available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Metabolism of protocatechuic acid influences fatty acid oxidation in rat heart: New anti-angina mechanism implication

Yan-guang Cao, Lin Zhang, Chen Ma, Bo-bo Chang, Yuan-Cheng Chen, Yi-qun Tang, Xiao-dong Liu, Xiao-quan Liu *

Center of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University, Nanjing City 210009, PR China

ARTICLE INFO

Article history:
Received 30 October 2008
Accepted 25 November 2008

Keywords:
Protocatechuic acid
Metabolism
Fatty acid oxidation
Anti-angina

ABSTRACT

Protocatechuic acid (PA), a structurally typical phenolic acid in danshen, shows anti-angina efficacy. But until now, besides scavenging of oxygen free radicals, the understanding of its anti-angina mechanism has been limited. In our study, based on a novel metabolic route of PA identified in rat heart and its influence on fatty acid oxidation (FAO), we proposed a new mechanism for its anti-angina. In detail, three metabolites, catechol methylated metabolite, acyl-coenzyme (CoA) thioester and glycine conjugation, were identified in rat heart. A novel metabolic pathway was confirmed based on several metabolic systems incubated with heart mitochondria, cytosol, microsomes and homogenate. Results indicated that PA was firstly methylated in microsomes and cytosol, which was regarded as the prerequisite step for further metabolism and could be inhibited by tolcapone, and then the resulting methylated metabolite (vanillic acid) diffused into mitochondria where it was converted into acyl-CoA thioester, in similar with FAO. In addition, part of the acyl-CoA thioester was transformed into glycine conjugation, a step also localized within mitochondria. Furthermore, based on isolated rat heart perfusion, it was found that PA markedly decreased FAO, which was shown by higher residual fatty acid level in perfusate ($p < 0.05$) and lower acy-CoA/CoA ratio in heart ($p < 0.05$). The FAO inhibiting effect of PA could be largely reversed by its methylation inhibitor tolcapone, indicating the effect was closely related with the identified metabolic pathway of PA in heart. The decrease of FAO may switch heart energy substrate preference from fatty acid to glucose, which is beneficial for ischemia heart.

1. Introduction

Although not as abundant as in liver, enzymes in heart mediating exogenous or endogenous substances metabolism have been widely reported [1,2]. Many researches have implicated the association of cytochrome P450 with heart pathology and pharmacology [3,4]. For drugs in treatment of heart diseases, including β-blockers, calcium antagonists, and angiotensin-converting enzyme inhibitors, all of them were reported to be subject to cytochrome P450-dependent oxidations. Thus, investigating their metabolism in heart will be very important to understand their efficacy and toxicity, such as verapamil [5] and dexrazoxane [6]. Although substantial numbers of herbal drugs are prescribed for heart diseases, such as *salvia miltiorrhiza* (danshen) and *Radix notoginseng*, little was known about their metabolism in heart. In our opinion,

* *Corresponding author*. Tel.: +86 25 83271260; fax: +86 25 86635699.
E-mail address: lxq_cpu@yahoo.com.cn (X.-q. Liu).
0006-2952/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2008.11.029

the investigation of these herbal drugs metabolism in heart may be helpful to understand their pharmacology or toxicology.

Danshen is a well-known Chinese herbal medicine and is used in clinics in China for angina pectoris and myocardial infarction [7,8]. Chemical constituents study generally divided the compounds in danshen into two groups, phenolic acids (hydrophilic) and diterpenoid tanshinones (lipophilic) [9]. For many years, the lipophilic compounds were regarded as the main contributing factors to danshen activities. Recently, these phenolic acids gained more attention as a large pool of natural antioxidants [10]. Especially in Danshen Injection (a danshen prescription), phenolic acids account for more than 90% of the constituents and nearly no lipophilic constitutes were detected [11]. Thus, the anti-angina efficacy of Danshen Injection should mainly contribute to these phenolic acids. Protocatechuic acid (PA), an active and typical phenolic acid in danshen, has shown obvious anti-angina effect [12,13]. Structurally similar with other phenolic acids in danshen, PA contains catechol group in benzene ring and carboxylic acid in side chain. Metabolism study indicated that these phenolic acids mainly underwent phase II reaction including methylation, glucuronidation, and sulfonation [14]. Recently, we accidentally found a glycine conjugative metabolite of PA in rat heart, a metabolite that was evidenced to only form within mitochondria according to previous reports [15,16]. Considering that coenzyme (CoA) thioesters formation is an obligatory step in the course of glycine conjugation, we supposed that there was some association between PA metabolism and heart fatty acid oxidation (FAO), because they both occur in mitochondria and need assistance of CoA factor. Additionally, a body of evidence has convincingly demonstrated that FAO reduction is beneficial for ischemia heart because it shifts heart energy substrate from fatty acid to glucose and enhances the oxygen utilizing efficiency for adenosine triphosphate (ATP) production [17,18]. The interesting and significant presumption promoted us to investigate the metabolic route of PA in heart and evaluate its relevance to heart FAO.

The present study was designed to investigate PA metabolism in rat heart *in vivo* and *in vitro*. The related metabolites were characterized and a novel metabolic pathway was identified based on several metabolic systems incubated with heart cytosol, microsomes, mitochondria and homogenate. Furthermore, the relevance of the metabolic pathway with FAO was also evaluated by isolated heart perfusion assay and a new mechanism for its anti-angina efficacy was proposed.

2. Materials and methods

2.1. Materials

Protocatechuic acid (PA, purity 98%) was gained from Qinze (Nanjing, China). Glycine, dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), benzoic acid and LiOH were purchased from Wanqing (Nanjing, China). Vanillic acid (VA, purity 98%), adenosine triphosphate (ATP), CoA, acyl-CoA, salicylsulfonic acid, palmitic acid, 2-phenylpropionic acid, s-adenosylmethionine (SAM), pivalic acid and dithiothreitol (DTT) were all supplied by Sigma–Aldrich (St. Louis, MO). Tolcapone and ranolazine (purity 99%) were both supplied by Qilu (Jinan, China). Triton X-100, ethylenediamine tetraacetic acid (EDTA), Tris–HCl and hydroxyethyl piperazine ethanesulfonic acid (HEPES) were all obtained from Shengxing (Nanjing, China). Benzylpenicillin was purchased from Xiansheng (Nanjing, China). Chloral hydrate, heparin and benzoic acid all gained from Nanjing Chemical Co. Ltd (Nanjing, China). All the other chemicals and solvents were of analytical grade. Free fatty acid kit and Coomassie Brilliant Blue R-250 Staining Solutions Kit were supplied by Jiancheng (Nanjing, China).

