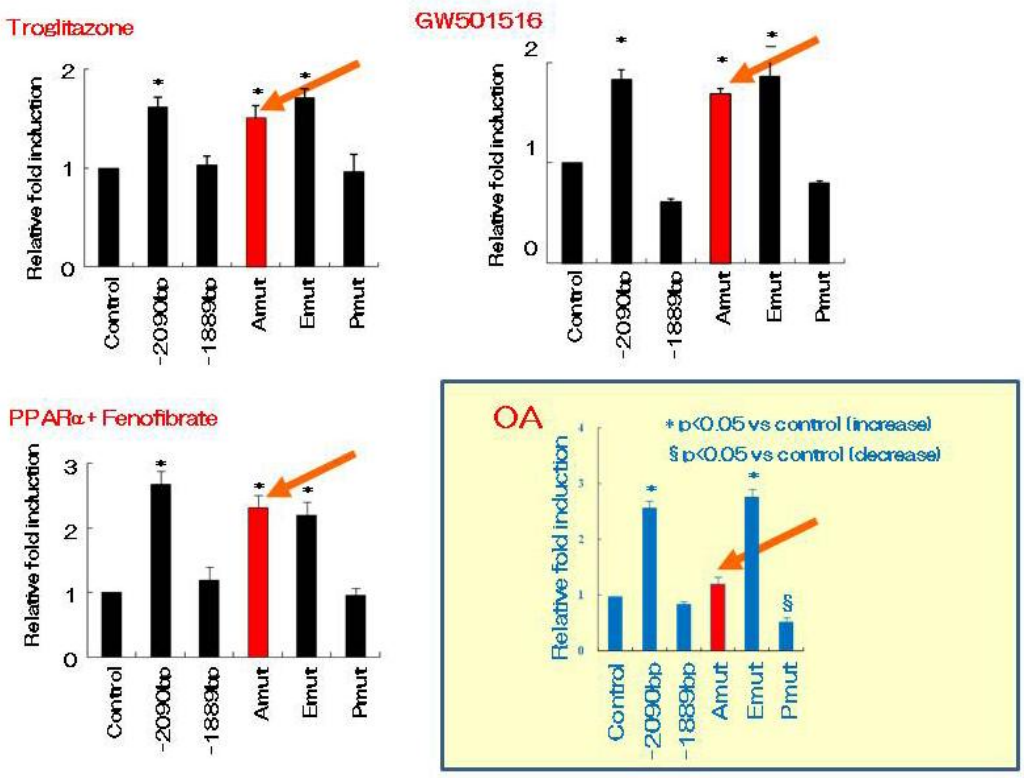

Fig. 4 Effect of specific PPAR ligands is mediated only by PPRE. Specific PPAR ligands, troglitazone (PPARγ), GW501516 (PPARδ) and fenofibrate(PPARα) could stimulate the AP-1-mutated promoter (Amut). Their stimulatory effects were only diminished in PPRE-mutated promoter (Pmut). Note that PPARα expression vector was co-transfected when tested the effect of PPARα ligand, because NMuLi cells do not express PPARα. These effects are totally different from oleic acid (OA) shown in the box. (Modified from ref. 27, with permission).

Pycnogenol^{R} suppresses lipid accumulation by facilitating the PLIN2 mRNA degradation in liver cells: We have been looking for putative compounds which suppress the PLIN2 expression. Although PLIN2 expression is suppressed by chemical compounds such as PI3 kinase inhibitors in macrophages (15) and NMuLi cells (Ikuyama S unpublished observation), this class of compounds is not suitable for clinical application. Therefore, we have been searching a candidate compound that fulfills our purpose among natural products. We focused on Pycnogenol^{R} (kindly provided by Horphag Research, Geneva, Switzerland), which is an extract from the bark of French maritime pine and has been used as a historical medicinal material for the treatment of scurvy, skin wound and sores (32). Various beneficial effects of French maritime pine bark extract have been disclosed to date, including the effects on cardiovascular disorders, diabetes and so on, and the underlying biological mechanisms

have also been investigated (33). Pycnogenol^{R} is a mixture of phenols, polyphenols, taxifolin and condensed flavonoids, and has a strong anti-oxidant effect (34). The Anti-inflammatory effects of Pycnogenol^{R}, such as effects on TNF-α-induced or NFκB-mediated gene expression, were also disclosed (35, 36). We expected that these biological properties were to be fit for suppression of PLIN2 expression considering the regulatory mechanism of the gene.

