Godard MP, Johnson BA, Richmond SR. 2005. Body composition and hormonal adaptations associated with forskolin consumption in overweight and obese men. *Obes. Res.* **13**: 1335–1343.

Grundy SM. 2004. Obesity, metabolic syndrome, and cardiovascular disease. *J. Clin. Endocrinol. Metab.* **89**: 2595–2600.

Han LK, Morimoto C, Yu RH, Okuda H. 2005. Effects of Coleus forskohlii on fat storage in ovariectomized rats. *Yakugaku Zasshi* **125**: 449–453.

Henderson S, Magu B, Rasmussen C, Lancaster S, Kerksick C, Smith P, Melton C, Cowan P, Greenwood M, Earnest C, Almada A, Milnor P, Magrans T, Bowden R, Ounpraseuth S, Thomas A, Kreider RB. 2005. Effects of coleus forskohlii supplementation on body composition and hematological profiles in mildly overweight women. *J. Int. Soc. Sports. Nutr.* **2**: 54–62.

Hori M, Oniki K, Nakagawa T, Takata K, Mihara S, Marubayashi T, Nakagawa K. 2009. Association between combinations of glutathione-S-transferase M1, T1 and P1 genotypes and non-alcoholic fatty liver disease. *Liver Int.* **29**: 164–168.

Ibdah JA, Perlegas P, Zhao Y, Angdisen J, Borgerink H, Shadoan MK, Wagner JD, Matern D, Rinaldo P, Cline JM. 2005. Mice heterozygous for a defect in mitochondrial trifunctional protein develop hepatic steatosis and insulin resistance. *Gastroenterology* **128**: 1381–1390.

Kamisoyama H, Honda K, Tominaga Y, Yokota S, Hasegawa S. 2008. Investigation of the anti-obesity action of licorice flavonoid oil in diet-induced obese rats. *Biosci. Biotechnol. Biochem.* **72**: 3225–3231.

Lara-Castro C, Fu Y, Chung BH, Garvey WT. 2007. Adiponectin and the metabolic syndrome: mechanisms mediating risk for metabolic and cardiovascular disease. *Curr. Opin. Lipidol.* **18**: 263–270.

Laurenza A, Sutkowski ME, Seamon KB. 1989. Forskolin: a specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action? *Trends Pharmacol. Sci.* **10**: 442–447.

Loria P, Lonardo A, Carulli L, Verrone AM, Ricchi M, Lombardini S, Rudilosso A, Ballestri S, Carulli N. 2005. Review article: the metabolic syndrome and non-alcoholic fatty liver disease. *Aliment. Pharmacol. Ther.* **22**: 31–36.

Ohnuma T, Anan E, Hoashi R, Takeda Y, Nishiyama T, Ogura K, Hiratsuka A. 2011. Dietary diacetylene falcarindiol induces phase 2 drug-metabolizing enzymes and blocks carbon tetrachloride-induced hepatotoxicity in mice through suppression of lipid peroxidation. *Biol. Pharm. Bull.* **34**: 371–378.

Okuda H, Morimoto C, Tsujita T. 1992. Relationship between cyclic AMP production and lipolysis induced by forskolin in rat fat cells. *J. Lipid Res.* **33**: 225–231.

Reagan-Shaw S, Nihal M, Ahmad N. 2008. Dose translation from animal to human studies revisited. *FASEB J.* **22**: 659–661.

Reeves PG, Nielsen FH, Fahey GC Jr. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **123**: 1939–1951.

Sallie R, Tredger JM, Williams R. 1991. Drugs and the liver. Part 1: Testing liver function. *Biopharm. Drug Dispos.* **12**: 251–259.

Stewart LK, Soileau JL, Ribnicky D, Wang ZQ, Raskin I, Poulev A, Majewski M, Cefalu WT, Gettys TW. 2008. Quercetin transiently increases energy expenditure but persistently decreases circulating markers of inflammation in C57BL/6J mice fed a high-fat diet. *Metab. Clin. Exp.* **57**: S39–S46.

Su GM, Sefton RM, Murray M. 1999. Down-regulation of rat hepatic microsomal cytochromes P-450 in microvesicular steatosis induced by orotic acid. *J. Pharmacol. Exp. Ther.* **291**: 953–959.

Unger RH, Clark GO, Scherer PE, Orci L. 2010. Lipid homeostasis, lipotoxicity and the metabolic syndrome. *Biochim. Biophys. Acta* **1801**: 209–214.