Analysis was conducted on a Shimazu LC-DAD-ESI/MS system which was coupled with Photodiode array detector and a 2010EV quadrupole mass spectrometer equipped with an ESI interface. The LC-MS/MS analysis was performed using a Shimazu LC-10AD HPLC system and API 3200 triple-quadrupole mass spectrometer equipped with turbo ion-spray interface (Foster City, CA, USA). Solid-phase extraction column was carried on HLB Cartridges and provided by Waters (Massachusetts, USA). Langendorff perfusion apparatus was provided by clinical pharmacology department of China Pharmaceutical University.

2.2. Animals

Male Sprague–Dawley rats (250–300 g) were purchased from China Pharmaceutical University (Nanjing, China) and maintained in a controlled housing environment with 12-h light/dark cycles and received standard laboratory chow and water ad libitum. Rats were acclimated (for at least 3 days) to the housing condition before experiments. All animal use was approved by China Pharmaceutical University on Animal Research and we tried our best to minimize the stress of rats.

2.3. Vanilloyl-glycine synthesis

Vanilloyl-glycine was synthesized according to previous method with some modifications [19]. Briefly, glycine methyl ester was firstly synthesized with glycine and methanol in dichloride. Then the glycine methyl ester was conjugated with VA in DMAP containing DCC. The methyl ester product was purified on silica gel column and then hydrolyzed by LiOH. The product was identified as vanilloyl-glycine by MS/MS and proton nuclear magnetic resonance spectroscopy (^{1}H NMR) (ACF-300 Bruker, Germany) in deuterochloroform. The predominant product ion was at m/z $[M-H]^-$ 224 and a series of product ions were at m/z 180 (224–44), 165 (224–59), 123 (224–101) and 100 in the MS^2 spectrum of the molecular ion. ^{1}H NMR data showed corresponding signals as –NH–CH_2–(s, 4.11), –OCH_3 (s, 3.71).

2.4. PA metabolites identification in rats

Rats were intravenously administrated of 20 mg/kg PA through femoral vein. After 30 min, rats were scarified by dislocation. Tissues (heart and liver) and blood were immediately gained. The tissues were washed with ice-cold saline and quickly frozen in liquid nitrogen. Plasma was prepared by 4000 $g \times 5$ min centrifugation of blood and stored at $-20\,^{\circ}C$

Table 1 – Heart subcellular fraction incubated in four metabolic systems.

Systems	Heart subcellular fractions			
	Homogenate	Mitochondria	Cytosol	Microsomes
1 Methylation[a]		√ (PA)[b]	√ (PA)	√ (PA)
2 CoA conjugation[c]		√ (PA + VA)	√ (PA + VA)	√ (PA + VA)
3 Glycine conjugation	√ (VA + PA)			
4 CoA conjugation + glycine		√ (VA + PA)		

'√' indicated experiment conducted; PA and VA represented protocatechuic acid and vanillic acid, respectively; all experiments were conducted at drug concentration of 20 μM; each fraction was carried out in concentration of 2 mg protein/ml.
[a] Indicated that all the experiments were conducted in another group with tolcapone (2 μM).
[b] Referred to the drug incubated for metabolism.
[c] Indicated that all the experiments were conducted in another group with pivalic acid (10 μM).

until analysis. In addition, another six rats were intraperitoneally (*i.p.*) injected of tolcapone (10 mg/kg, a typical catechol-O-methyltransferase (COMT) inhibitor) 10 min prior to PA administration. The metabolie vanilloyl-glycine and PA were both quantified in tissues and compared between groups with and without tolcapone pretreatment.

For samples analysis, frozen tissues were homogenized in saline at 1:2 (g/ml). The tissues homogenate and plasma were prepared according to previous method [14] and analyzed by HPLC-DAD-MS. For possible acyl-CoA thioester formation, we prepared samples mainly followed previously reported method [20].

2.5. *Studies of PA metabolism in heart subcellular fractions*

2.5.1. *Subcellular fractionation*

Heart homogenate preparation, heart mitochondria, cytosol and microsomes were prepared according to method described by Tracy [21]. Each fraction was carried out in concentration of 2 mg protein/ml.

2.5.2. *Incubation systems*

System 1: COMT-mediated *O*-methylation system. The reaction mixtures consisted of Tris–HCl buffer 50 mM (pH 7.4), $MgCl_2$ 4 mM, SAM 200 mM and experiments were carried out according to previous report [22].

System 2: Acyl-CoA thioesters formation system. The reaction was conducted according to previous method [23]. Briefly, incubations of drugs with subcellular fractions (1.0 mg protein/ml) were carried out in 150 mM Tris–HCl buffer (pH 7.4) containing 0.05% Triton X-100, 1 mM DTT, 2 mM EDTA, 1.2 mM CoA, 6.2 mM $MgCl_2$, and 2.5 mM ATP in a final volume of 0.5 ml. After a 3 min pre-incubation, reactions were initiated by addition of ATP and allowed to proceed for 30 min at 37 °C, at which time the incubations were stopped by the addition of 50 μL of perchloric acid (7%).

System 3: Glycine conjugation assay was followed previous method [24]. The system consisted of 2 mM glycine, 10 mM glucose, 10 mM HEPES (pH 7.4) and 200 IU/ml benzylpenicillin.

System 4: One mixture system was developed: 2 mM glycine into CoA conjugation system.

2.5.3. *Incubation assays*

In order to elucidate the metabolic subcelluar sites and metabolic route of PA in rat heart, we incubated heart subcellular fractionations in different metabolic systems. Heart cytosol, microsomes and mitochondria were all incubated in methylation reaction system with PA (20 μM). Heart cytosol, microsomes and mitochondria were all incubated in acyl-CoA thioester conjugation system with PA (20 μM) or VA (20 μM). Heart homogenate was also incubated in glycine conjugation system with VA (20 μM) or PA (20 μM). Mitochondria were incubated in mixture system with VA (20 μM) or PA (20 μM). In addition, we conducted all our methylation reaction and acyl-CoA thioester formation experiments in another two groups with tolcapone (2 μM) and pivalic acid (10 μM), respectively. The detail experiments were summarized in Table 1.

