DOI:10.16438/j.0513-4870.2003.01.015

Differential expression of lipid metabolism related genes in HepG2 cells treated with neotoralactone

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Abstract: Aim To study the biological effect and molecular mechanism of neotoralactone on lipid metabolism in HepG2 cells. **Methods** Animal experiments were carried out to examine the biological effects of the compound. Differentiation assay was carried out to determine the effect of the compound on the 3T3-L1 preadipocyte. Finally, a high-density cDNA microarray containing about 10 000 human genes and ESTs were used to analyze the gene expression profiles of the HepG2 cells with and without neotoralactone treatment. **Results** Preliminary results showed that neotoralactone can decrease the increase of body weight of rats fed with high nutrient diet. As the differentiation assay showed that neotoralactone has minimal effect on the obese 3T3-L1 preadipocyte cell line, HepG2 heptocarcinoma cell line was used as the model system because one of the proposed mechanisms of weight reduction effect of *Cassia obtusifolia* is the alteration of lipid metabolism primarily taken place in the liver. The results showed that neotoralactone regulates a panel of genes related to lipid metabolism. **Conclusion** The effects of neotoralactone may be due to the regulation of a panel of important genes related to lipid metabolism.

Key words: *Cassia obtusifolia*; neotoralactone; cDNA microarray; HepG2

CLC number: R965 **Document code:** A **Article ID:** 0513-4870(2003)01-0062-05

新决明内脂诱导 HepG2 细胞内脂质代谢相关基因的差异表达

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摘要: 目的 研究新决明内脂与细胞内脂质代谢有关的生物学效应及分子机理。方法 首先用动物实验检查该化合物的生物学效应。再用细胞学方法研究该化合物对 3T3-L1 前脂肪细胞分化的作用。最后用含有10 000个人类基因和 ESTs 的高密度 cDNA 微阵列去研究新决明内脂如何改变 HepG2 细胞的基因表达图谱。结果 新决明内脂可减少大鼠的体重增加率, 但对 3T3-L1 前脂肪细胞分化的作用有限。HepG2 肝细胞基因表达图谱结果显示新决明内脂调节了 46 个与脂质代谢、蛋白代谢、细胞增生与凋亡等功能有关的基因。结论 新决明内脂的生理效果可能与一系列与脂质代谢有关的重要基因有关。

关键词: 决明子; 新决明内脂; cDNA 微阵列; HepG2

Obesity, defined as an increase in the mass of adipose tissue, is the most prevalent nutritional disorder in industrialized countries and a growing problem in developing countries[1]. Therefore, it has become an acute need to develop new and effective therapeutic strategies for controlling obesity. Neotoralactone (NT, patent-pending), a derivative of toralactone, is a compound extracted from the seed of *Cassia obtusifolia* (Figure 1). *Cassia obtusifolia* is a traditional Chinese medicine claimed to have body weight reduction effect and

Received date: 2002-06-05.
Foundation item: City University of Hong Kong through an internal research grant (CityU Project No. 9360044).
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has been used as one of the main ingredients for many body weight reduction medicines in the market[2,3]. A study on the subchronic toxicity of *C. obtusifolia* seed found that treatment of ten rats with diets containing 0, 0.15%, 0.50%, 1.50% or 5.00% *C. obtusifolia* seed for 13 weeks led to decreased body weights[3]. However, the physiological effect and molecular mechanism of the herbal medicine is not well understood.

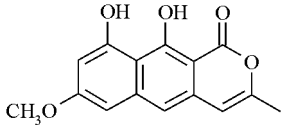

Figure 1 Chemical structure of toralactone

In this study, animal experiments were carried out to examine the biological effects of the single, purified compound NT. A differentiation assay of 3T3-L1 preadipocyte was employed to examine the effect of NT on adipocyte differentiation. As the effect was shown to be minimal, the HepG2 human heptocarcinoma cell line was selected as the model system because one proposed mechanism of the weight reduction effect of *Cassia obtusifolia* is the alteration of lipid metabolism primarily taking place in the liver. A high-density cDNA microarray[4,5] containing about 10 000 human genes and EST (expression sequence tag, EST) was used to analyze the gene expression profiles of the HepG2 cells in the absence and the presence of NT treatment. The results indicated that the effects of NT may be due to the regulation of a panel of important genes related to lipid metabolism.

