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Naringin at a nutritional dose modulates expression of genes related to lipid metabolism and inflammation in liver of mice fed a high-fat diet

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Abstract. Epidemiological and clinical studies a role for flavanones (predominately found in citrus fruits) in the prevention of cardiovascular disease. Previously, we have shown that a nutritional dose of naringin exerts anti-atherogenic properties together with non HDL-cholesterol lowering effect in a murin model of dietary-induced hypercholesterolemia [1]. The goal of the present study was to explore possible molecular mechanisms of naringin at the hepatic level. To this end, we analyzed the hepatic transcriptome using a microarray approach in response to naringin supplementation (0.02%) in mice fed a high-fat high-cholesterol diet. Naringin was observed to increase hepatic lipid content (triglyceride and cholesterol) without significant liver dysfunction (ALAT and ASAT activities) or histopathological alterations. Naringin supplementation also significantly improved insulin sensitivity as evaluated by the HOMA index and nutrigenomics revealed that naringin modulated the expression of 1,766 genes. These genes encode proteins involved in different cellular processes, such as lipid metabolism, inflammation and insulin signaling. In conclusion, this study revealed that the hypolipemic and anti-atherogenic effects induced by a nutritional-level naringin supplementation in high-fat high-cholesterol diet could be related to changes in hepatic lipid metabolism and inflammatory response, revealing new *in vivo* targets of this flavanone.

Keywords: Naringin, liver, high-fat high-cholesterol diet, hypercholesterolemic mouse model, transcriptomic

1. Introduction

Epidemiological studies have established the protective role of fruit and vegetable consumption against disease development, particularly with regard to cardiovascular diseases (CVD) [2, 3]. It has long been considered that this beneficial effect is attributable to the antioxidant vitamins and carotenoids present in these foods. However, as shown by several

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meta-analysis, large intervention studies with these antioxidants brought disappointing results [4–7] and recently much attention has been paid to polyphenols, bioactive compounds specifically provided by vegetal foodstuffs that may be involved in healthy effects of fruits and vegetables. This is substantiated by epidemiological studies consistently showing a protective effect of some polyphenol-rich foods (fruits, tea, wine, and cocoa or chocolate) against CVD [8–11]. Furthermore numerous studies conducted on animal models with isolated flavonoids [12, 13] and on humans with flavonoid-rich foods have shown an improvement in some systemic and functional biomarkers related to CVD risk [14–16].

Among flavonoids, flavanones are found at high concentrations almost exclusively in citrus fruit and citrus-based products [17]. The main flavanones are naringin (NAR) in grapefruit and hesperidin in orange [19]. Some recent findings emerging either from epidemiological and clinical studies have driven interest onto the impact of flavanones on CVD prevention. Indeed, a recent prospective study showed that the dietary intake of flavanones was associated with a lower risk of death due to coronary heart disease and stroke (CHD) [10, 18]. Furthermore, a daily intake of flavanones ranging from 50 to 73 mg [17] has been shown to reduce the rate of CHD mortality by 15%. A possible role for citrus flavanones in cardiovascular protection was strengthened by recent clinical trials showing their beneficial effects on blood pressure and microvascular reactivity [20, 21]. In animal experiments, naringin (NAR) and naringenin, the aglycone form of NAR, have been shown to exhibit lipid lowering effects, to reduce plasma markers of endothelial dysfunction and to improve insulin sensitivity when added to high-fat high-cholesterol (HF-HC) or cholesterol-rich diets [22–24]. The improvement in hepatic and plasma lipid profiles induced by NAR or naringenin consumption has been related to effects on both cholesterol and fatty acid metabolism. In fact, NAR has been shown to reduce the activity of two hepatic enzymes involved in cholesterol synthesis and storage: 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) and acylcholesterol acyltransferase (ACAT) respectively [25]. In addition, naringenin also up-regulates the expression of genes involved in fatty acid oxidation and down-regulated those related to lipogenesis in the liver [23, 26]. Such effects on hepatic lipid metabolism have been associated with a reduction in atherogenic lipoprotein secretion and in plasma lipid concentrations. These findings highlighted some interesting properties of NAR that could contribute to its anti-atherogenic effects [22–27]. However the physiological relevance of these studies must be interpreted carefully, as most of them have been carried out using supra-nutritional and pharmacological doses of NAR or of naringenin.