2.6. *Potential toxicity excluded*

Many reports indicated that many xenobiotic acyl-CoA thioester caused tissue damage due to covalent proteins adducts [23,25]. In order to examine the possible toxicity of PA, possible covalent adducts were analyzed in kidney and liver, in which PA were activated into acyl-CoA thioester. Briefly, 9 rats were randomly grouped into three groups and intravenously injected of PA (40 mg/kg), 2-phenylpropionic acid (130 mg/kg, positive control) and benzoic acid (120 mg/kg, negative control), respectively. Rats were sacrificed at 1 h and liver and kidney were immediately removed and frozen in liquid nitrogen. The proteins adducts were hydrolyzed according to previous method [25] and the samples were analyzed according to method described below.

2.7. *Isolated heart perfusion assay*

In order to evaluate the relevance of the metabolic pathway of PA with FAO, we conducted assays based on isolated rat heart perfusion according to previous method [26]. SD rats (250–300 g), which had been fed ad libitum, were killed after treatment with chloral hydrate (*i.p.*, 350 mg/kg) and heparinised (*i.p.*, 100 U/kg). Hearts were rapidly excised and placed in ice-cooled Krebs–Henseleit solution (K–H) (NaCl 118 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, 2.5 mM $CaCl_2$, 5 mM glucose, 100 mU/L insulin). After cannulating the aorta, constant pressure (80 mmHg) Langendorff perfusion was commenced. The perfusate, a modified Krebs–Henseleit solution, was maintained at 37 °C and continuously bubbled with a gas mixture of 95% O_2/5% CO_2. Hearts were immersed in a chamber containing perfusate at a constant 37 °C.

Isolated hearts were perfused with K–H solution containing 0.3 mM palmitic acid and 3% albumin. In all experiments, hearts were initially perfused in the absence of drug. After 5 min, hearts were randomly assigned to an untreated control group, ranolazine (10 μM, positive control) group, PA (40 μM), PA (40 μM) plus tolcapone (5 μM) and VA (40 μM) group. Drugs were present for the remainder of the experiments. Each heart was perfused with 100 ml circulating perfusate. The concentrations of residual fatty acid in perfusate were analyzed at 15 min and 30 min with assay kit. After 30 min perfusion, hearts were collected and immediately frozen in liquid nitrogen, then the collected heart were homogenated in 9% salicylsulfonic acid for CoA and acyl-CoA analysis. Based on the analysis of residual free fatty acid in perfusate, acyl-CoA/CoA ratios in heart, we evaluated the level of FAO among groups.

2.8. Analysis methods

The simultaneous analysis of PA and vanilloyl-glycine was conducted on HPLC at 260 nm. In detail, the samples were pretreated with 1 M HCl and then extracted by 5 times volume acetoacetate. The mobile phase was a mixture of water (ammonium acetate, 5 mM) and methanol (15:85, v/v) at an isocratic flow rate of 1.0 mL/min. The possible acyl-CoA thioester was detected by HPLC-MS as *in vivo* metabolites searching method described above [23]. Briefly, frozen rat heart (or liver) (0.6 g) was homogenized in ice-cold 1.5 ml of potassium phosphate buffer (0.05 M, pH 5). The heart (or liver) homogenate was immediately denatured by 0.75 ml of $HClO_4$ (7%), mixed vigorously, then centrifuged (10,000 *g*, 10 min). Supernatants were neutralized with 1 N NaOH and analyzed by HPLC-MS.

Benzoic acid was also analyzed on HPLC according to previous method [27]. 2-phenylpropionic acid was analyzed on HPLC-MS with the reported method [28]. CoA, acyl-CoA were analyzed on HPLC according to previous method [29]. Free fatty acid analysis was conducted with purchased free acid-kit. Protein content was determined by coomassie brilliant blue method.

2.9. Data analysis

All results were expressed as the mean ± S.D. Comparison of the means was made using a two-tailed student's *t*-test. The acceptable level of significance was established at $p < 0.05$ except when otherwise indicated.

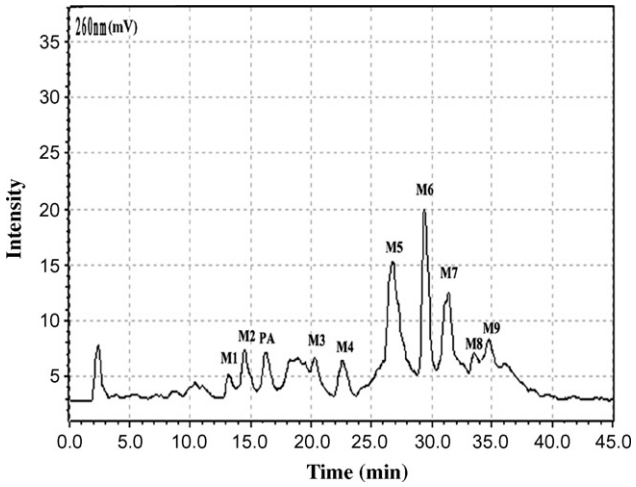

Fig. 1 – LC-UV (260 nm) chromatogram obtained from rat plasma 30 min after intravenously injected 20 mg/kg protocatechuic acid.

3. Results

3.1. PA metabolites identification in rats

The *in vivo* metabolites of PA in rats were characterized by the LC-DAD-MS. Their structures were tentatively proposed from their respective retention time, molecular ion according to previous report [14]. The proposed metabolites labeled M1-M9 in Fig. 1 were summarized in Table 2. M9 was proposed as 3 position methylated metabolite of PA, which was further and definitely determined by comparison their retention times and UV absorption with the authentic compound VA. Besides retention times and UV absorption characters (maximum absorption are 206 and 256 nm), the metabolite M6 were also determined with MS–MS fragment ions (Fig. 2) according to the chemically synthesized vanilloyl-glycine and results definitely evidenced that M6 was vanilloyl-glycine, which was more abundant than its isomer M8, in consistent with the 3 position

Table 2 – LC-MS data and identification of metabolites in rat plasma, liver and heart.

Peak no.	Retention time (min)	$[M-H]^-$	Plasma	Heart	Liver	Proposed metabolites
M1	13.23	329	+	–	+	PA monoglucuronide
M2	14.55	329	+	–	+	PA monoglucuronide
PA	16.38	153	+	+	+	PA
M3	20.49	329	+	–	+	PA monoglucuronide
M4	22.81	343	+	–	+	Methylated PA monoglucuronide
M5	27.01	343	+	–	+	Methylated PA monoglucuronide
M6	29.48	224	+	+[a]	+	Methylated PA monoglycine
M7	31.39	343	+	–	+	Methylated PA monoglucuronide
M8	33.67	224	+	–	+	Methylated PA monoglycine

'+' Indicated metabolites were found; '–' referred to no metabolite were found; PA referred to protocatechuic acid.
[a] Indicated the metabolite peak detected was very small.