Experimental section

Materials Human clone set (Unigem V2.0) was obtained from Incyte Genomics. Trizol reagent and Superscript II reverse transcriptase (Invitrogen), Cy3 and Cy5 labeling kit (Amersham Pharmacia Biotech), microcon spin column (Millipore), methylisobutylxanthine (MIX), insulin, dexamethasone (DEX), and Oil Red O stain (all from Sigma) were used as received. Scanarray 4 000 was from GSI Lumonics.

Evaluation of weight reduction effect of NT on rats Twenty female rats (Rattus norvegicus) were equally separated into two groups (group 0 and group 1); with similar body weight (≈43 g). All the rats were fed with high nutrient diet for 35 days before experiment and continuously fed with high nutrient diet for another 30 days during the whole experiment. Group 0 was the control and Group 1 was treated with NT (50 mg·kg^{-1}·d^{-1}) for 30 days. The body weight of the rats was measured during the whole experiment.

3T3-L1 differentiation assay 3T3-L1 preadipocytes (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum until 2 days after confluence. Differentiation was induced by addition of inducing medium (methylisobuthylisobutylxanthine 0.5 mmol·L^{-1}, DEX 1 μmol·L^{-1}, insulin 1 g·mL^{-1}, and 10% fetal bovine serum (FBS) in DMEM). After 2 days, the medium was replaced with DMEM containing insulin 1 μg·mL^{-1} and 10% FBS. Then, the cells will be fed every other day with 10% FBS in DMEM without insulin[6,7]. During the course, 3T3-L1 cells were treated with NT 30 μg·mL^{-1} by adding the drug to the culture medium and incubated for 8 days. Cells without treatment were used as control.

Oil Red O staining[7] was used to evaluate the change in cytoplasmic triglyceride of 3T3-L1 adipocytes after NT treatment. 3T3-L1cell monolayers were fixed with 10% formalin, stained with 0.5% Oil Red *O*-isopropyl alcohol solution for photography and destained with isopropanol. The absorbance (A) of the destaining isopropanol was measured at 510 nm. Finally, the ratio of differentiation was counted by the formula, A (sample)/A (control) *100%.

HepG2 cell culture and cDNA microarray hybridization HepG2 cells (ATCC) were cultured in DMEM supplemented with 10% FBS. NT 30 mg·L^{-1} was added to the culture medium of confluent HepG2 cells, and then incubated for 24 hours. Cells without treatment were used as control. The cells were harvested and total RNA was isolated with TRIZOL reagent according to the manufacturer's instruction. The quantity and quality of the extracted total RNA was checked by a biophotometer and agarose gel electrophoresis, respectively 150 μg of total RNA samples were reverse-transcribed into cDNA using SuperScript II reverse transcriptase in the presence of either of the two distinct fluorescent dyes, Cy3-dUTP and Cy5-dUTP, for the control and treated samples, respectively. The dye-labeled cDNA samples were mixed in 1∶1 ratio and hybridized at 65 ℃ overnight with a home-made cDNA microarray containing 10k genes and ESTs from a human cDNA library (Incyte Genomics). After washing, the microarrays were scanned using a fluorescence scanner (ScanArray 4 000, GSI Lumonics) and the microarray images were analyzed with ScanAnalyze software[4,5,8].

Results and discussion

1 Effect of NT on rats and 3T3-L1 differentiation assay

Table 1 shows that the body weights of NT treated rats were lower than those of the control rats. During the course of NT treatment, the treated rats displayed an average of 10%－35% decrease in body weight gain compared with those of the untreated rats. The results suggest that NT showed body weight reduction effect in rats. The 3T3-L1 pre-adipocyte cells were treated with NT during the period when the cells were induced to differentiate into adipocytes. After 8 days of induction, there was no obvious difference in morphology and oil red O staining assay between NT treated 3T3-L1 cells and the control cells (data are not shown). It suggests that NT does not affect the pathways in adipocyte differentiation and the target of NT may not be at the pre-adipocyte/adipocyte level.