Previously we have shown that NAR supplementation at a nutritional dose (0.02%) reduced dietary-induced atherosclerosis in wild type mice fed a HF-HC diet [1]. This protective effect was associated with a reduction in plasma non HDL-cholesterol and with an improvement of the atherogenic index (total cholesterol/HDL ratio). Furthermore, as shown by a FPLC lipoprotein profile analysis, NAR particularly affected plasma VLDL cholesterol. The objective of the present work was to further elucidate the mechanisms underlying the effects of NAR on lipid metabolism, focusing at the hepatic level. In the present work we examined effects of NAR in mice fed a diet rich in cholesterol, saturated fat and sucrose, a diet known to induce insulin resistance. We aimed to assess to what extent and by which mechanisms dietary NAR is able to modulate alterations of the hepatic metabolism induced by this atherogenic and diabetogenic diet using transcriptomic approach.

2. Materials and methods

2.1. Mice and diets

We used a dietary-induced model of atherosclerosis, namely wild-type mice (C57BL/6J) (Charles River laboratories, L'Arbresle, France) on a HF-HC diet [2–29]. Male mice were housed in a temperature-controlled ($22 \pm 0.8^{\circ}$C) pathogen-free environment on a 12-h light-dark cycle and with free access to food and water. All animal experiments were performed according to the French Ministry of Agriculture section of Health and Animal Protection (approval number 33-04476), the Institutional Ethics Committee of the INRA (decree number 87-848) and approved by Valorization Unit of the University of Bordeaux 2 under the agreement number: R-45GRETA-F1-04. All procedures were carried out in compliance with standards for use of laboratory animals. Mice were fed a standard breeding diet A03 (Safe, Epinay-sur-Orge, France) before the

beginning of the experiment. After a three week adaptation period, 8-week-old mice were randomly divided into two groups ($n = 15$ per group) and fed *ad libitum* for 18 weeks a HF-HC diet (15% fat, 1.25% cholesterol, 0.5% cholic acid in Cocoa Butter Diet with 75% Purina Mouse Chow #5015, TD.90221, Harlan Teklad, USA) supplemented or not with 0.02% (w/w) of NAR (Sigma, Saint-Quentin L'Abresles, France). The dose used in the present study corresponds to a human equivalent dose of 100 mg NAR, which according to the UK Food Standard Agency, is the amount of NAR provided by the consumption of 1.5 servings of grapefruit juice or by one fruit. No significant difference in weight between the two groups was observed. At the end of the 8-week period, mice were fasted for 4 h, and blood was drawn from retro-orbital veins in the morning in tubes containing EDTA. Plasma samples were obtained by centrifugation at 2400 *g* for 20 min at 4°C, separated and divided into aliquots, then stored frozen (–80°C) until analyzed. At the end of the 18-week period, non-fasted mice were sacrificed under pentobarbital anesthesia. The organs were washed with physiologic saline solution maintained at 37°C by direct injection in the heart's left ventricle.

2.2. Liver lipids, histological examination and plasma hepatic enzymes

To establish the hepatic lipid content, livers from non-fasted mice of each study group were harvested, minced and extracted according to Folch [30], then analyzed for total cholesterol (TC) and triglycerides (TG) using commercial enzymatic kits (BioMérieux, Marcy l'Etoile, France). Some liver samples were also fixed in 10% buffered formalin and embedded in paraffin, sliced and stained with hematoxylin/eosin (Sigma, Saint-Quentin Fallavier, France) to evaluate liver structure. Finally, fasted plasma levels of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were determined using an automated analyzer (Modular, Roche Diagnostics, Meylan, France).