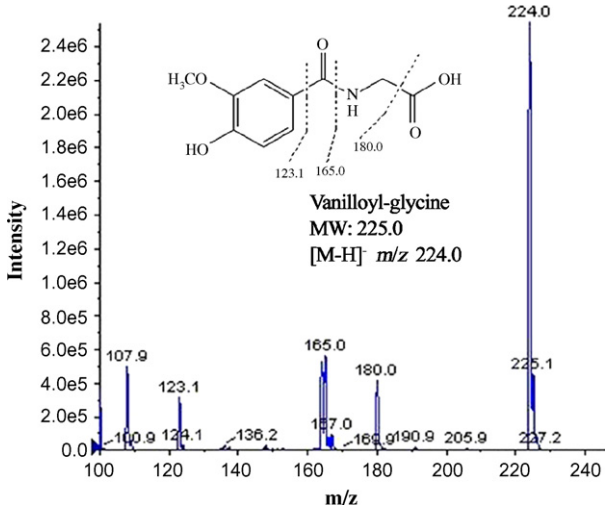

Fig. 2 – MS/MS ions spectra of M6, proposed to be vanilloyl-glycine conjugate.

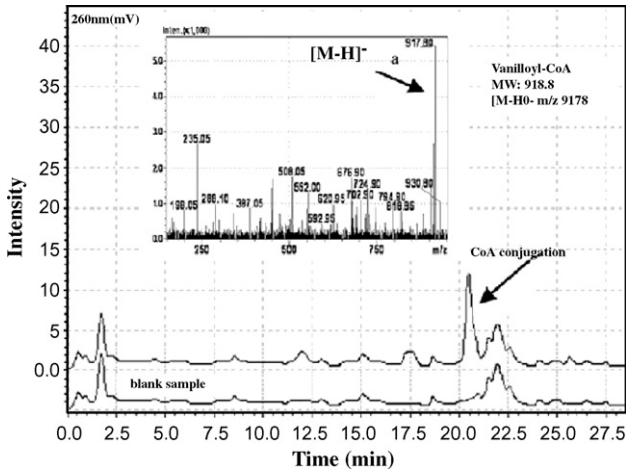

Fig. 3 – The LC-UV (260 nm) chromatogram obtained from rat heart homogenate at 30 min after intravenously injected 20 mg/kg protocatechuic acid.

preference for COMT methylation [30]. As shown in Table 3, in tolcapone pretreated rats, M8 and M6 were both obviously lowered, further supporting their structures presumption.

As shown in Table 2, all these metabolites found in plasma could be detected in liver. But in heart, only M9 (VA) and M6 (vanilloyl-glycine) were detected. As shown in Table 3, M6 was largely decreased in tolcapone pretreated rats, both in liver and heart. Previous report suggested that formation of acyl-CoA thioesters was an obligatory step for glycine conjugation of xenobiotic carboxylic acids [31]. Thus, glycine conjugation metabolite M6, which had been thoroughly determined, strongly supported the formation of acyl-CoA thioester in heart. Besides, we had directly monitored the acyl-CoA thioester metabolite in heart based on HPLC-MS. As shown in Fig. 3, chromatographic comparison with blank and molecular ion demonstrated the formation of the vanilloyl-CoA thioester in heart. Minor acyl-CoA thioester was detected in tolcapone treated rats, both in liver and heart, which was also in line with its structure presumption.

3.2. *Studies of PA metabolism in heart subcellular fractions*

Subcellular incubation results were summarized in Table 4. Results indicated that PA could be methylated into VA in cytosol and microsomes, but not in mitochondria. The formation of VA was completely inhibited by tolcapone, suggesting the methylation was most likely catalyzed by COMT. Mitochondria incubation in acyl-CoA thioester formation system with PA could not result in corresponding acyl-CoA thioester, indicating that PA could not directly conjugate with CoA in mitochondria. In contrast, the methylated metabolite VA in the system formed vanilloyl-CoA thioester, indicating methylation was a prerequisite step for acyl-CoA thioester formation. Additionally, the acyl-CoA formation was completely inhibited by pivalic acid, which implied acyl-CoA synthetase (ACS) participation. Whatever in microsomes or in cytosol under acyl-CoA formation system, no acyl-CoA thioester metabolite was detected for both PA and VA, suggesting acyl-CoA thioester of PA and VA did not form in heart microsomes and cytosol. As shown in Table 4, VA incubated with mitochondria in mixture system resulted in both metabolites of vanilloyl-CoA thioester and glycine conjugation, indicating that mitochondria were also the heart subcellular site for glycine formation. Taken together, it was known that PA was firstly methylated in microsomes and cytosol, and then the methylated metabolite VA was further metabolized into acyl-CoA thioester in mitochondria, where some acyl-CoA thioester was biotransformed into glycine conjugation (Fig. 4).

3.3. *Potential toxicity excluded*

In PA treated rats, no PA and methylated metabolite were detected in the tissue hydrolysis residual, both liver and

Table 3 – The quantification results of protocatechuic acid (PA) and vanilloyl-glycine (M6) in tissues with and without tolcapone inhibiting after 20 mg/kg PA dosing (n = 5).

Tissues	Without tolcapone		With tolcapone		M6 decrease (%)
	PA (μg/g)	M6 (μg/g)	PA (μg/g)	M6 (μg/g)	
Plasma	24.4 ± 2.5	3.0 ± 0.3	50.7 ± 14.0	0.9 ± 0.1	69.7%
Liver	1.2 ± 0.1	1.7 ± 0.2	1.7 ± 0.2	0.8 ± 0.2	51.4%
Heart	2.1 ± 0.7	0.5 ± 0.1	6.6 ± 2.5	0.3 ± 0.0	30.1%

M6 decrease indicated the extent of M6 decrease by catechol-O-methyltransferase inhibitor tolcapone (10 mg/kg).

Table 4 – Subcelluar incubation results in four metabolic systems.