Table 1 Comparison of the body weight change between obese rats with and without NT treatment. The duration of NT treatment is 30 days (n=10, $\bar{x}\pm s$)

Group	High nutrient diet fed rats control/g (without NT treatment)	Net gain in body weight/g Group 0	High nutrient diet fed rats (treated with 50 mg·kg^{-1}·d^{-1} NT oral intake	Net gain in body weight/g Group 1	Increase in body weight* /%
Body weight/g before high nutrient diet treatment	42.8±2.4	－	43±3	－	－
Body weight/g 35 days after high nutrient diet treatment	81±7	0	81±8	0	－
Body weight/g 10 days after NT treatment	117±122	35.9	109±8	27.5	10.4
Body weight/g 20 days after NT treatment	154±14	73.5	136±22	55.3	22.5
Body weight/g 30 days after NT treatment	180±19	99.0	152±16	70.6	35.1

* Calculation based on: (Net body weight gain for Group 0 － Net body weight gain for Group 1)/Body weight after 35 days obese treatment

2 HepG2 cells and cDNA microarray hybridization

HepG2 cells were selected as an alternate model to 3T3-L1 since one possible mechanism of NT action is the alteration of lipid metabolisms in liver. The gene expression profiles of HepG2 cells treated with NT were obtained by using a high-density cDNA microarray (Figure 2). The fluorescence intensities were normalized and the Cy3/Cy5 ratio for each spot was calculated. The genes with ratios over 2 and below 0.5 were considered as up-regulated and down-regulated genes, respectively, following NT treatment. When comparing NT-treated HepG cells with untreated HepG2 cells, a total of 46 genes and ESTs showed differential expressions: 12 genes and 1 EST were up regulated and 21 genes and 12 ESTs were down regulated, respectively (Table 2).

Table 2 Genes up-regulated in HepG2 cells treated with neotoralactone

Unigene ID	Gene name	Genbank	Ratio
	Lipid metabolism related genes		
Hs. 73849	Apolipoprotein CIII	APOC3	2.2
Hs. 1955	Serum amyloid A4	SAA4	2.1
Hs. 177516	High density lipoprotein binding protein	HDLBP	0.18
Hs. 48876	Farnesyl-diphosphate farnesyltransferase 1	FDFT1	2.5
Hs. 76038	Isopentenyl diphosphate delta isomerase	IDI1	2.2
	Protein metabolism related genes		
Hs. 44532	Diubiquitin	UBD	7.8
Hs. 89545	Proteasome (prosome, macropain) subunit, beta type, 4	PSMB4	2.8
Hs. 790	Microsomal glutathione S-transferase 1	MGST1	2.8
Hs. 71618	Polymerase (RNA) II (DNA directed) polypeptide L (7.6 kD)	POLR2L	2.8
Hs. 14839	Polymerase (RNA) II (DNA directed) polypeptide G	POLR2G	2.4
Hs. 299465	Ribosomal protein S26	RPS26	2.4
Hs. 179774	Proteasome (prosome, macropain) activator subunit 2	PSME2	2.2
Hs. 172280	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c	SMARCC1	0.37
Hs. 83765	Dihydrofolate reductase	DHFR	0.36
Hs. 14611	Dual specificity phosphates 11	DUSP11	0.30
Hs. 74267	Ribosomal protein L15	RPL15	0.29
Hs. 75280	Glycyl-tRNA synthetase	GARS	0.19
	Proliferation and apoptosis genes		
	Cancer-related genes		
Hs. 1174	Cyclin-dependent kinase inhibitor 2A	CDKN2A	2.3
Hs. 11	Carcinoembryonic antigen-related cell adhesion molecule 3	CEACAM3	0.40
Hs. 349109	Insulin-like growth factor II	IGF2	0.37
Hs. 335842	Retinoic acid receptor responder (tazarotene induced) 3	RARRES3	0.34
Hs. 5300	Bladder cancer associated protein	BLCAP	0.28
Hs. 76095	Immediate early response 3	IER3	0.21
Hs. 75412	Arginine-rich, mutated in early stage tumors	ARMET	0.21
	Other genes		
Hs. 9615	Myosin, light polypeptide 9, regulatory	MYL9	0.48
Hs. 80552	Dermatopontin	DPT	0.47
Hs. 2785	Keratin 17	KRT17	0.46
Hs. 146381	RNA binding motif protein, X chromosome	RBMX	0.45
Hs. 93659	Protein disulfide isomerase related protein	ERP70	0.37
Hs. 89643	Transketolase	TKT	0.37
Hs. 56045	Src homology three (SH3) and cysteine rich domain	STAC	0.34
Hs. 107164	Spectrin, beta, non-erythrocytic 1	SPTBN1	0.33
Hs. 30376	Hypothetical protein	HSPC194	0.24