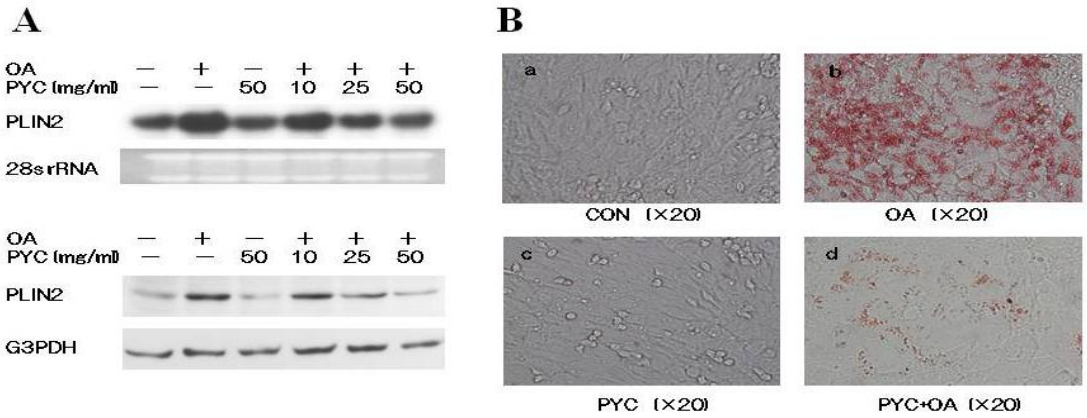

Fig. 5 Pycnogenol^{R} suppresses the PLIN2 expression and intracellular lipid accumulation in NMuLi liver cells. A) Pycnogenol^{R} (PYC) suppressed oleic acid induced increase in the PLIN2 mRNA (upper panel) and protein (lower panel). B) Pycnogenol^{R} suppressed oleic acid-induced lipid accumulation (Oil red O staining); (Modified from ref. 27, with permission).

As expected, we found that Pycnogenol^{R} suppressed oleic acid-induced PLIN2 mRNA expression in parallel with its protein level (Fig.5A). In concert with this effect, Pycnogenol^{R} suppressed oleic acid-induced lipid accumulation in NMuLi cells (Fig.5B). Considering possible biological effects of Pycnogenol^{R} as mentioned above and functional importance of AP-1 in the PLIN2 promoter, we presumed that Pycnogenol^{R} would suppress the PLIN2 gene expression at the transcriptional level. In contrast to our presumption, Pycnogenol^{R} did not inhibit the PLIN2 promoter activity (27). In addition, AP-1 binding activity in nuclear extracts prepared from Pycnogenol^{R}-treated cells was also not reduced (27). These results suggested that Pycnogenol^{R} exerted other action on the PLIN2 mRNA expression. Therefore, we assessed a half-life of PLIN2 mRNA in the presence or absence of Pycnogenol^{R}. As shown in Fig. 6, the half-life of PLIN2 mRNA was significantly reduced in the presence of Pycnogenol^{R}; an estimated half-life was 6 hours in the presence of Pycnogenol^{R} while 11 hours in the absence of Pycnogenol^{R}. Thus, Pycnogenol^{R} facilitates the PLIN2 mRNA degradation, reduction in PLIN2 protein, thus leading to suppression of lipid accumulation in the cells.