Reactions		Homogenate	Mitochondria	Cytosol	Microsomes
PA	System 1	/	–	+[a]	+[a]
	System 2	/	–	–	–
	System 3	–	/	/	/
	System 4	/	–	/	/
VA	System 2	/	+[b]	–	–
	System 3	+	/	/	/
	System 4	/	++	/	/

PA and VA referred protocatechuic acid and its catechol methylated metabolite vanillic acid, respectively; '+' indicated the corresponding metabolites were found in the metabolic system; '–' referred to no corresponding metabolite was found; '/' represented no experiment was done; '++' referred to both vanilloyl-CoA thioester and vanilloyl-glycine were detected; Systems 1–4 indicated the COMT-mediated O-methylation system, acyl-CoA thioesters formation system, glycine conjugation system and mixture system, respectively.

[a] Indicated the methylation was inhibited by tolcapone.

[b] Indicated the acyl-CoA thioester formation was inhibited by pivalic acid.

kidney, suggesting no protein adducts formed despite of acyl-CoA thioester formation. Similar with PA, benzoic acid did not form any protein adducts. In contrast, we detected large amount of 2-phenylpropionic acid in the residual of liver, which demonstrated that 2-phenylpropionic acid caused liver toxicity due to protein adducts in accordance with previous report [25].

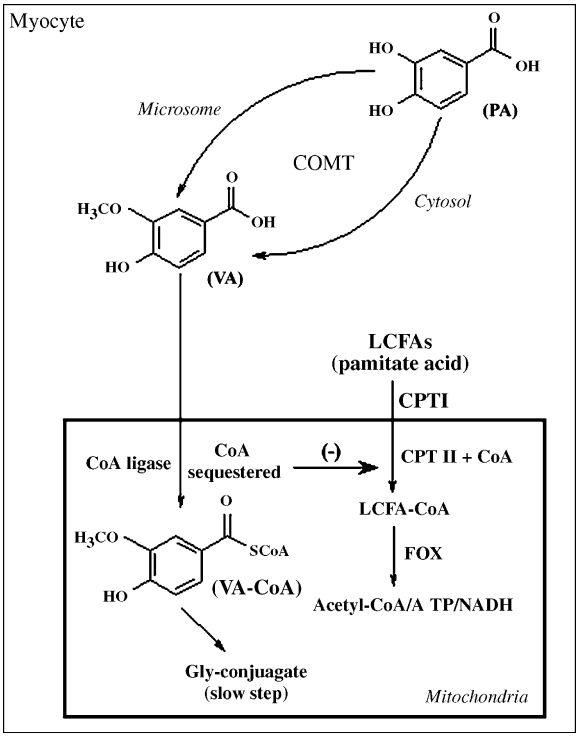

Fig. 4 – The summary of protocatechuic acid (PA) metabolism in subcellular of mytocyte and the proposed mechanism for its fatty acid oxidation inhibition. PA firstly is methylated into vanillic acid (VA) in microsomes and cytosol, then the resulting methylated VA is converted into vanilloyl-CoA (VA-CoA) in mitochondria. COMT referred to catechol-O-methyltransferase, LCFAs indicated long-chain fatty acids and FOX represented fatty acid β-oxidation. CPT referred to carnitine palmityl transferase. The symbol "(-)" indicated inhibiting effect.

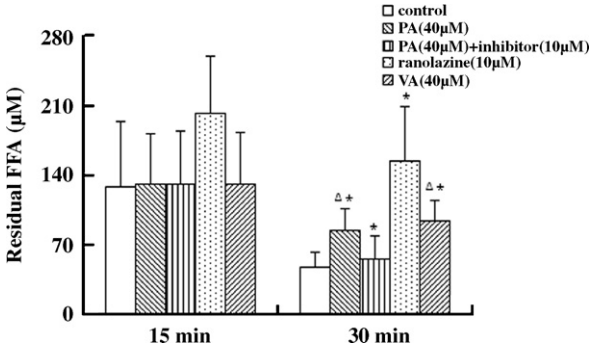

Fig. 5 – The influence of PA on residual fatty acid concentration in perfusate at 15 min and 30 min of isolated heart perfusion. Residual free fatty acid (FFA) indicated residual fatty acid in perfusate. 15 min and 30 min referred to isolated heart perfusion times. PA and VA represented protocatechuic acid and vanillic acid, respectively. Asterisk (*) indicated significant difference compared with control at 30 min ($p < 0.05$), Δ indicated significant difference compared with ranolazine group at 30 min ($p < 0.05$).

3.4. *Isolated heart perfusion assay*

As Fig. 5 indicated, compared with control, no obvious difference was found among all the groups at 15 min except for ranolazine. At 30 min, more residual palmitic acid in perfusate was found in both PA and VA group ($p < 0.05$, *vs* control), demonstrating both PA and VA significantly inhibited palmitic acid oxidation. But their efficacy was largely weaker than ranolazine, a well-known partial FAO inhibitor [32]. As Fig. 5 shown, the inhibiting effect of PA on FAO was totally reversed by its methylation inhibitor tolcapone, implicating relevance of its metabolism to the FAO inhibiting effect. The efficacy of VA was just a little higher than equal concentration of PA and no significance was detected. The close efficacy between PA and VA and the rapid methylation of PA collectively suggested methylation might not be the rate-limited and capacity-limited step for PA to inhibit FAO, despite

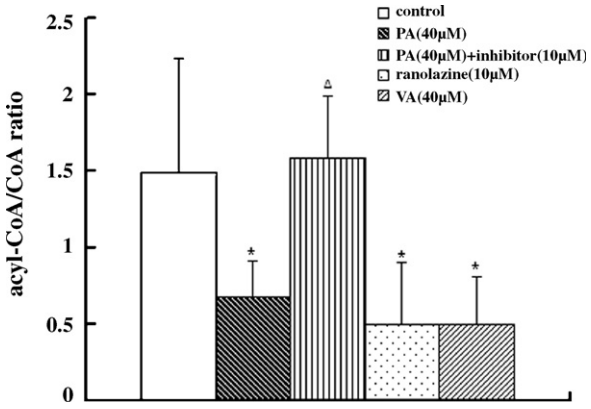

Fig. 6 – The influence of PA on acyl-CoA/CoA ratio in heart after 30 min of isolated heart perfusion. PA and VA represented protocatechuic acid and vanillic acid, respectively. Asterisk (*) indicated significant difference compared with control ($p < 0.05$), Δ indicated significant difference compared with PA group ($p < 0.05$).

the inhibition was totally reversed by methylation inhibitor tolcapone.