Virgona N, Taki Y, Umegaki K. 2010. A rapid HPLC with evaporative light scattering method for quantification of forskolin in multi-herbal weight-loss solid oral dosage forms. *Pharmazie* **65**: 322–326.

Virgona N, Yokotani K, Yamazaki Y, Shimura F, Chiba T, Taki Y, Yamada S, Shinozuka K, Murata M, Umegaki K. 2012. *Coleus forkohlii* extract induces hepatic cytochrome P450 enzymes in mice. *Food Chem. Toxicol.* **50**: 750–755.

Wendel AA, Purushotham A, Liu LF, Belury MA. 2008. Conjugated linoleic acid fails to worsen insulin resistance but induces hepatic steatosis in the presence of leptin in ob/ob mice. *J. Lipid Res.* **49**: 98–106.

Yang Y, Cheng JZ, Singhal SS, Saini M, Pandya U, Awasthi S, Awasthi YC. 2001. Role of glutathione S-transferases in protection against lipid peroxidation. *J. Biol. Chem.* **276**: 19220–19230.

応用薬理 Ōyō Yakuri
Pharmacometrics **82** (5/6) 67-73 (2012)

Coleus forskohlii エキス中の肝シトクローム P450 誘導物質の推定

横谷　馨倫[1,2], 千葉　剛[1], 佐藤　陽子[1], 窪田　洋子[3], 渡邉　泰雄[3], 村田　容常[2], 梅垣　敬三[1]*

[1]〒162-8636　東京都新宿区戸山 1-23-1　独立行政法人　国立健康・栄養研究所　情報センター
[2]〒112-8610　東京都文京区大塚 2-1-1　お茶の水女子大学大学院　人間文化創成科学研究科
[3]〒362-0806　埼玉県北足立群伊奈町小室 10281　日本薬科大学　薬学科

Estimation of Components which Induce Mice Cytochrome P450 in *Coleus forskohlii* Extract

Kaori Yokotani[1,2], Tsuyoshi Chiba[1], Yoko Sato[1], Yoko Kubota[3], Yasuo Watanabe[3], Masatsune Murata[2], Keizo Umegaki[1]*

[1]*Information Center, National Institute of Health and Nutrition; 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8636, Japan*
[2]*Department of Nutrition and Food Science, Ochanomizu University; 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan*
[3]*School of Medical Pharmaceutical Sciences, Nihon Pharmaceutical University; 10281 Komura, Ina-Machi, Kita-Adachi-gun, Saitama 362-0806, Japan*

Received May 18, 2012, Accepted June 15, 2012

Drug-herb interaction is a major concern for the safety use of herbal products. In our previous mice study, we found that *Coleus forskohlii* extract (CFE) markedly induced hepatic cytochrome P450 (CYP), especially CYP2B, 2C and 3A type at the dose added to weight loss supplements. Also, we showed that forskolin, an active constituent in CFE, was not involved in the CYP induction *in vivo*. The present study was designed to estimate the compounds inducing CYP. CFE was fractionated into 4 (diethyl ether-, ethyl acetate-, acetone-, the remainder), and the effect of those fractions on CYP was examined in two systems: test materials were fed to mice for 2 weeks (*in vivo* system), and those were directly added to CYP3A enzyme assay (*in vitro* system). It was found that CYP inducing activity *in vivo* was mainly distributed in the diethyl ether-fraction, which also showed a direct inhibition of CYP3A activity *in vitro*. The water soluble fraction showed neither CYP induction *in vivo* nor CYP3A inhibition *in vitro*. It was also suggested the existence of several compounds inducing CYP, some of which was eliminated during the fractionation procedure. CFE-induced CYP induction *in vivo* was well correlated with an increase in liver weight, and was related to direct inhibition of CYP enzyme activity *in vitro*. Combination of these characteristics would be useful for further study to identify the active constituents in CFE materials that induce CYP.

Keywords: *Coleus forskohlii* / hepatic CYP / forskolin / herb / weight loss supplement

緒　言

インド地方の熱帯や亜熱帯地域に自生するシソ科の植物 *Coleus forskohlii* は，伝統医学アーユルベーダにおいて，心血管疾患や中枢神経系疾患，呼吸器疾患，腎疾患などの治療に用いられてきた(de Souza, et al., 1983). *Coleus forskohlii* の薬理作用は，根に含まれるジテルペンの forskolin がアデニル酸シクラーゼを活性化して cAMP 濃度を上昇させることにより発現すると考えられている(Bauer, et al., 1993; Baumann, et al., 1990). Forskolin による cAMP 濃度の上昇は，ホルモン感受性リパーゼを活性化して脂肪分解を促進することから(Allen, et al., 1986), *Coleus forskohlii* エキス (CFE) は，ダイエット効果が期待できるサプリメント素材として多用されている (Godard, et al., 2005). 近年のダイエットブームと，ハーブ類は「天然だから安全」との消費者のイメージから，CFE を含むサプリメントの利用者は益々