3 Functional Analysis of Differentially Expressed Genes

The 33 differentially expressed genes may be classified into 4 clusters according to their functions (Table 2), including (1) lipid metabolism, (2) amino

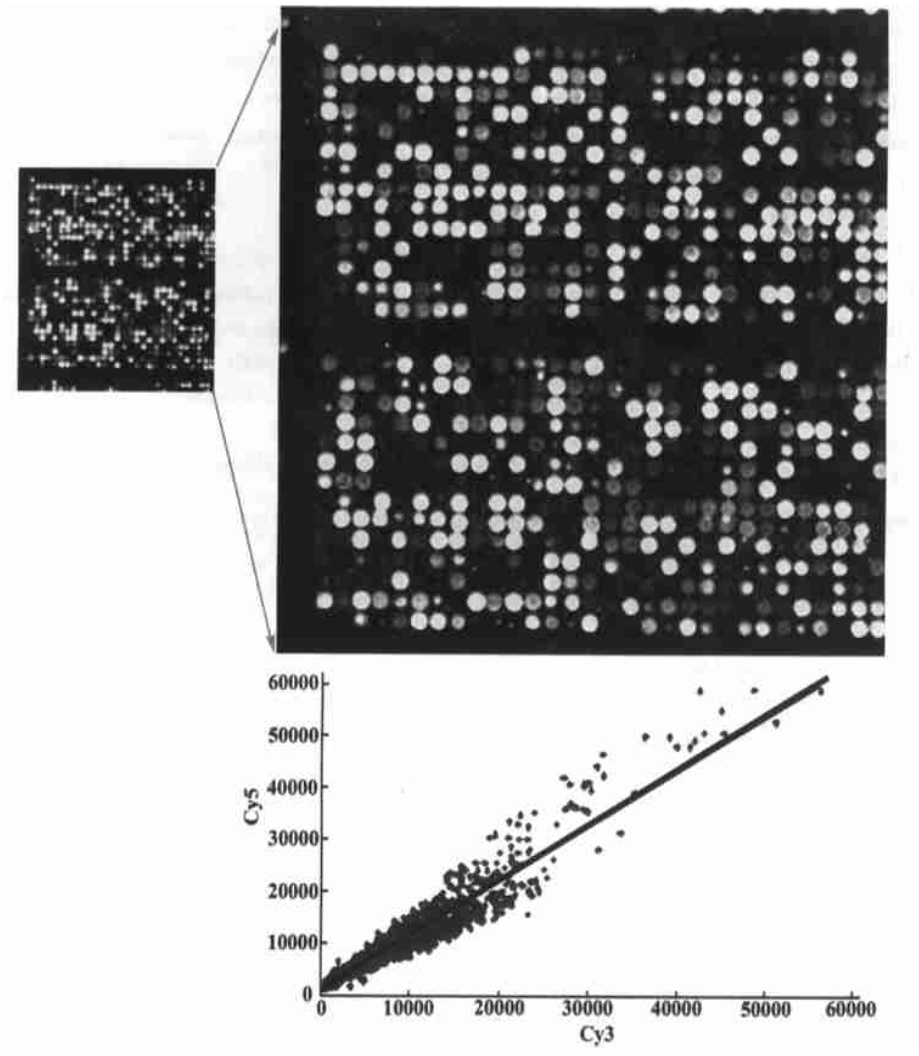

Figure 2 Merged image of Cy3 and Cy5 images. Red spot represents the up-regulated genes, green spots represent the down-regulated genes, and yellow spots indicate that level of expression of this gene is equivalent in both samples. (Bottom right) Scatter plot of Cy3 vs. Cy5 signals. Linear distribution of spots indicates the signals of two fluorescent dyes were balanced in the experiment

acid (and protein) and nucleic acid metabolism, (3) proliferation, apoptosis and tumor, and (4) other functions.