2.3. Plasma glucose and insulin

Glycemia was determined according to the manufacturer (BioMérieux, Marcy l'Etoile, France). Mouse insulin was assayed in duplicate in the same test battery by using a quantitative sandwich enzyme immunoassay technique (R&D Systems, Lille, France). Intra- and inter-assay coefficients of variation (CV) were <5% and <9%, respectively, with a detection limit of 80 pg/mL insulin. Homeostatic model assessment for insulin resistance (HOMA) was calculated as a surrogate for insulin resistance, as described elsewhere (Mather, 2009).

2.4. Microarray analysis

A fraction of the liver from both batches of mice ($n = 4$ treated mice and $n = 4$ untreated mice) was collected and immediately put into RNA*later* (Sigma, France). Transcriptomic analysis was performed on livers using pangenomic oligonucleotide Op Arrays$^{(TM)}$ (Operon, Cologne, Germany).

2.4.1. RNA extraction and fluorescent cDNA labeling

Liver samples were homogenized in lysis buffer for total RNA extraction using the SV Total RNA Isolation System (Promega, Madison, WI, USA) as recommended by the manufacturer. Total RNAs were extracted from 8 livers: 4 from mice fed control diet and 4 from mice fed the diet supplemented with NAR and the quality of total RNA was monitored in 1% agarose gel subjected to electrophoresis. With the ChipShot™ Direct Labeling System kit (Promega), cDNAs were obtained from 5 μg of total RNA with 1 μL of random primers, 1 μL of oligo(dT), and labeling was performed with Cy™3- or Cy™5-dCTP (Amersham Biosciences, Orsay, France). The labeled cDNAs were purified by application to an equilibrated filter cartridge using the ChipShot™ Membrane Clean-Up System (Promega). Quantities and labeling efficiencies of labeled cDNAs were determined by measuring the absorbance at 260, 550 and 650 nm using a ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.4.2. Hybridization

Hybridization was carried out on the Operon mouse microarray (Operon). Array-Ready OligoSet Mouse Genome version 4.0 contains 35,852 longmer probes representing approximately 24,000 genes. Eight microarrays were used for a total of 4 independent comparisons. Hybridization was carried out in a Ventana hybridization system (Ventana Medical Systems, S.A, Illkirch, France) at 42°C for 8 h. Slides were

subsequently washed twice in 2X saline sodium citrate (SSC) and 0.1X SSC at room temperature. The buffer remaining on the slide was removed by rapid centrifugation (4000 *g*, 15 sec). The fluorescence intensity was scanned using an Agilent Micro Array Scanner G2505B (Agilent Technologies, Inc., Santa Clara, CA, USA).

2.4.3. Image and data analysis

Image and data analysis were performed as previously described [14]. The signal and background intensity values for each spot in both channels were obtained using ImaGene 6.0 software (Biodiscovery, Inc, Proteigene, Saint-Marcel, France). Data were filtered using the ImaGene "empty spot" option, which automatically flags low-expressed and missing spots to remove them from the analyses. After base-2 logarithm transformation, data were corrected for systemic dye bias by Lowess normalization using GeneSight 4.1 software (BioDiscovery, Inc, Proteigene). Ratios were then filtered in accordance with their variability among the 4 comparisons, and genes with high variability were removed from the analysis. Statistical analyses were performed using the free R 2.1 software (http://www.r-project.org). The log ratio between NAR-supplemented and control samples was analyzed with Student's *t* tests to detect differentially expressed genes in the two nutritional conditions, and probability values were adjusted using a Bonferroni correction for multiple testing at 1% to eliminate false positives. Genes selected by these criteria are referred to as "differentially expressed genes".

To validate data of microarray study, real-time quantitative PCR was performed on the same RNA for a subset of genes identified as differentially expressed (RAPGEF1, FABP4, DGAT2, LCAT). High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to reverse transcribe RNA to cDNA. The primers were identified using Primer Express software (Applied Biosystems, Foster City, CA, USA). The qRT-PCR was carried out on Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the Power SYBR®Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA). After initial denaturation of 10 min at 95°C, a two-step cycling conditions were: 15 seconds denaturation at 95°C and annealing/extension at 60°C for 30 seconds, cycled 40 times. The expression levels were calculated using the $\Delta\Delta$Ct method.