*Correspondence author: 梅垣　敬三
〒162-8636　東京都新宿区戸山 1-23-1
独立行政法人　国立健康・栄養研究所　情報センター
Tel: 03-3203-5721　Fax: 03-3202-3278
E-mail: umegaki@nih.go.jp

増加してくると想定される．CFE を安全かつ有効に利用するためには，CFE の安全性を確保することが重要になる．Forskolin の経口投与における LD_{50} は，ラットで 2,550 mg/kg，マウスでは 3,100 mg/kg である．また，CFE の腹腔内投与による LD_{50} はマウスで 375 mg/kg と報告されている (de Souza et al., 1983)．現時点で CFE の経口摂取によるヒトでの有害事象の報告は見当たらない．しかし，CFE 自体に有害性がなくても，医薬品等の併用による相互作用が，安全性に影響する可能性が考えられる．例えば，セントジョーンズワートは，CYP3A 誘導と P 糖タンパク活性化により，併用医薬品の薬効を減弱させることが明らかにされている (Mannel, 2004)．そこで著者らは CFE と医薬品の相互作用を検討する目的で，先ず CFE の薬物代謝酵素の誘導の有無を検討した．その結果，CFE は肝シトクローム P450 (CYP) を強く誘導すること，その誘導作用は CYP2B とともに医薬品代謝の 70%を占める CYP2C と CYP3A (Rendic, et al., 1997; Tompkins, et al., 2007) で著しいことを認めた (Virgona, et al., 2011)．CFE の CYP 誘導は，投与量 60 mg/kg から認められ，この量は Regan-Shaw ら (Reagan-Shaw, et al., 2008) の方法によるヒト摂取量への換算では約 5 mg/kg となり，一般的なサプリメントからの摂取量の範囲内であった．また，CFE の活性成分である forskolin をマウスに投与しても CYP の誘導はほとんど認められなかったことから，forskolin 以外の成分が CYP 誘導に関与していることが示唆された (Virgona et al., 2011)．Ding ら (Ding, et al., 2005) は，肝細胞を用いた *in vitro* 系において CFE による CYP 誘導を報告しているが，彼らは forskolin が CYP 誘導作用を持つことを示しており，著者らの *in vivo* の結果とは異なっていた．これまでヒトを含めて *in vivo* において CFE と医薬品の相互作用を示した報告は認められない．

CFE 中の CYP 誘導成分，すなわち医薬品との相互作用に影響する成分が特定できれば，その成分の製品からの除去，あるいは減量などによって，医薬品との相互作用の影響を回避する方策を立てることができる．ちなみに，セントジョーンズワート中の相互作用に関連する成分は hyperforin と考えられ，hyperforin 含量の少ないセントジョーンズワート製品では，経口避妊薬との相互作用を認めないことがヒト試験において報告されている (Will-Shahab, et al., 2009)．

そこで本研究では，CFE 中に存在する CYP 誘導成分を明らかにする目的で，CFE を溶媒により 4 分画し，それらの画分の CYP 誘導作用を，マウスの *in vivo* 評価系および肝マイクロソームを用いた *in vitro* 評価系において検討し，CFE 中に含まれる CYP 誘導物質を推定した．

方　法

1．試薬と飼料

実験に利用した CFE は，既報 (Virgona et al., 2011) と同様に (株) 常磐植物化学研究所より提供を受けた．その CFE はインドのバンガロールで採取した *Coleus forskohlii* 乾燥根を粉砕し，超臨界抽出法により forskolin が豊富に含まれる画分 (20–30%) を調製し，その後に 10% forskolin になるようにデキストリンを添加したものである．一般成分は，水分 5.6%，タンパク質 0.3%，脂質 22.7%，灰分 2.2%，炭水化物 69.2%であった．CYP2B，CYP2C，CYP3A の測定試薬は，シグマ・アルドリッチ (株) (St Louis, MO, USA) より，NADPH はオリエンタル酵母工業 (株) より購入した．*In vitro* の CYP 阻害実験に利用した P450-Glo™ CYP3A4 測定キット (Luciferin-PPXE) はプロメガ (株) (Madison, WI, USA)，その他の試薬は全て和光純薬工業 (株) より購入した．

飼料は Table 1 に示した AIN93G を基本とした Patten ら (Patten, et al., 2004) の組成のものを利用し，これに CFE ならびに CFE の各画分を添加した．飼料に添加した CFE ならびに CFE の各画分は，CFE として 1% (w/w) 相当として調製した．飼料の原材料となるコーンスターチ，カゼイン，α-セルロース，ミネラル (AIN93G)，ビタミン (AIN93G) 等はオリエンタル酵母工業 (株) より購入した．

Table 1　Composition of experimental diets.

