EFFECTS OF MULBERRY HEARTWOOD EXTRACT ON GENES RELATED TO LIPID METABOLISM

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ABSTRACT

Scientific evidence exists that extracts from different parts of *Morus nigra* (mulberry tree), mainly fruits, leaves and root bark, exhibit various beneficial effects, such as antiviral, antihyperglycemic, antiatherogenic and hypotensive activity. Although it is known that the heartwood has a specific phytochemical composition, the biological effects of this part of the plant are not yet clarified. This study examines the effect of 40% ethanol infusion from mulberry heartwood on the expression of three genes related to lipid metabolism: CCAAT/enhancer binding protein- α (C/EBP α) gene, adipogenic transcriptional factors peroxisome proliferator-activated receptor gamma (PPAR γ) and fatty acid-binding protein (aP2) genes in adipocytes. Results obtained present that treatment with two concentrations of mulberry ethanol extract decreased expression of aP2 messenger RNA as compared to controls. Based on these results we concluded that heartwood mulberry extract had beneficial effects on lipid metabolism and could be a potential source for search of active compounds for treatment of metabolic disorders related to adipose tissue metabolism.

Keywords: *Morus nigra*, heartwood extract, adipocytes, gene expression, lipid metabolism

INTRODUCTION

In the last few decades medicinal plants are on the focus of scientific studies as potential sources of new active compounds with beneficial biological effects. Scientific hypothesis often generate from the folk knowledge about the usage of the plants. *Morus nigra* L. (mulberry tree) is traditionally applied by the folk medicine for various complaints. According to some authors extracts from mulberry leaves exhib-

it diuretic, hypoglycemic, and hypotensive activities, whereas the fruits and root bark are known to possess antiinflammatory, antitussive, and antipyretic properties (5,20,30,33). Along with the knowledge about the medicinal properties of the plant, mulberry is known to have also other traditional applications – its heartwood is used as a material for barrels manufactured for storage of alcoholic beverages and respectively for their aging. Although, the ethanol extracts of mulberry heartwood have specific phytochemical composition (8), to our knowledge their biological effects still remain unrevealed.

Several studies report that extracts from mulberry leaves and fruits have a potential to reduce significantly blood cholesterol, triglycerides, VLDL and LDL levels (1,25,34). These reports allow us to suggest that the mulberry tree could be potentially considered as a source of remedies for treatment of obesity and diabetes.

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Obesity is associated with adipocyte differentiation and fat accumulation (16). The molecular basis of adipocyte differentiation is a subtle coordinated work of the adipogenic transcriptional factors such as CCAAT/enhancer binding protein- α (C/EBP α) and nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR γ). They play a key role for the expression of adipocyte-specific genes, such as adipocyte fatty acid-binding protein (aP2) which is one of the genes involved in lipid accumulation into the cells (7). The two transcriptional factors alone or in cooperation regulate the expression of genes for synthesis of enzymes involved in triacylglycerol synthesis, glucose transporters, acylCoA synthetases (ACSS) and the fatty acid binding proteins (FABPs) family. The adipocyte cytosolic aP2, which is a member of FABPs, binds with high affinity to hydrophobic ligands such as saturated and unsaturated long-chain fatty acids and eicosanoids such as hydroxyeicosatetraenoic acid, leukotrienes and prostaglandins 2 (3,13,19).

The aim of this study was to determine the ability of *M. nigra* (MN) heartwood ethanol extract to modulate the expression of genes involved in lipid metabolism such as C/EBP α , PPAR γ and aP2 in a differentiated 3T3-L1 cell line.

MATERIALS AND METHODS

Heartwood processing and extract preparation

Heartwood samples were subjected to fumigation following the popular technology for aging of beverages: the wooden chips were boiled for 10 minutes and then saturated with cold water for 24 hours. Finally, the material was dried for 15 minutes at 150–190°C. Ethanol infusion from *Morus nigra* heartwood was prepared following the traditional recipe for coloring high alcoholic beverages: 2g dried material from heartwood was placed in 1L 40% ethanol for 40 days.

Cell culture differentiation and treatment

Mouse 3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza), supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin mixture to final concentration of 100U/ml each at 37°C in a humidified chamber containing 5% CO₂. Two days after reaching 80% confluence (designated as day 0), cell differentiation was stimulated with differentiating medi-

um (DM) containing 10% FBS and a mixture of 5 μ g/mL insulin/0,5 mM 3-isobutyl-1-methylxanthine (IBMX)/0,25 μ M dexamethasone (AppliChem, Biochemica, Germany) for 4 days (day 1 to day 4). After 4 days, the medium was replaced with post DM, containing 10% FBS and 5 μ g/mL insulin for 4 days (day 5 to day 8). At day 8 cells were incubated for 16 hours with MN heartwood ethanol extract at final concentration in the medium of 0.5 or 1% (groups 0.5 MN and 1 MN respectively). At the end of the experiment cells were collected for RNA extraction.

Quantitative real-time PCR analysis

Total RNA was extracted from differentiated 3T3-L1 cells using TRI Reagent according to the manufacturer's protocol (Ambion). Complementary DNA was synthesized using RevertAid™ First Strand Synthesis Kit with oligo (dT)₁₈ primers and RevertAid™ reverse transcriptase (Fermentas). The synthesis reaction was performed on GeneAmp PCR System 9700 (Applied Biosystems). Reaction conditions in final volumes of 10 μ L were provided according to the manufacturer's guidelines. After synthesis each sample of cDNA was dissolved in 30 μ L TE-buffer (pH 8.0) (AppliChem, Biochemica, Germany). Two-step real-time PCR analysis was performed (ABI PRISM 7500, Applied Biosystems) to estimate the gene expression level in cultured cells. Maxima SYBR Green qPCR Kit (Fermentas) was used for sample analysis. The cDNA was amplified using forward and reverse primers of target genes (Table 1) commercially synthesized (Invitrogen Alpha DNA, Canada). Beta-actin was used as endogenous control. Amplification products were examined for nonspecific amplification by including an additional denaturation step in the real-time thermal cycler protocol. All measurements were performed at least in triplicate. Gene expression levels were calculated by $2^{-\Delta\Delta C_t}$ method (24) and expressed as relative units (RU) mRNA compared to the untreated controls where the level of gene expression of interest was considered to be equal to 1.