Differentially expressed genes were classified according to their role(s) in Gene Ontology cellular processes using GOstat software (http://gostat.wehi.edu.au/). Furthermore, differentially expressed genes were placed into cellular pathways using KEGG (Kyoto Encyclopedia of Genes and Genomes) database (http://www.genome.jp/kegg/tool/map_pathway2.html) and Pathway Miner analysis software (BioRag: Bio Resource for Array Genes; http://www.biorag.org), which combines the pathway analysis capabilities of three different tools (KEGG, GenMAPP and Biocarta) through a Fisher Exact test [31]. MetaCore pathway analysis was also used to determine the transcription factors affected by NAR supplementation. Finally, Gene Ontology (GO) analysis (http://www.geneontology.org) was also performed to describe the associated biological processes of the differentially expressed genes overall.

2.5. Statistical analyses

A one-way ANOVA coupled with a multiple comparison test was used to compare effects of diets on biochemical parameters. Values are expressed as means ± SEM. $P < 0.05$ was taken to imply statistical significance.

3. Results

3.1. NAR induces changes in hepatic lipid levels of mice fed HF-HC diet

Compared to untreated mice fed HF-HC diet, NAR supplementation significantly increased both TC and TG liver contents expressed either per *g* of tissue (+43% and +63% respectively, Table 1) or per *mg* of hepatic protein content (data not shown). Nevertheless, NAR supplementation did not cause discernable hepatocyte damage, as evaluated by measurements of

Table 1
Hepatic lipid contents after an 18-week supplementation period with 0.02% naringin in wild-type mice fed a high fat-high cholesterol diet

	Control	NAR
Liver triglycerides (mg/g)	21.0 ± 6.2	34.3 ± 2.2*
Liver cholesterol (mg/g)	2.3 ± 0.5	3.3 ± 0.4*

Values are means ± SEM ($n = 15$ per group), $^*P < 0.03$ versus control.

Table 2
Insulin sensitivity after an 18-week supplementation period with 0.02% naringin in wild-type mice fed a high fat-high cholesterol diet

	Control	NAR
Glycaemia (mg/dL)	86 ± 12	105 ± 24
Insulinemia (ng/mL)	0.71 ± 0.35	0.18 ± 0.04***
HOMA index	10.91 ± 1.95	4.34 ± 0.46***

plasma ALAT (19 ± 4 vs 20 ± 6 IU/L, $P > 0.05$) and ASAT activities (70 ± 5 vs 68 ± 5 IU/L, $P > 0.05$) and by histological examination (data not shown) of the liver.

3.2. NAR improves insulin sensitivity in mice fed HF-HC diet

Mice fed HF-HC diet supplemented with 0.02% NAR presented normalized insulinemia, known to be elevated by the high fat diet, without modification in glycaemia (Table 2). Calculation of HOMA index showed a significant improvement in insulin-resistance (Table 2).

3.3. NAR modulates expression of genes in liver of mice fed HF-HC diet

In the transcriptomic analysis performed in the present study, 1,766 genes were differentially expressed, with an average-fold change of 1.52, following NAR supplementation compared to controls (Student *t*-tests using a Bonferroni correction at 0.01%). Among these differentially expressed genes, 1,036 genes were up-regulated and 730 were down-regulated (supplementary Table 1). Real-time quantitative PCR was performed using same RNA samples on 4 genes identified as differentially expressed in the liver and, for the majority of genes studied; the expression values were concordant with data from microarray analysis (supplementary Figure 1).