Ingredient	Diet
	g
Cornstarch	690.686
Sucrose	40
Casein	140
Sunflower oil	30
Cellulose	50
Vitamin mixture (AIN93G)	10
Mineral mixture (AIN93G)	35
L-Cystine	1.8
Choline hydrogen tartrate	2.5
Tertiary butylhydroquinone	0.014
Total	1000

2．CFE の分画

CFE は既報 (Umegaki, et al., 2007) の方法に準じて 4 分画した (Fig. 1)．具体的には，CFE 粉末 100 g を水 2,000 mL に溶解し，ジエチルエーテル 2,000 mL を加えて分液ロートにて十分に振盪し，ジエチルエーテル層を減圧濃縮乾固して画分 1 を調製した (17.11 g)．一方，水層には酢酸エチル 2,000 mL を加え，同様に振盪して酢酸エチル層を減圧濃縮乾固して画分 2 を調製した (4.57 g)．残った水層を減圧濃縮乾固し，アセトン 1,000 mL を加えて 35℃で 4 時間撹拌し，吸引濾過した．このアセトン層を減圧濃縮乾固して画分 3

を調製した (1.03 g). またアセトン不溶部の残渣を乾固して画分 4 とした (76.05 g). 各画分中に含まれていた forskolin の含有率は，画分 1 が 41.8%，画分 2 が 39.7%，画分 3 が 18.8%，画分 4 が 0.001%であった.

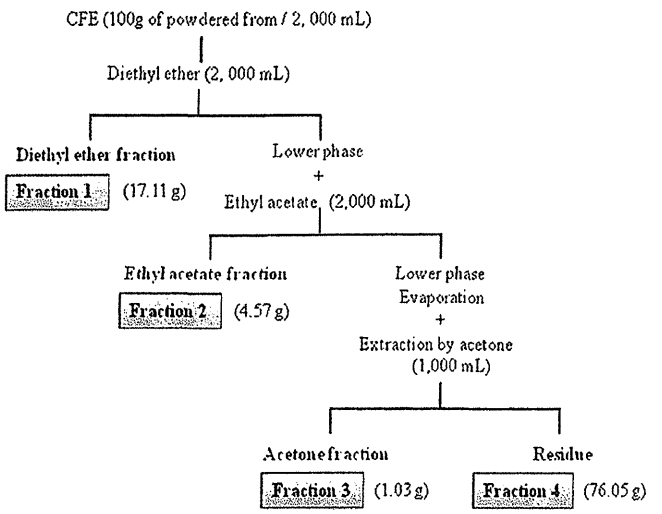

Fig. 1 Fractionation of *C. forskohlii* extract

3. 動物実験

ICR 系雄マウス 4 週齢（日本クレア（株））を利用し，室温 23±1℃，12 時間の明暗サイクルの環境で飼育した. 5 日間の予備飼育の後に，対照食，CFE 食，CFE の各画分食，全画分混合物食を 2 週間摂取させた. 前述のように飼料に添加した CFE ならびに CFE の各画分は CFE として 1% (w/w) に調製し，飼料と水は自由摂取させた. 飼料中 1 kg の被検物質の添加量は，画分 1 が 1.73 g，画分 2 が 0.48 g，画分 3 が 0.10 g，画分 4 が 7.7 g，画分 1-4 の混合物と未分画 CFE が 10 g/kg であり，これらの添加量に相当するコーンスターチ量を飼料から減量した. マウスは一夜絶食させた後，ペントバルビタール麻酔下で開腹し，直ちに肝臓を摘出して重量を測定し，CYP 測定まで-80℃にて保存した. 以上の動物実験は（独）国立健康・栄養研究所実験動物倫理委員会の承認を得，同委員会のガイドラインに準じて行った.