The genes related to lipid metabolism are the most interesting in this research. The up regulation of apolipoprotein C-III (APOC3), which has the function of inhibiting lipoprotein lipase and hepatic lipase and also decreases the uptake of lymph chylomicrons by hepatic cells, is considered to be one possible cause of the body reduction effect of NT[9]. When the lipoprotein lipase and hepatic lipase are inhibited, the catabolism of triglyceride particles would be delayed. Thus, the cells cannot absorb and utilize the triglyceride and may cause body weight reduction effect. This mechanism is similar to the weight loss mechanism of Xenical[10]. Serum amyloid A4, constitutive (SAA4), an apolipoprotein component of non-acute phase high-density lipoprotein, was also up regulated. It may also play a role in helping the cells produce apolipoprotein CIII[11]. As a feedback mechanism, some genes related to cholesterol metabolism may need to be regulated to compensate for the reduced production of cholesterol. These include the down regulation of high-density lipoprotein binding protein (HDLBP), with the function of protecting cells from over-accumulation of cholesterol[12], and up-regulation of farnesyl-diphosphate farnesyltransferase 1 (FDFT1), involving in the first step of cholesterol biosynthesis and as a control point in sterol and isoprene synthesis[13]. Similarly, isopentenyl diphosphate delta isomerase (IDI1) encodes a peroxisomally localized enzyme, catalyzes the interconversion of isopentenyl diphosphate (IPP) to its highly electrophilic isomer, dimethylallyl diphosphate

(DMAPP), the substrate for the successive reaction leading to the synthesis of farnesyl diphosphate and, ultimately, cholesterol[14]. Figure 3 summarizes the possible biochemical pathways of the lipid metabolism and the differentially expressed genes that may play functional roles in the effect of NT. For the genes related to protein, amino acid and nucleic acid metabolism, there are 7 genes up regulated and 5 genes down regulated. These suggested that the NT also affects the cells in various biochemical pathways and the transcription and translation of the cells. For the cluster of genes related to proliferation, apoptosis and tumor, there are 1 gene up regulated and 6 genes down regulated. This is expected because NT may also affect cell cycle and HepG2 is a tumor cell line. However, there is no direct evidence to suggest that NT can cause either proliferation or growth arrest of the cells.

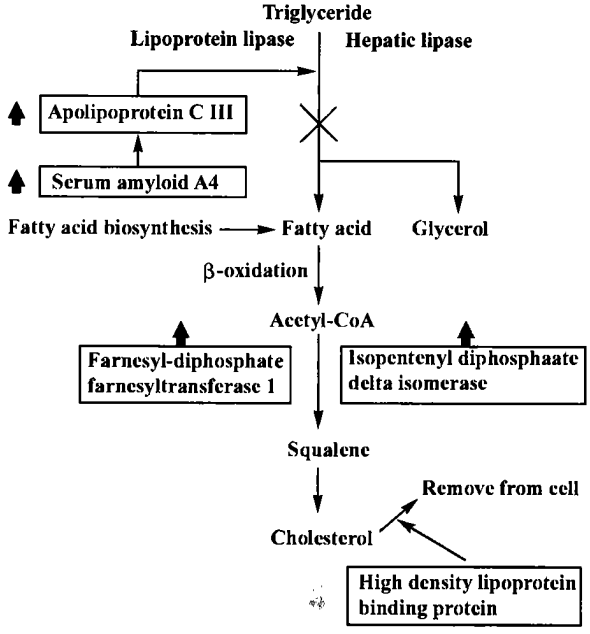

Figure 3 Biochemical pathways involving APOC, FDFT1, IDI1 and HDLBP genes. Regulation of these genes results in decreased catabolism of triglyceride and a feedback accumulation of cholesterol

Conclusion

In this paper, the *in vivo* effect of NT on body weight of obese rat and the *in vitro* cell based assay in 3T3-L1 adipocytes were investigated. The global gene expression profiles in HepG2 cells after NT treatment were analyzed using cDNA microarrays. The results of animal experiment suggested that NT has weight reduction effect. The results of differentiation assay suggested that NT has minimal effect on adipocyte differentiation. The microarray results indicate that NT regulates several groups of genes involving in cellular metabolisms and proliferation and other functions, and that the weight reduction effects of NT may be due to the regulation of a panel of important genes related to lipid metabolism pathways.

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