行政院國家科學委員會補助專題研究計畫成果報告

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※順式-肉桂酸在老鼠肝細胞的粒腺體代謝途徑之研究 ※

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計畫主持人：孫芳明

共同主持人：

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中 華 民 國 90 年 10 月 23 日

行政院國家科學委員會專題研究計畫成果報告

順式-肉桂酸在老鼠肝細胞的粒腺體代謝途徑之研究

The study of the possible metabolic pathway of cis-cinnamic acid by the intact mitochondria of rat liver' cells

計畫編號：NSC 89-2313-B-041-020

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主持人：孫芳明　嘉南藥理科技大學保健營養系

中文摘要

在此研究中經由老鼠肝細胞之粒腺體（In Vitro)及老鼠的活體實驗（In Vivo)證實，順式-肉桂酸在老鼠肝臟的代謝途徑可能有兩種方式：一是先形成高能的順式-肉桂酸CoA、經類似順式-脂肪酸的β-氧化（β-oxidation)再與甘氨酸（glycine)形成馬尿酸排出體外；另外一個可能的代謝途徑則是由特殊的異構酵素（isomerase)將順式-肉桂酸轉變為逆式-肉桂酸，而此新形成的逆式-肉桂酸再進行β-氧化並形成馬尿酸排出體外；老鼠肝臟代謝順式-肉桂酸的效率與苯甲酸和逆式-肉桂酸的效率並無明顯差異，顯示出老鼠肝臟負責代謝順式-肉桂酸的酵素系統不須經過誘導即存於肝細胞中。

關鍵辭：順式-肉桂酸、β-氧化、逆式-肉桂酸、異構酵素。

Abstract

The present study demonstrated that there might be two different catabolic pathways for rat liver to metabolize cis-cinnamic acid. In the first proposed pathway, cis-cinnamic acid was converted to cis-cinnamoyl-CoA followed by β-oxidation to form benzoyl-CoA. This step was the same as the β-oxidation of unsaturated cis-fatty acids. The benzoyl-CoA was then conjugated with glycine to generate hippuric acid. The other possible catabolic pathway for rat liver to metabolize cis-cinnamic acid was the isomerization of cis-cinnamic acid to trans-cinnamic acid. The tans-cinnamic acid produced from this step was then catabolized by β-oxidation and conjugation with glycine to form hippuric acid. There was no significant difference in the catabolic efficiencies for cis-cinnamic acid、trans-cinnamic acid、and benzoic acid in rat liver suggesting the enzyme systems for the catabolism of cis-cinnamic acid are constitutive present in rat liver's cells.

Keywords: cis-cinnamic acid、β-oxidation、trans-cinnamic acid、isomerase。

Introduction

Cinnamic acid is present in all kinds of plant derived foods, herbs, and Chinese medicines [1,2]. Two forms of cinnamic acids, trans-cinnamic acid and cis-cinnamic acid, have been found to exist in the plant cells [3]. However, the trans-cinnamic acid has been shown to be the predominate form in nature (>99%), since it is much more stable than the cis-isomer [4]. Extensive studies have been reported for the metabolic pathways of trans-cinnamic acid in animals [5,6,7]. cis-Cinnamic acid also plays an important role in the physiology of plant cells and fungi for the significant differences in the activities of bioactive enantiomers/diastereomers produced by or active against living organisms which are vitally important to pharmaceutical, flavor, and food industries [8]. However, due to the stability and purity of this unavailable cis-cinnamic acid, the mechanisms of cis-cinnamic acid on nutritional, toxicological, and metabolic pathway in eukaryotic cells are virtually unknown [9].

The metabolic procedure of trans-cinnamic acid is similar to the metabolic process of short-chain fatty acids in rat liver cell's mitochondria [10]. The first step is the forming of trans-cinnamoyl-CoA. trans-Cinnamoyl-CoA undergoes β-oxidation yielding benzoyl-CoA. Benzoyl-CoA is then in turn conjugated with glycine giving hippuric acid [11]. This reactions in sequence are of the great historical importance in biochemistry of fatty acids catabolism [6], and has been tested as a clinical index for the presence of fatty liver [12]. Because of the different chemical properties between trans-, and cis-cinnamic acids, cis-cinnamic acid may present different biological and nutritional effects on eukaryotic cells when compared with trans-cinnamic acid. Thus, it would be very interesting and necessary to investigate the catabolic pathway of cis-cinnamic acid in animal liver's cells.