4. 酵素活性測定法

肝マイクロソームは既報 (Umegaki, et al., 2002) に従って調製し，CYP 含量は Omura ら (Omura, et al., 1964) の方法，CYP2B 活性 (Pentoxyresorufin *O*-dealkylase)，CYP2C 活性 ((*S*)-warfarin 7-hydroxylase)，CYP3A 活性 (testosterone 6β-hydroxylase) はそれぞれ HPLC 法 (Umegaki et al., 2002) により測定した. 被検物質の CYP3A 活性に対する *in vitro* での影響は，CFE を 2 週間投与したマウス肝マイクロソームを CYP 酵素として用い，CYP3A 測定用キット (P450-Glo™ CYP3A4) により評価した. CYP3A の阻害活性は，酵素反応により生成した発光シグナルをルミノメーター (GloMax™96, Promega Co.) で測定し，コントロールに対する割合 (%) で算出した. この測定系の CYP3A に対する特異性は，ketoconazole の阻害により確認した.

タンパク量は，Pierce™ BCA タンパク測定キット (Thermo Fisher Scientific Inc., Rockford, IL, USA) により定量した.

5. 統計処理

各データは平均±標準誤差で示した. 統計処理は Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) を用い，一元配置分散分析を実施後，Tukey の多重比較検定により各群間の有意差を検定した. 肝臓重量と CYP の関係については，Pearson の相関係数を求めた. $P<0.05$ 以下を有意とした.

結　果

1. *In vivo* における CFE 画分の肝 CYP の誘導作用

1% CFE 添加食またはそれに相当する CFE の各画分の添加食を 2 週間混餌投与したとき，マウスの体重と摂餌量は，対照群と各 CFE 添加飼料群で差異はなかった (Table 1). 肝重量/体重比は，CFE 画分群の中で画分 1 群が最も高値であった. この画分 1 群の肝重量/体重比は，全画分混合物群（画分 1 から 4 の混合）と有意な差異はなかったが，未分画 CFE 群よりも有意に低かった. 肝 CYP の総量，CYP2B，CYP2C，CYP3A の活性は，肝重量/体重比とほぼ同様の変化を示し，4 つの画分群の中では画分 1 群が最も高く，全画分混合物群が画分 1 群よりも高い傾向を示した (Fig. 2). 全画分混合物群と未分画 CFE 群を比べると，CYP2B 活性と CYP2C 活性では大きな違いが認められたが，CYP 含量と CYP3A 活性の違いは比較的小さかった. Table 1 の肝重量/体重比と Fig. 2 の CYP 含量や各 CYP 活性には，ほぼ同様の変化が認められ，有意な正相関を示した (Fig. 3).

2. *In vitro* における CFE 画分の肝 CYP3A 活性の阻害作用

CFE ならびに各画分の CYP 阻害作用を *in vitro* において検討した (Fig. 4). CYP サブタイプは高い活性が認められた CYP3A とし，測定系への被検物質の添加量は一定濃度とした (2.5, 7.5, 25 μg/mL). その結果，分画していない CFE ならびに画分 1，2，3 は，それぞれ濃度依存的に CYP3A 活性を阻害し，画分 4 は阻害作用を示さなかった. 画分 4 において CYP 阻害活性がなかったことは，Fig. 2 の *in vivo* において CYP 誘導作用を認めなかった結果とよく一致した. この被検物質濃度を一定にした *in vitro* 条件では，分画していない CFE に比べて，画分 1，画分 2，画分 3 の CYP 阻害濃度は低い値となった.

考　察

著者らのこれまでの研究から，CFE による肝 CYP 誘導には，CFE の活性成分である forskolin 以外の成分が関与していると考えられた (Virgona et al., 2011). そこで，本研究では CFE をジエチルエーテル，酢酸エチル，アセトンを用いて 4 つに分画し，マウスの *in vivo* 実験系と肝マイクロソー

Table 2 Body weight, liver weight and food intake of mice fed diets containing either fractionated or unfractionated *C. forskohlii* extract (CFE).

	Control	Fractionated extracts F1	F2	F3	F4	Sum of F1-4	Unfractionated Extract
Final body weight (g)	32.7 ± 0.8	34.9 ± 1.3 [1.1]	32.6 ± 1.0 [1.0]	33.3 ± 0.9 [1.0]	32.9 ± 0.8 [1.0]	34.9 ± 0.8 [1.1]	31.8 ± 0.6 [0.97]
Liver weight (% /body weight)	4.30 ± 0.11	5.80 ± 0.12 [1.3][a]	5.11 ± 0.022 [1.2]	4.48 ± 0.086 [1.0]	4.27 ± 0.043 [0.99]	6.57 ± 0.27 [1.5][a]	9.92 ± 0.65 [2.3][abc]
Average dairy food intake (g)	4.5 ± 0.12	4.5 ± 0.22 [0.99]	4.5 ± 0.14 [1.0]	4.5 ± 0.063 [1.0]	4.5 ± 0.067 [0.99]	4.5 ± 0.10 [0.99]	4.5 ± 0.20 [1.0]

Male ICR mice were fed diets containing either fractionated or unfractionated *C. forskohlii* extract (CFE) at the dose equivalent of 1% (w/w) CFE for 2 weeks.