To decipher biological processes affected by NAR supplementation, the list of differentially expressed genes was subjected to gene-annotation enrichment analysis using GOstat bioinformatics resources. The list of GO groups of biological processes, which are highly represented, is presented in the supplementary Table 2. This analysis revealed that differentially expressed genes are implicated in developmental process, cell differentiation or anatomical structure. Classification of the differentially expressed genes using KEGG tool showed that several genes related to lipid metabolism were altered by NAR supplementation (i.e. fatty acid metabolism, arachidonic and linoleic acid metabolism) (Fig. 1). Among the signalling pathways, it appeared that insulin and Mitogen-Activated Protein Kinase (MAPK) signalling contain highest number of genes which expression is affected by NAR. Finally, expression of genes involved in cytokine-cytokine receptor interaction was also modulated. Further analysis by using MetaCore, allowed us to identify HNF4α ($P = 1.2\text{E}{-}300$) as well as NF-κB ($P = 3.17\text{E}^{-186}$) as potential transcription factors involved in the observed alterations of gene expression.

4. Discussion

In this study we hypothesized that a change in plasma lipid concentrations and a subsequent reduction in the development of atherosclerosis (observed in a previous work following a nutritional NAR supplementation in wild-type mice on a HF-HC diet) [1] may originate from the effects of NAR on hepatic lipid metabolism. However, we observed an enhanced hepatic lipid accumulation in NAR supplemented mice, although the integrity of livers in the NAR-fed mice was not compromised, as revealed by the histological examination and the lack of changes in the plasma ALAT and ASAT activities. Thus, we investigated functional changes associated with NAR supplementation and determined at the transcriptional level its molecular target candidates in diabetogenic mice (fed HF-HC sucrose-rich diet).

4.1. NAR modulates expression of genes involved in lipid biosynthesis and β-oxidation

We observed that NAR supplementation was associated with an increase in hepatic TC and TG levels. Transcriptomic analysis of the liver revealed an up-regulation of the expression of genes coding ATP-citrate lyase (ACLY × 1.53) which is involved in *de novo* lipogenesis as donor of acetyl-CoA for the acetyl-CoA carboxylase reaction [32]. We also found that NAR supplementation led to the up-regulation of mRNA encoding two isoforms of acyl-coenzyme A:diacylglycerol transferases (DGAT1 × 1.35, DGAT2 × 1.75) which are involved