The present study was designed to investigate the overall metabolic pathways of cis-cinnamic acid in rat liver. Also, the difference in mechanisms and efficiency of rat's liver to breakdown benzoic acid、trans-cinnamic acid、and cis-cinnamic acid were studied.

Materials and Methods:

Chemicals:

trans-Cinnamic acid, benzoic acid and other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) with the purities of 95-99.5%. The cis-cinnamic acid was prepared following by the method developed by Sun et al. 2000. Male rats which weighted 85-134 g when used for assay were purchased from Animal Center of National Cheng-Kung University (Tainan, Taiwan).

In Vitro Assay of the metabolism of cis-cinnamic acid in rat liver's mitochondria:

Isolation of liver mitochondria:

Male rats fasted 18-22 hours were decapitated and the liver were removed, blotted and weighted. Mitochondria were isolated from liver of fasted rats as described by Griffith et al. [13]. The protein concentration of this mitochondria suspension was determined by Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

Assay of CoA ligase:

The assay conditions for the ligase is from Man and Brosnan [14].

In Vivo Assay of the metabolism of cis-cinnamic acid in rat liver:

Male rats fasted 18-22 hours were removed into Metabolic Cages. A 1 ml of benzoic acid, trans-cinnamic acid or cis-cinnamic acid (400 mM) was administered into rat's body ip. The urine sample was collected for 8 hours. After 12 hours, rats were decapitated and the livers were removed. The urine sample were freeze dried, then the pellet was redissolved by adding 0.5 ml of methanol. The suspension was centrifuged at 12,000 rpm at 30 ℃ for 1 min. The supernatants were saved in a –20 ℃ freezer for HPLC analysis.

HPLC Analysis of Ligase Activity:

The HPLC used was a Hitachi HPLC system (L7100 multisolvent delivery system) coupled with a L7455 photodiode array detector (PDA) and a C_{18}-ODS analytical reverse phase column (0.46 mm OD x 250 mm, Vercopak Co., Taiwan) were used to separate substrates and metabolic product. Acyl-CoA was eluted with 0.1 M potassium phosphate buffer, pH 5.0:methanol (65:35) at a flow rate of 1 ml/min. Hippuric acid was eluted with methanol:water (35:65) at a flow rate of 1 ml/min. The chromatographs were extracted at 254 nm for quantification of substrates and products.

Results and Discussions:

The changes of substrates and product during the 12 hours incubation period for the in Vivo

experiments are shown in Table 1. Most of the administered benzoic acid and trans-cinnamic acid are metablized to hippuric acid and excreted into urine after 12 hours. For cis-cinnamic acid, 41.6 μg of cis-cinnamic acid and 125.4 μg of trans-cinnamic acid were found in the liver sample. No product (hippuric acid) can be identified in blood and liver sample by HPLC.

The results from the in Vivo study further support that the acyl-CoA was involved in the catabolism of benzoic acid and trans-cinnamic acid. Moreover, the end product-hippuric acid was detected for benzoic acid, trans-cinnamic acid in rat's urine sample (Table 1). Unfortunately, there was no evidence for the formation of cis-cinnamoyl- CoA in the Vitro study. However, hippuric acid was also found in the urine sample for the in Vivo study of the catabolism of cis-cinnamic acid in rat liver (Table 1). The recoveries for both benzoic and trans-cinnamic acids were over 65% suggesting that β-oxidation was involved in the catabolism of trans-cinnamic acid and benzoyl-CoA was the key intermediate in their catabolism route in rat's liver. This finding was coincided with the results published by Nutley et al. in which trans-cinnamic acid was converted to its acyl-CoA esters. The trans-cinnamoyl-CoA was further metabolized by a cycle of β-oxidation to form a molecule of acetyl-CoA and benzoyl-CoA. The benzoyl-CoA was in turn conjugated with glycine giving hippuric acid. Since more than 70% of cis-cinnamic acid was also metabolized to hippuric acid indicating that cis-cinnamoyl-CoA was the first intermediate in the catabolic pathway. The cis-cinnamoyl-CoA was hydrated and isomerized to form L-3-hrdroxycinnamoyl-CoA which was catabolized by β-oxidation to form benzoyl-CoA. Once the benzoyl-CoA was formed it was further converted to hippuric acid (Fig. 2). The presence of a trace amount of trans-cinnamic acid in the liver sample of the in Vivo study of the metabolism of cis-cinnamic acid suggests there might be an another pathway for rat to metabolized cis-cinnamic acid. That is an isomerase may present in rat liver to transform cis-cinnamic acid to trans-cinnamic acid before the CoA ester was formed (Fig. 2).