Values are expressed as means and SE for 5 mice.

[a]: Significant difference from control group at $p<0.05$.

[b]: Significant difference from fraction 1 group at $p<0.05$.

[c]: Significant difference from fraction 1-4 mixture group at $p<0.05$.

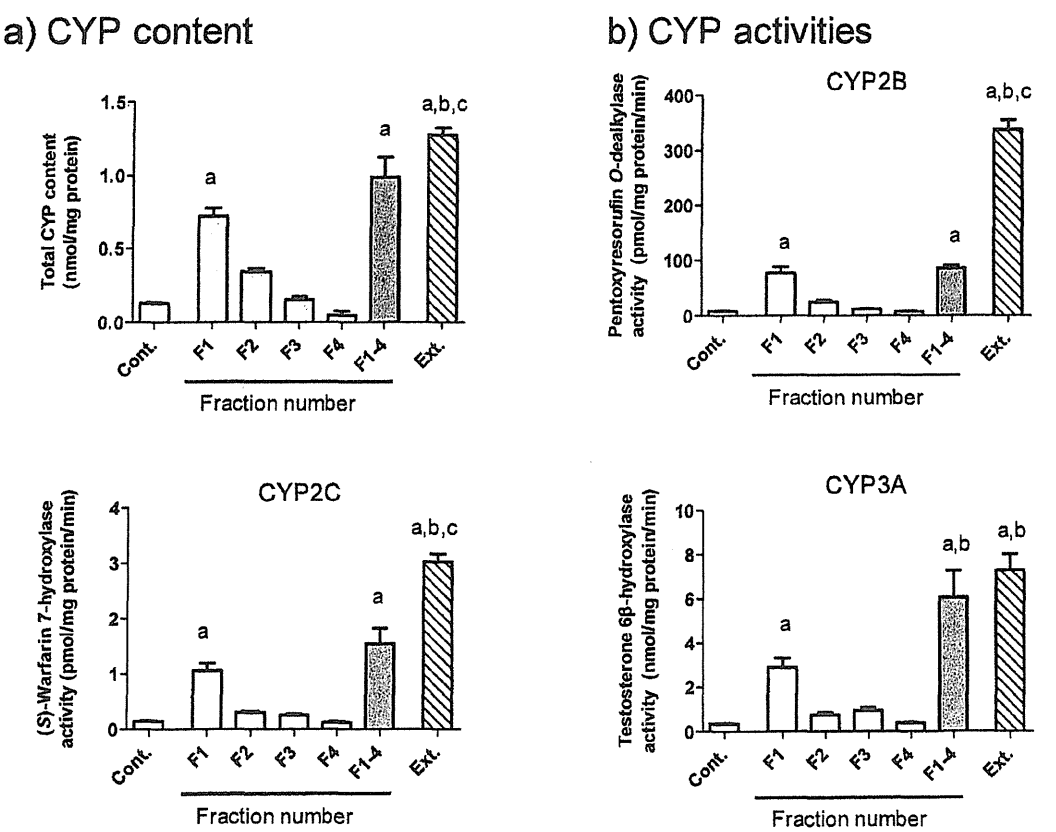

Fig. 2 Hepatic CYP in mice fed diets containing either fractionated or unfractionated *C. forskohlii* extract (CFE).

Each value is the means and SE for 5 mice. Cont., control; F, fraction; Ext., unfractionated extract.

[a]: Significant difference from control group at $p<0.05$.

[b]: Significant difference from fraction 1 group at $p<0.05$.

[c]:Significant difference from fraction 1-4 mixture group at $p<0.05$.