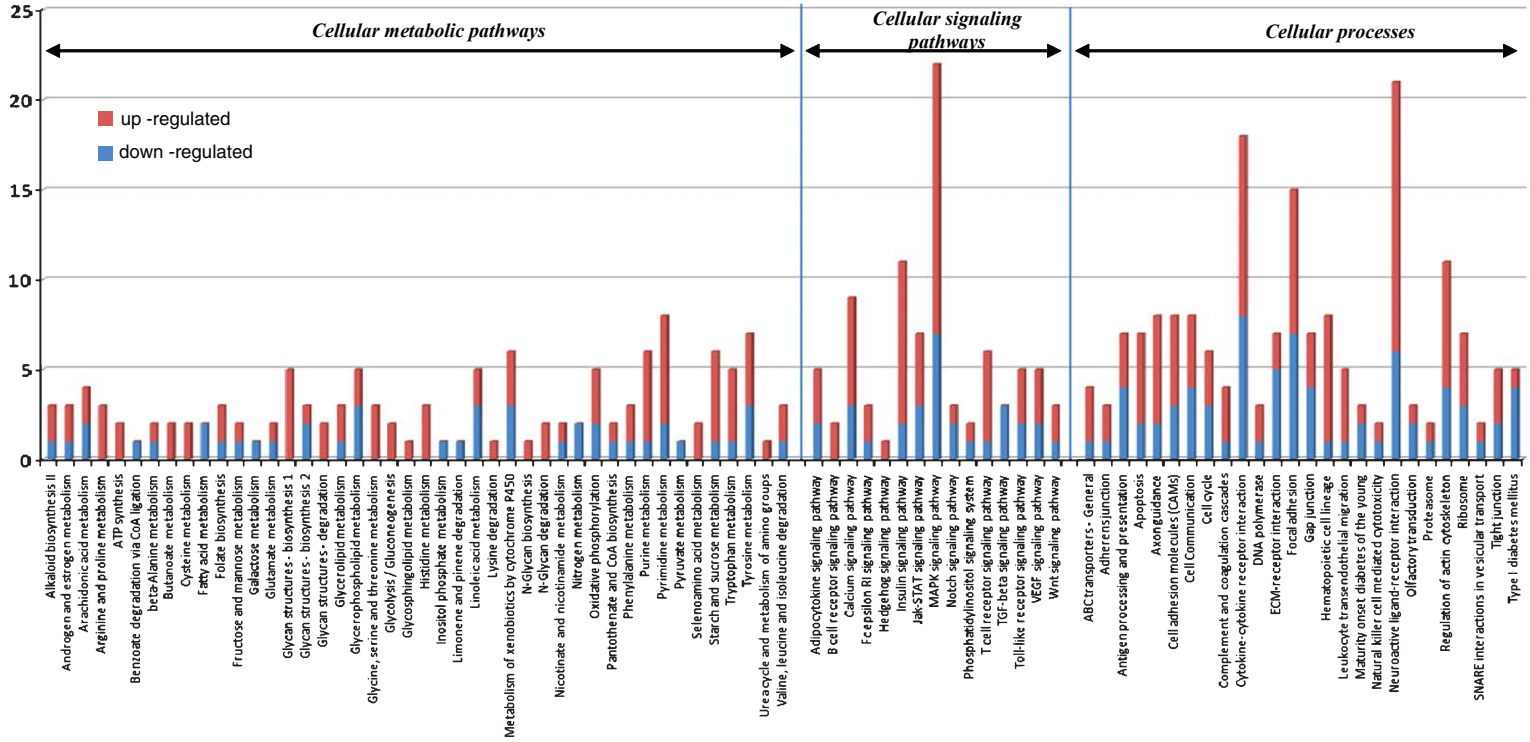

Fig. 1. Pathways affected by an 18-week supplementation period with 0.02% naringin in the livers of mice fed a high fat-high cholesterol diet. Differentially expressed genes were analyzed across pathways using KEGG.

in the final step of TG synthesis [33]. In addtion, the expression of the major lipid droplet-associated protein expressed in hepatocytes, adipose differentiation-related protein (ADRP) or adipophilin (ADFP), increased after NAR feeding (ADFP × 1.85). At the surface of lipid droplets, the cellular organelles enclosing neutral lipid depots (triglycerides and cholesterol ester), ADFP protects lipid droplets from lipolysis. The direct consequence is a decrease in the release of lipids so decreasing their availability for lipoprotein synthesis. Consistent with this, mRNA levels encoding the lysosomal acid lipase A, an enzyme known to break down lipids such as cholesteryl esters and triglycerides, were reduced in the liver of NAR-supplemented mice (Lipa × 0.76). Furthermore, the mRNA levels of the peroxisomal β-oxidation key enzyme acyl-CoA oxydase (Acox × 0.77), which is known to be induced by high-fat diet, were also down-regulated in the liver of NAR-fed mice. These results suggest a lower degradation rate for lipids in the liver of those animals, supporting the increased hepatic TG and cholesterol levels in the NAR-fed animals. Moreover, we showed a decrease in plasma VLDL levels in our model [1]. A lack of available neutral lipids in hepatocytes could be the main pathway for reduction in plasma VLDL levels. Indeed, this may lead to a degradation of apoB [34] and a subsequent decrease in VLDL assembly [35] as we did not observe any change in microsomal transfer protein expression in contrast to published *in vitro* data [36]. Interestingly, adfp–/– mice had a VLDL secretion rate significantly higher compared to adfp+/+ mice [37].