As we know, acyl-CoA/free CoA ratio is a modulator and marker for heart energy metabolism. Lower acyl-CoA/free CoA ratio activates pyruvate dehydrogenase (PDH), which is a key and irreversible step in carbohydrate oxidation [33]. As shown in Fig. 6, the acyl-CoA/CoA ratios were largely decreased by PA and VA, implying the shifting from fatty acid oxidation to carbohydrate oxidation. In similar with results from residual fatty acid analysis, the effect of PA was totally reversed by its methylation inhibitor tolcapone.

4. Discussion

In this study, the metabolic profile of PA in rat heart was firstly characterized and a metabolic route was investigated *in vivo* and *in vitro* including several subcellular metabolic incubation systems. Results indicated that PA was firstly methylated in heart cytosol and microsomes, and then the resulting methylated metabolite (VA) diffused to mitochondria and formed vanilloyl-CoA thioester there, which was similar with the process of FAO. Additionally, part of acyl-CoA thioester was further transformed into glycine conjugation, a step also localized within mitochondria. Based on isolated heart perfusion assay, we demonstrated the partial FAO inhibiting effect of PA, which was shown by higher residual fatty acid in perfusate and lower acyl-CoA/CoA ratio in heart. Importantly, the partial FAO inhibiting effect was totally reversed by methylation inhibitor tolcapone, suggesting that the effect was closely related with PA methylation, which had evidenced as a prerequisite step for the identified metabolic pathway of PA in heart. Thus, we concluded that the identified metabolic pathway of PA influenced FAO in heart. A body of evidence had demonstrated that metabolic manipulation, by shifting the energy substrate preference away from fatty acid metabolism and towards glucose metabolism, would enhance the oxygen utilization efficiency and was an effective strategy for ischemia therapy [17,18].

The enzymes responsible for the formation of acyl-CoA thioester are members of the ACS family, which are located in various cell compartments and exhibit wide tissue distribution, with highest activity associated with liver and adipose tissue [34]. Similar with P450, ACS in liver gain more attention due to their importance to whole body energy supply. To date, knowledge is still limited about ACS in heart. Our results demonstrated that ACS for carboxylic acid-containing xenobiotics metabolism also expressed in rat heart. Subcellular fraction results indicated the ACS responsible for PA metabolism located in mitochondria, neither cytosol nor microsomes. Although cytosolic CoA concentration is about 10 times higher than that in mitochondria matrix, CoA sequestration in mitochondria matrix is also regarded as an effective strategy for FAO inhibition, which is beneficial for ischemia heart [17,18]. According to our results, we proposed that the FAO inhibition of PA maybe contribute, at least in part, to CoA sequestration in mitochondria matrix, which was summarized in Fig. 4. The magnitude of CoA sequestration is dependent on the amount of vanilloyl-CoA thioester formation in mitochondria, which is influenced by the rate of conjugation and deconjugation of the thioesters, including direct hydrolysis by acyl-CoA thioesterase [35] or acyltransfered to other metabolites [36]. Although we did not quantitatively and dynamically evaluate the correlation of acyl-CoA thioester formation with its FAO inhibition effect, the relatively low activity of N-acyltransferases in heart mitochondria may cause low rate of acyl-CoA thioester deconjugation and enhance its abundance in mitochondria. In consistent with this, the glycine conjugation formed in heart was low in our results (Table 2). In addition, it is generally noted that the substrates of ACS are relatively hydrophobic and similar with natural substrate fatty acid [33]. The lipophilicity preference of ACS is in concordance with our results that the methylation metabolite VA, but not PA, formed acyl-CoA thioester in mitochondria.

Structurally similar with other phenolic acids in danshen, PA contains catechol group in benzene ring and carboxylic acid in side chain. As for their anti-angina mechanism, until now, researches mainly focused on their anti-oxidant and anti-radical property based on catechol group [37,38]. However, the rapid and extensive methylation of the catechol group may largely discount their antioxidant activity *in vivo*. Here we gave a new explanation for PA anti-angina property based on the side chain carboxylic acid group, which was dependent on catechol methylation. In our opinion, although methylation of PA largely lowers their antioxidant and antiradical activity, it may bring another action that partially inhibits FAO for its anti-angina efficacy. As for phenolic acids in danshen, the metabolic modulation of PA gives us a new clue to understand the activity of these compounds *in vivo*. Actually, some people had investigated the activity of the methylated metabolites of these phenolic acids and endothelial NADPH oxidase inhibiting efficacy was reported [39]. All these results give us a more clear description of the actually active forms of danshen *in vivo* for its anti-angina efficacy.

Current pharmacotherapies for angina include dilating coronary, reducing heart rate and arterial blood pressure.

These traditional hemodynamic approaches are effective as monotherapy, but have little or no additional benefit when used in combination [40–42]. Until now, many reports have investigated the anti-angina mechanism of danshen focusing on hemodynamic improvement, including coronary artery dilatation, calcium antagonist action [43], inhibiting prostaglandin synthesis and platelet adhesion and aggregation [44]. Here we gave a new mechanism about FAO inhibition for PA, which may achieve metabolic manipulation effect for ischemia heart. One of the attractions of metabolic therapies is that they act independently of the traditional mechanisms and are effective as adjunctive therapy [45]. According to our results, metabolic modulation of PA may give greatly synergic effect towards these hemodynamic actions of danshen previously reported. It is well known that herbal medicine always achieve their therapeutic effect based on synergic actions of many components [46], here we provided a pharmacodynamically synergic action for danshen investigation. As our results shown in Figs. 5 and 6, the metabolic modulation efficacy of PA was not as powerful as positive drug ranolazine. However, in our opinion, the relatively weaker metabolic modulation of PA will be enough to give obviously additional benefit to the anti-angina effect of Danshen.