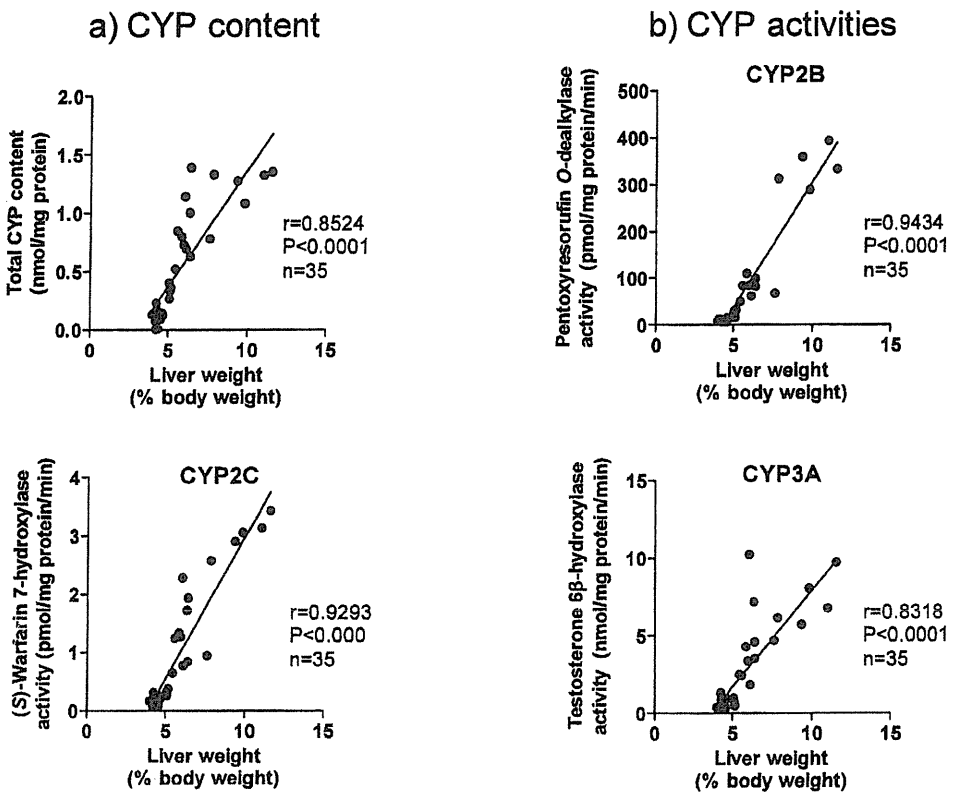

Fig. 3 Correlation between the liver weight/body weight and hepatic CYP content or activities.
Data was from Table 1 and Fig. 2.

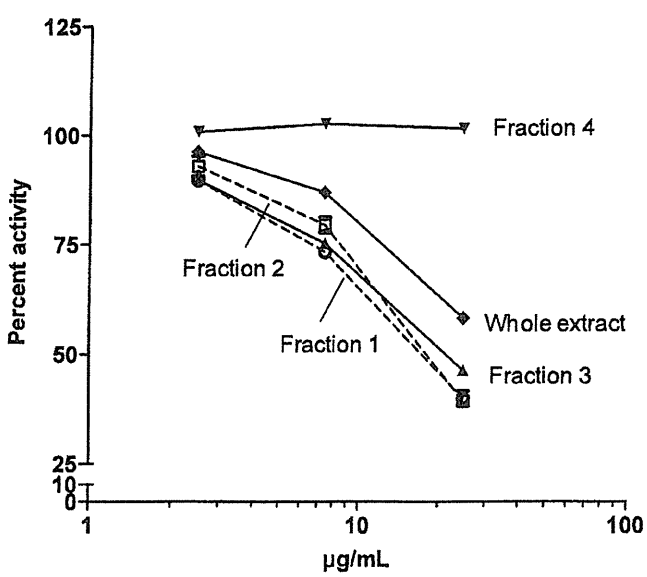

Fig. 4 Inhibition of CYP3A activity by CFE whole extract or its fractionated samples.
CFE (whole extract) or fractionated samples were added into CYP3A assay medium at the concentration of 2.5-25 μg/mL. CFE-treated mouse liver microsome was used as CYP3A enzyme.
Values are expressed as means and SE of duplicate determinations.