Overall, while major factors involved in lipid biosynthesis and storage were up-regulated, mRNA levels for proteins involved in pathways related to lipid utilization were down-regulated. These metabolic pathways are known to lead to the development of hepatic steatosis. Indeed, hepatic lipid accumulation is a hallmark of non-alcoholic fatty liver disease and is closely associated with insulin resistance in both rodents and humans [38], even in lean subjects with normal glucose tolerance [39]. In most cases, decreasing liver lipid accumulation was associated with improved insulin sensitivity. This is in agreement with Mulvihill et al. who used a pharmacological dose of naringenin, which is more bioavailable than NAR [24]. On the other hand, a recent study [40] showed increased insulin sensitivity and corrected insulin resistance in obese mice without affecting hepatic steatosis, indicating a clear dissociation between hepatic steatosis and insulin resistance, as was the case in our study. It has previously been reported that oil rich in polyphenols, such as olive oil, increased hepatic fat content [41] without impairment of insulin sensitivity. Moreover the retention of lipids in the liver is not necessarily damaging [42] as we demonstrated in livers from NAR-supplemented mice on HF-HC diet. Rather, the accumulation of lipids is perhaps indicative of a protective effect against extensive hepatic fibrosis and impaired hepatocellular function as suggested by Matsuzawa et al. [43]. Previously published works suggest an increase in oxidative stress as an important factor contributing to liver steatosis induced by a high-fat high-sucrose diet [44]. In our experiment, mice were fed for 18 weeks a high saturated fat (15% Cocoa Butter) sucrose rich diet. The limited extent of the lipid peroxidation in our experimental conditions may be due to the use of saturated fat, along with an adequate vitamin E supply and the absence of alternations in hepatic enzymes. This does not totally exclude the occurrence of oxidative stress but suggests the preponderal role of direct action of SFA and sucrose on insulin resistance induction.

4.2. *NAR exerts insulin-like activity at molecular level*

An alternative explanation of the reduced VLDL fraction in plasma could be related to the effect of NAR on the insulin-signaling pathway. From *in vivo* [23] and *in vitro* studies [36, 45–47], naringenin has been shown to exhibit insulin-like activity. *In vitro,* naringenin reduced apoB secretion from HepG2 cells by activating both the phosphoinositol 3-kinase (PI3K) pathway and the MAP kinase pathway. One of the most relevant results from our transcriptomic study was the change found in the mRNA levels of proteins related to the insulin-signalling pathway. Indeed, in the present study, AKT1 substrate 1 (Akt1s1) gene expression was up-regulated 5.34-fold in mice fed the HF-HC diet supplemented with NAR when compared to HF-HC control. This gene, also known as PRAS40, has been recently identified as a regulator and substrate of both mTORC1 and PKB/Akt, playing a major role as a novel physiological target of *in vivo* insulin action and a potential marker for insulin resistance [47]. Thus, phosphorylation of PRAS40 is increased by insulin in target tissues, and this response is reduced under conditions of high fat diet-induced insulin resistance.

Furthermore, another highly differentially expressed genes after NAR supplementation was Rap guanine nucleotide exchange factor 1 (Rapgef1 × 1.98), a gene encoding for a component of the CAP/TC10 pathway involved in insulin signalling [48]. Defects in the RAPGEF1 protein are thought to contribute to insulin resistance and type 2 diabetes [49].

We also observed that NAR significantly affected the expression of the MAPK signaling pathway, which mediates part of the effects of insulin. Overall, these results argue for an insulin-like effect of NAR in the liver of HF-HC-fed mice, which is supported by the improvement in the HOMA index that indicates an elevation of insulin sensitivity in response to NAR supplementation. While the full signalling pathway used by NAR to exert its insulin-like activity is not fully known, our data strongly suggest the involvement of the PRAS-PKB/Akt and mTORC1 systems as well as CAP/TC10 and also MAPK pathway in such functions. Such an insulin-like effect of NAR could in turn reduce apoB-containing lipoprotein secretion and explain the decrease in plasma VLDL observed in our previous work [1]. Moreover, bioinformatic analysis of nutrigenomic data, using Metacore software, allowed us to identify different transcription factors whos activity could be regulated by NAR and in consequence regulate expression of identified differentially expressed genes. Among these transcription factors, HNF4α has been identified as a central transcription factor regulating hepatic genes expression following NAR supplementation ($P < 0.05$). Interestingly, HNF4α regulates genes involved in glucose transport and metabolism and has been associated with diabetes onset, supporting an additional influence of NAR on carbohydrate metabolism [50].