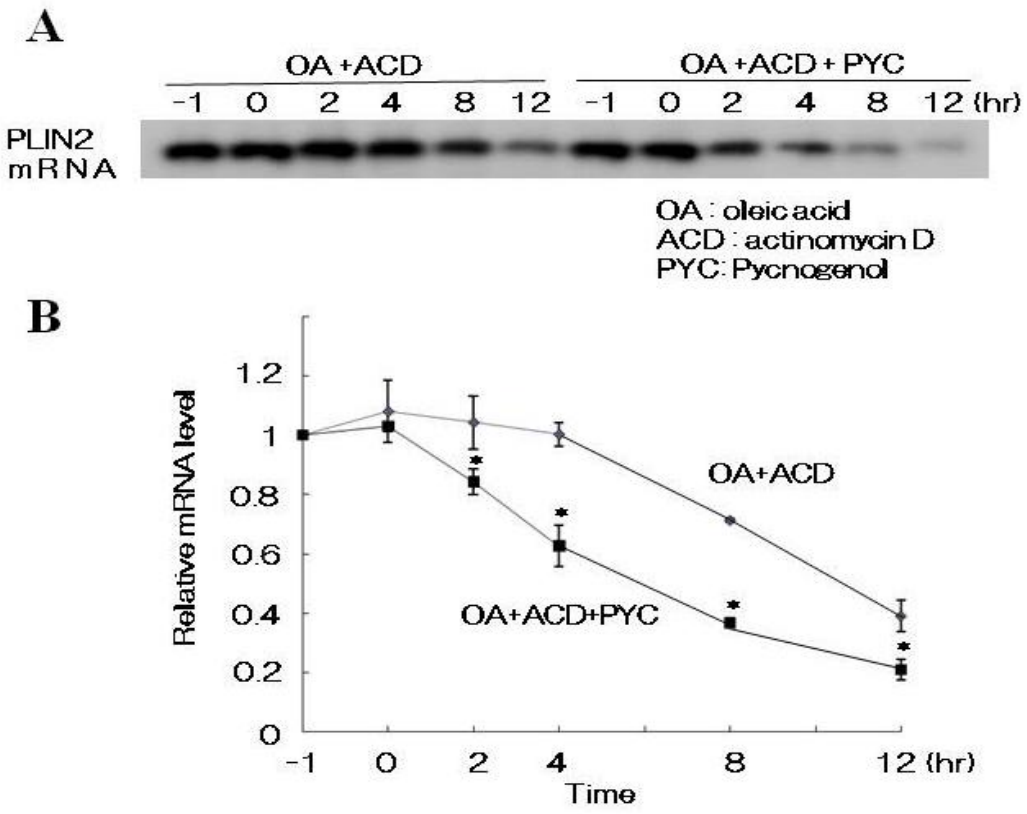

Fig. 6 Pycnogenol^{R} facilitates the PLIN2 mRNA degradation. PLIN2 mRNA level was assessed by Northern blot analysis (A) and Real-time PCR (B). (Modified from ref. 27, with permission).

Although dominant biological effects of Pycnogenol^{R} have been attributed to its antioxidant property (34), its suppressive effect of the PLIN2 expression could not be explained by the antioxidant action, and seems to be specific. Curcumin and astaxanthin, which are well-known antioxidant substances (37, 38), failed to suppress the oleic acid-stimulated PLIN2 mRNA expression (27). In addition, DNA binding of AP-1 and NFκb, typical redox-sensitive transcription factors, was not inhibited by Pycnogenol^{R} (12, 27). We presume that Pycnogenol^{R} could modify the activity of probable stabilizing or destabilizing factors of the PLIN2 mRNA, although this surmise has not been proved.

CONCLUSION:

In this review, we described "depository genes" such as PLIN family proteins that act on the lipid accumulation. In particular, molecular mechanism of the PLIN2 expression was elaborated based on the results of our investigation. And we raised the idea that PLIN2 could be a possible molecular target for prevention or therapy of the pathological conditions associated with excessive intracellular lipid accumulation, like fatty liver disease or atherosclerosis. In

this regard, the agents which suppress the PLIN2 expression should be guaranteed safety-wise. We have not yet found possible candidate chemical compounds which meet this purpose. Functional food like Pycnogenol^{R}, could be a promising natural compound although the clinical usefulness should be strictly verified.

Acknowledgements: The authors thank Horphag Research, Geneva, Switzerland, for generous gift of Pycnogenol^{R} powder.

Competing interests: The authors do not have any conflict of interest which should be disclosed.

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