For xenobiotic-CoA thioester metabolites from carboxylic acid-containing drugs, most researches focused on their toxicity because the reactive intermediates always formed irreversible adducts with proteins [24,23]. These adducts can change the protein function or serve as immunogens that lead to immunotoxic reactions [47]. The toxicity of xenobiotic-CoA thioesters has been extensively investigated. However, as for some drugs, despite of acyl-CoA thioesters formation, no obvious protein adducts were detected, such as clofibric acid [48] and simvastatin [49]. In order to exclude potential toxicity due to acyl-CoA thioester, we analyzed potential adducts in liver and kidney and no protein adducts of PA were detected in our results. Until now, no paper has deeply investigated the factors that influence the intermediates toxicity. In our opinion, several factors may relate to it, such as the intermediates formation subcelluar sites and drug retention time in cell. Because the acyl-CoA thioester cannot be excreted directly from cell, if formed, they should be transformed into amino acid-conjugations by acyl-CoA:glycine N-acyltransferases or form protein adducts or directly hydrolyzed. The acyl-CoA:glycine N-acyltransferases locates in mitochondria matrix, which is more effective to metabolize the thioesters formed in mitochondria than other subcellular sites. The abundant glycine conjugation detected in our results implied the effective detoxification of acyl-CoA thioester, which was also formed in mitochondria. Although we excluded the protein adducts of PA after single dose administration, we cannot rule out the possibility under long-time administration and further investigation is still needed.

Acknowledgement

The authors gratefully acknowledge financial support from the National Natural Science Foundation of the People's Republic of China (No. 30772609).

Conflict of interest

The authors state no conflict of interest.

REFERENCES

[1] Delozier TC, Kissling GE, Coulter SJ, Dai D, Foley JF, Bradbury JA, et al. Detection of human CYP2C8, CYP2C9, and CYP2J2 in cardiovascular tissues. Drug Metab Dispos 2007;35:682–8.
[2] Thum T, Borlak J. Gene expression in distinct regions of the heart. Lancet 2000;355:979–83.
[3] Elbekai RH, El-Kadi AO. Cytochrome P450 enzymes: central players in cardiovascular health and disease. Pharmacol Ther 2006;112:564–87.
[4] Gottlieb RA. Cytochrome P450: major player in reperfusion injury. Arch Biochem Biophys 2003;420:262–7.
[5] Walles M, Thum T, Levsen K, Borlak J. Verapamil metabolism in distinct regions of the heart and in cultures of cardiomyocytes of adult rats. Drug Metab Dispos 2001;29:761–8.
[6] Schroeder PE, Wang GQ, Burczynski FJ, Hasinoff BB. Metabolism of the cardioprotective drug dexrazoxane and one of its metabolites by isolated rat myocytes, hepatocytes, and blood. Drug Metab Dispos 2005;33:719–25.
[7] Wu T, Ni J, Wu J. Danshen (Chinese medicinal herb) preparations for acute myocardial infarction. Cochrane Database Syst Rev 2008;CD004465.
[8] Adams JD, Wang R, Yang J, Lien EJ. Preclinical and clinical examinations of Salvia miltiorrhiza and its tanshinones in ischemic conditions. Chin Med 2006;1:3.
[9] Wang X, Morris-Natschke SL, Lee KH. New developments in the chemistry and biology of the bioactive constituents of Tanshen. Med Res Rev 2007;27:133–48.
[10] Zhu YZ, Huang SH, Tan BK, Sun J, Whiteman M, Zhu YC. Antioxidants in Chinese herbal medicines: a biochemical perspective. Nat Prod Rep 2004;21:478–89.
[11] Liu AH, Lin YH, Yang M, Guo H, Guan SH, Sun JH, et al. Development of the fingerprints for the quality of the roots of Salvia miltiorrhiza and its related preparations by HPLC-DAD and LC-MS(n). J Chromatogr B Analyt Technol Biomed Life Sci 2007;846:32–41.
[12] Rao MR, Liu GY, Gao CZ, Liang MD, Zhu SH. Effects of protocatechuic acid and propranolol on myocardial metabolism and infarct size in dogs with acute myocardial infarction. Zhongguo Yao Li Xue Bao 1988;9:27–30.
[13] Rao MR, Liang MD. Effects of protocatechuic acid on myocardial oxygen consumption and tolerance to anoxia in animals (author's transl). Zhongguo Yao Li Xue Bao 1980;1:95–9.
[14] Xu M, Zhang Z, Fu G, Sun S, Sun J, Yang M, et al. Liquid chromatography-tandem mass spectrometry analysis of protocatechuic aldehyde and its phase I and II metabolites in rat. J Chromatogr B Analyt Technol Biomed Life Sci 2007;856:100–7.
[15] Nandi DL, Lucas SV, Webster Jr LT. Benzoyl-coenzyme A:glycine N-acyltransferase and phenylacetyl-coenzyme A:glycine N-acyltransferase from bovine liver mitochondria. Purification and characterization. J Biol Chem 1979;254:7230–7.
[16] Webster LT, Siddiqui UA, Lucas SV, Strong JM, Mieyal JJ. Identification of separate acyl-CoA:glycine and acyl-CoA:L-glutamine N-acyltransferase activities in mitochondrial fractions from liver of rhesus monkey and man. J Biol Chem 1976;251:3352–8.