Statistical Analysis

Data are presented as mean $\pm$ standard error of mean (SEM). Differences between means of groups were analyzed using Student's *t*-test or one-way ANOVA with Dunnett's multiple comparison test

Table 1. Sequences of primers used for RT-PCR analysis

Genes	Nucleotide sequence
β -Actin	F: 5'-ACG GCC AGG TCA TCA CTA TTG-3' R: 5'-CAA GAA GGA AGG CTG GAA AAG- 3'
C/EBP α	F: 5'-AGC AAC GAG TAC CGG GTA CG-3' R: 5'-TGT TTG GCT TTA TCT CGG CTC-3'
aP2	F: 5'-AGT GAA AAC TTC GAT GAT TAC ATG AA-3' R: 5'-GCC TGC CAC TTT CCT TGT G-3'
PPAR γ	F: 5'-AAA AAC CCT TGC ATC CTT CAC AAG CAT-3' R: 5'-TCA ATC GGA TGG TTC TTC GG-3'

(GraphPad Prism 5.0). Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

The effects of MN heartwood extract on three genes involved in lipogenesis are summarized in figure 1. Compared to controls, the transcription of C/EBP α and aP2 genes was significantly inhibited upon treatment with the extract in both concentrations. Significant decrease of PPAR γ levels was estimated only in the cells treated with the lower concentration (0.5%) of MN extract. Variations in the effects of the two applied concentrations were estimated for C/EBP α and PPAR γ . The mRNA levels of these genes were significantly lower in cells incubated in 0.5% final concentration of MN heartwood extract (0.5 MN) as compared with 1 MN treated cells. On

the other hand both concentrations of MN extract equally inhibited aP2 mRNA expression.

DISCUSSION

In this study we investigated the effects of MN heartwood ethanol extract on the expression of three genes directly involved in adipocyte differentiation and lipid accumulation: PPAR γ and C/EBP α are transcriptional factors that are known to influence the development of fat cells, activating the expression of genes that characterize the adipocyte phenotype, such as aP2 (31). These genes are notably active during the last stages of the differentiation when morphological changes in the cell shape it and accumulation of lipids occurs. In our study the differentiation of the cells to mature adipocytes was verified by observation of lipid droplets accumulation in cytoplasm, followed by Oil Red O staining of triacylglycerols (2) (data are not presented). After differentiation the cells were treated with two concentrations of the extract which were selected based on the cytotoxicity test performed previously (27).

Our results indicate that mRNA expression of C/EBP α and aP2 genes was significantly reduced by the two applied concentrations as compared to controls, whereas PPAR γ expression was down regulated by the lower concentration only (fig. 1). Even though this is the first report about the effects of mulberry tree heartwood extracts on lipid metabolism, these results are not surprising. Several studies demonstrated the potential of plant-derived compound or of total extracts to suppress adipogenesis in 3T3-L1 cells (6,9,10,16,21,32). Authors reported that polyphenols, phenolic acids and alkaloids added to the nu-

Fig. 1. Effects of MN heartwood ethanol extract on transcription of C/EBP α , PPAR γ and aP2 genes

tritional medium significantly reduced mRNA expression of PPAR γ and C/EBP α thus inhibiting cell differentiation and lipid accumulation. The inhibition of aP2 gene expression is associated with lower triacylglycerol accumulation in mature fat cells (32). These effects are verified *in vivo* in experimental animals (14,32,35). Some authors reported that leaves and fruits extracts of mulberry species have hypolipidemic effects probably due to the active components rich content (4,22,25).

Mulberry species are rich in polyphenols and it is known that different parts of the tree, especially steams, roots and heartwood, have their specific polyphenol composition (8,11,15,36). Recently we estimated highest total polyphenol content of mulberry heartwood ethanol extract, compared with other arboreal species (28). Results obtained in numerous studies, including human intervention studies, associate polyphenol rich diets with improved lipid and cardiovascular profiles as well as increased total plasma antioxidant capacity (9,12,17,23,25,26). Based on this knowledge and on the results presented above it could be suggested that polyphenols modulate gene expression, therefore contributing to reducing lipogenesis in differentiated fat cells. Aiming to explore possible concentration-dependent effects we applied two different concentrations of the extract. Interestingly, the manifested effects were stronger in the lower concentration treated cells. It could be assumed that accumulation of polyphenols and probably of other, yet unidentified compounds, might contribute to the above described metabolic effects. At this stage of the investigation we can speculate that the inhibitory effects on lipogenesis are mainly due to the action of polyphenols. Unlike other parts of the plant, the mulberry heartwood contains high amounts of tannins, alkaloids and other specific components which are likely be extracted together with polyphenols. We may suggest that in the higher concentration these compounds may have already insulin-like properties contributing for the diminishing of the inhibitory effect of the extract on the lipogenesis (29). This hypothesis remains to be verified by examining the effect of the extract on different stages of preadipocytes differentiation and glucose uptake. Further examination of the phytochemical composition of the extract may provide more answers to these questions.

In conclusion, our study expands the existing knowledge about the biological effects of mulberry tree. Based on the presented data heartwood ethanol extract from mulberry could be considered a promising source of active compounds with possible beneficial effects for prevention and treatment of obesity-related metabolic disorders.

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