Regarding potential molecular mechanisms of naringenin, it has been previously reported that this flavanone could induce the activity of both PPARα and PPARγ through PGC1A activation [51, 52]. However, our bioinformatic analysis performed on the identified differentially expressed genes did not revealed PPARs as a potential transcription factors modulated by naringin in mice. The discrepancy between these two observations could be explained by the fact that the previous cited studies have been performed *in vitro*, using naringenin aglycone at concentrations as high as 200μM. These experimental conditions do not take into account the low absorption of naringenin observed *in vivo* (with circulating levels in the microM range), nor the high conjugation activity, occurring both in the intestine and liver, that generates conjugated metabolites of naringenin which constitute the major forms of naringenin in plasma [53, 54].

4.3. Modulation of inflammatory gene expression by NAR

Further analysis of microarray data revealed modulation of inflammatory gene expression by NAR. It has been reported that cholesterol intake by apoE Leiden mice switches the liver from a resilient state to an inflammatory, pro-atherosclerotic state and modulates expression of pro-inflammatory and pro-atherogenic genes [55]. In our study, bioinformatic analysis identified NF-κB as a potential transcription factor which activity could be regulated by NAR and, in consequence, modulates expression of NF-κB dependant genes. We observed an up-regulation of genes encoding sphingosine-1-phosphate receptor (Edg8 × 2.34) involved in the decrease in T-cell-mediated immune responses [56], serglycin (Srgn × 2.20) known to be important for the retention of key inflammatory mediators inside storage granules and secretory vesicles [57], Ca^{2+}/calmodulin-dependent protein kinase (Camk1d × 1.95) which can inhibit neutrophil differentiation and maturation [58], A20 (or tumor necrosis factor alpha induced protein 3, Tnip2 × 2.66) and protein phosphatase 1A (Ppm1a × 1.89) acting as a IKKβ phosphatase [56–60], both of which are known to turn off inflammation signaling by inhibition of NFκB signalling a key regulator of inflammation and immune response [61]. Overall, changes in expression of these genes suggest a potential anti-inflammatory action of NAR.

Further effects of NAR on inflammatory processes were found related to the enzyme platelet-activating factor acetylhydrolase, also known as lipoprotein associated phospholipase A2 (Lp-PLA_2 × 0.76). This enzyme cleaves oxidized phosphatidylcholine molecules produced during the oxidation of LDL, generating the soluble proinflammatory and proapoptotic lipid mediators. It has previously been shown that Lp-PLA_2, down-regulated in our study, plays a role in the development of atherosclerotic necrotic cores [62]. Moreover, the liver was causatively related to lesion initiation in the aorta because pro-atherogenic genes (genes encoding candidate inflammatory components reportedly or putatively involved in early lesion formation) were found to be up-regulated by dietary lipids

[55]. Consequently, the modulation of expression of genes in the liver of mice fed HF-HC diet supplemented by NAR could be related to the reduced plaque progression observed in a previous study [1].

In summary, in the present work we show that NAR is able to counteract, at least in part, the induction of insulin resistance caused by a HF-HC sucrose-rich diet. However, NAR did not prevent diet induced lipid accumulation in the liver. Rather, transcriptomic-based analysis of the liver of NAR supplemented mice on HF-HC sucrose-rich diet revealed changes in the expression of genes that are modulated by consumption of this flavanone at nutritionally relevant dose. Firstly, several aspects of lipid metabolism and lipoprotein assembly and secretion were affected by NAR leading to hepatic lipid accumulation. Compared to previously published results, obtained under normal diet fed mice or hepatocytes cultured in standard conditions, our observations were made under conditions of enhanced FAs and cholesterol influx to the liver. This is of particular interest because it indicates the specific response to NAR in response to a nutritionally relevant metabolic challenge. As such, it may be hypothesized that the insulin-like potential of NAR could, at least in part, explain its action on hepatic lipid metabolism at the molecular level. Secondly, our transcriptomic data indicate the potential for the anti-inflammatory action of NAR. Thus, increased lipid retention in the liver with, the reduced plasma levels in non-HDL lipoproteins and the reduction in HF-HC diet-induced inflammation may help explain the antiatherogenic effect of NAR, which has previously been reported in the same animals.

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Supplementary material

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