[17] O'Meara E, McMurray JJ. Myocardial metabolic manipulation: a new therapeutic approach in heart failure? Heart 2005;91:131–2.
[18] Lee L, Horowitz J, Frenneaux M. Metabolic manipulation in ischaemic heart disease, a novel approach to treatment. Eur Heart J 2004;25:634–41.
[19] Jung YJ, Lee JS, Kim YM. Synthesis and in vitro/in vivo evaluation of 5-aminosalicyl-glycine as a colon-specific prodrug of 5-aminosalicylic acid. J Pharm Sci 2000;89: 594–602.
[20] Park JW, Jung WS, Park SR, Park BC, Yoon YJ. Analysis of intracellular short organic acid-coenzyme A esters from actinomycetes using liquid chromatography-electrospray ionization-mass spectrometry. J Mass Spectrom 2007;42:1136–47.
[21] Tracy TS, Wirthwein DP, Hall SD. Metabolic inversion of (R)-ibuprofen, Formation of ibuprofenyl-coenzyme A. Drug Metab Dispos 1993;21:114–20.
[22] Kadowaki M, Ootani E, Sugihara N, Furuno K. Inhibitory effects of catechin gallates on o-methyltranslation of protocatechuic acid in rat liver cytosolic preparations and cultured hepatocytes. Biol Pharm Bull 2005;28:1509–13.
[23] Li C, Olurinde MO, Hodges LM, Grillo MP, Benet LZ. Covalent binding of 2-phenylpropionyl-S-acyl-CoA thioester to tissue proteins in vitro. Drug Metab Dispos 2003;31:727–30.
[24] Quistad GB, Staiger LE, Schooley DA. The role of carnitine in the conjugation of acidic xenobiotics. Drug Metab Dispos 1986;14:521–5.
[25] Li C, Grillo MP, Benet LZ. In vivo mechanistic studies on the metabolic activation of 2-phenylpropionic acid in rat. J Pharmacol Exp Ther 2003;305:250–6.
[26] Wang P, Fraser H, Lloyd SG, McVeigh JJ, Belardinelli L, Chatham JC. A comparison between ranolazine and CVT-4325, a novel inhibitor of fatty acid oxidation, on cardiac metabolism and left ventricular function in rat isolated perfused heart during ischemia and reperfusion. J Pharmacol Exp Ther 2007;321:213–20.
[27] Arin MJ, Diez MT, Resines JA. Rapid and simple method for the determination of urinary benzoic and phenylacetic acids and their glycine conjugates in ruminants by reversed-phase high-performance liquid chromatography. J Chromatogr 1992;582:13–8.
[28] Cremin Jr JD, McLeod KR, Harmon DL, Goetsch AL, Bourquin LD, Fahey Jr GC. Portal and hepatic fluxes in sheep and concentrations in cattle ruminal fluid of 3-(4-hydroxyphenyl)propionic, benzoic, 3-phenylpropionic, and trans-cinnamic acids. J Anim Sci 1995;73:1766–75.
[29] Corkey BE. Analysis of acyl-coenzyme A esters in biological samples. Methods Enzymol 1988;166:55–70.
[30] Funaki T, Onodera H, Ushiyama N, Tsukamoto Y, Tagami C, Fukazawa H, et al. The disposition of the tolcapone 3-O-methylated metabolite is affected by the route of administration in rats. J Pharm Pharmacol 1994;46:571–4.
[31] Tanaka Y, Shimomura Y, Hirota T, Nozaki A, Ebata M, Takasaki W, et al. Formation of glycine conjugate and (−)-(R)-enantiomer from (+)-(S)-2-phenylpropionic acid suggesting the formation of the CoA thioester intermediate of (+)-(S)-enantiomer in dogs. Chirality 1992;4:342–8.
[32] Bhandari B, Subramanian L. Ranolazine, a partial fatty acid oxidation inhibitor, its potential benefit in angina and other cardiovascular disorders. Recent Patents Cardiovasc Drug Discov 2007;2:35–9.
[33] Eaton S, Middleton B, Sherratt HS, Pourfarzam M, Quant PA, Bartlett K. Control of mitochondrial beta-oxidation at the levels of [NAD+]/[NADH] and CoA acylation. Adv Exp Med Biol 1999;466:145–54.
[34] Knights KM, Drogemuller CJ. Xenobiotic-CoA ligases: kinetic and molecular characterization. Curr Drug Metab 2000;1:49–66.
[35] Gerber LK, Aronow BJ, Matlib MA. Activation of a novel long-chain free fatty acid generation and export system in mitochondria of diabetic rat hearts. Am J Physiol Cell Physiol 2006;291:C1198–207.
[36] Knights KM, Sykes MJ, Miners JO. Amino acid conjugation: contribution to the metabolism and toxicity of xenobiotic carboxylic acids. Expert Opin Drug Metab Toxicol 2007;3:159–68.
[37] Zhao GR, Zhang HM, Ye TX, Xiang ZJ, Yuan YJ, Guo ZX, et al. Characterization of the radical scavenging and antioxidant activities of danshensu and salvianolic acid B. Food Chem Toxicol 2008;46:73–81.
[38] Jiang RW, Lau KM, Hon PM, Mak TC, Woo KS, Fung KP. Chemistry and biological activities of caffeic acid derivatives from Salvia miltiorrhiza. Curr Med Chem 2005;12:237–46.
[39] Steffen Y, Gruber C, Schewe T, Sies H. Mono-O-methylated flavanols and other flavonoids as inhibitors of endothelial NADPH oxidase. Arch Biochem Biophys 2008;469:209–19.
[40] Hunt SA, Baker DW, Chin MH, Cinquegrani MP, Feldman AM, Francis GS, et al. ACC/AHA guidelines for the evaluation and management of chronic heart failure in the adult: executive summary a report of the American College of Cardiology/American Heart Association Task Force on practice guidelines (committee to revise the 1995 guidelines for the Evaluation and Management of Heart Failure): developed in collaboration with the International Society for Heart and Lung Transplantation; endorsed by the Heart Failure Society of America. Circulation 2001;104:2996–3007.
[41] Bristow MR, Gilbert EM, Abraham WT, Adams KF, Fowler MB, Hershberger RE, et al. Carvedilol produces dose-related improvements in left ventricular function and survival in subjects with chronic heart failure. MOCHA investigators. Circulation 1996;94:2807–16.
[42] Colucci WS, Packer M, Bristow MR, Gilbert EM, Cohn JN, Fowler MB, et al. Carvedilol inhibits clinical progression in patients with mild symptoms of heart failure. US Carvedilol Heart Failure Study Group. Circulation 1996;94:2800–6.
[43] Lam FF, Yeung JH, Chan KM, Or PM. Relaxant effects of danshen aqueous extract and its constituent danshensu on rat coronary artery are mediated by inhibition of calcium channels. Vascul Pharmacol 2007;46:271–7.
[44] Nie R, Xia R, Zhong X, Xia Z. Salvia miltiorrhiza treatment during early reperfusion reduced postischemic myocardial injury in the rat. Can J Physiol Pharmacol 2007;85:1012–9.
[45] Stanley WC, Sabbah HN. Metabolic therapy for ischemic heart disease: the rationale for inhibition of fatty acid oxidation. Heart Fail Rev 2005;10:275–9.
[46] Li XJ, Zhang HY. Synergy in natural medicines: implications for drug discovery. Trends Pharmacol Sci 2008;29:331–2.
[47] Boelsterli UA. Xenobiotic acyl glucuronides and acyl CoA thioesters as protein-reactive metabolites with the potential to cause idiosyncratic drug reactions. Curr Drug Metab 2002;3:439–50.
[48] Bronfman M, Morales MN, Amigo L, Orellana A, Nunez L, Cardenas L, et al. Hypolipidaemic drugs are activated to acyl-CoA esters in isolated rat hepatocytes. Detection of drug activation by human liver homogenates and by human platelets. Biochem J 1992;284(Pt 1):289–95.
[49] Prueksaritanont T, Ma B, Fang X, Subramanian R, Yu J, Lin JH. beta-Oxidation of simvastatin in mouse liver preparations. Drug Metab Dispos 2001;29:1251–5.