Table 1: In Vivo assay of the metabolism of benzoic, cis-, and trans-cinnamic acid in rats liver

Determination / Substract	Hippuric acid in urine	Hippuric acid in blood	Hippuric acod in liver	Total Recovery
Control	0.32 mg	ND	ND	-
Benzoic acid	33.41 mg	ND	ND	68.46 %
trans-cinnamic acid	49.06 mg	ND	ND	67.76 %
cis-cinnamic acid	47.23	ND	ND	65.23 %

ND: Not Detected

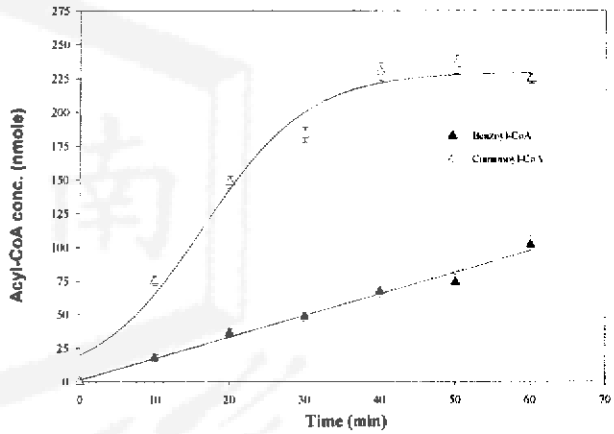

Figure 1. Kinetics of acyl-CoA formation by isolated rat liver cell's mitochondria. The reaction mixture contained the following components at the conc. Shown in paremheses: KCL (100 mM), Tris-HCl (50 mM, pH 8.0), ATP (5 mM), CoASH (0.5 mM), $MgCl_2$ (5 mM), substrate (benzoate, trans-cinnamate, or cis-cinnamate 1mM), and mitochondria protein 3.81 mg.

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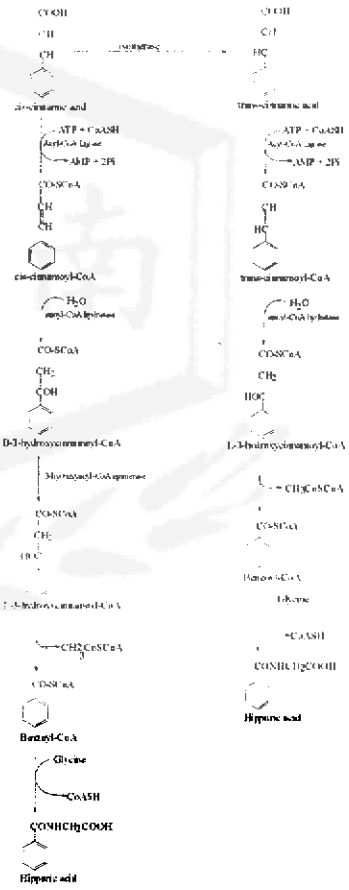

Figure 3: The proposed catabolic pathways for cis-cinnamic acid in rat's liver.

成果自評：

在此實驗中我們除了按照預訂的進度完成了實驗，並在有限的資源下以活體實驗來進一步的確認在 in Vitro 實驗的結果，唯一遺憾的是無法使用同位素，如果在此實驗中能使用 ^{14}C 的肉桂酸，我們將會得到更完整的關於順式-肉桂酸在老鼠肝臟代謝途徑的資訊。