ムを用いた *in vitro* 実験系においてCYP誘導成分の検索を試みた．*In vivo* の試験結果から，CYP誘導成分は画分1(エーテル画分) に多く含まれ，画分2，3になるほどその活性が低くなり，画分4にはほとんど含まれていないことが明らかとなった．また，*in vitro* の CYP3A に対する直接作用を検討した結果では，CFE ならびに画分1から3が CYP の阻害活性を示し，画分4には阻害活性は認められなかった．以上の結果から，CFE の誘導成分はエーテル可溶で水に難溶の特性を有していることが推定された．画分4に活性がないことは，*in vitro* の CYP に対する直接作用でも確認された．CFE で認めた *in vivo* における CYP 誘導と *in vitro* における CYP 活性阻害は，*in vitro* の CYP 活性の測定に利用した基質と CYP 誘導成分が，競合阻害，非競合阻害，不競合阻害のいずれかの阻害様式を示した結果と考えられる．本研究では酵素の阻害様式については検討していないが，競合阻害と考えると，*in vivo* と *in vitro* で認めた現象は解釈しやすい．

マウスに forskolin を投与した前報の実験結果から，CFE 中の CYP 誘導成分は forskolin ではないことが示されている (Virgona et al., 2011)．本実験において，各画分中の forskolin の含有率は，画分1が 41.8%，画分2が 39.7%，画分3が 18.8%，画分4が 0.001%であった．画分1と2では forskolin の含有率はほぼ同程度であったが，CYP 含量と活性から示された CYP 誘導作用は，画分1に対して画分2でおよそ 1/2 であった．この各画分中の forskolin 含量と CYP 誘導作用の関係が一致しない結果からも，CFE 中に含まれる CYP 誘導成分は forskolin 以外の成分と考えられる．

CYP の誘導において，各画分を合わせた群が画分1群よりも高かったことは，検索している CYP 誘導成分が画分2や3にもある程度分散していることから概ね説明することができる．しかし，各画分を合わせた群が，未分画群よりも有意に低いこと，また，その影響の程度が CYP 含量や CYP3A に比べて CYP2B や CYP2C で著しいことは説明できない．これは CYP 誘導成分が，分画操作において一部除去されている可能性や CYP 誘導作用を有する成分が複数存在している可能性を示している．例えば，CFE 中には forskolin の類縁体である 1,9-dideoxyforskolin，7-deacetyl-1,9-dideoxyforskolin の存在が明らかにされている (Inamdar, et al., 1989)．また，1,9-dideoxyforskolin については，肝 CYP 誘導作用を有することが報告されている (Ding et al., 2005)．今回は検討できなかったが，これらが CYP 誘導物質であるかもしれない．

CFE 中の CYP 誘導成分の検索において，今回示した *in vitro* の CYP 活性阻害系は微量の試料で迅速に検討できる利点がある．しかし，*in vitro* で CYP 活性を阻害する成分であっても，それが *in vivo* において CYP を誘導するとは限らない．実際，イチョウ葉エキス中に含まれるプロアントシアニジンは *in vitro* において CYP2B 活性を阻害したが，*in vivo* では CYP2B の誘導は示さなかったケースもあった (Sugiyama, et al., 2004)．このようなことから，*in vitro* における阻害作用を認めた成分について，*in vivo* における CYP 誘導を必ず確認する必要がある．*In vivo* で CYP 含量や活性を測定することが確実であるが，肝 CYP 含量や活性は，食餌条件や栄養状態によって変動し (Guengerich, 1995)，試料の保存状態によっても低下する可能性がある．従って，より簡単な CYP 誘導の評価指標が求められる．本研究で示したように CFE による CYP 誘導は，肝重量/体重比の増加と極めて強い相関を示した．この肝重量/体重比の増加は肝臓重量の増加に由来するものであり，肝重量の測定は極めて簡単で，CYP 活性測定のように，マイクロソームの調製や酵素活性の測定における特殊な技術は必要でない．つまり，肝重量/体重比を測定すれば，CFE による肝 CYP の誘導レベルを容易に把握することが可能である．今後の CYP 誘導成分の検索実験では，微量で検討できる *in vitro* の CYP 活性阻害系，ならびに簡単で安定した CYP 誘導の評価指標となる肝重量/体重比を測定する *in vivo* の評価系を組み合わせて実施することが効率的と考えられる．

ハーブ類は複数の成分から構成される天然物である．そのために収穫時期や地域，抽出方法によって素材中の個別成分の含有量が変動すると考えられる．CFE については，forskolin が薬理活性を示す成分として同定されていることから，市場に流通している CFE は，10% forskolin を含有した素材が主流である (Virgona, et al., 2010)．しかし，これまでの著者らの検討から CFE による CYP 誘導には，forskolin 以外の成分が関連することが明らかとなっている．CFE 中の forskolin の薬理活性は明確である．従って，CFE を安全に利用するためには，今後の CYP を誘導する未知成分の同定，ならびにその成分の原材料からの除去あるいは低減，またその成分を含めた CFE の規格基準の作成が必要であろう．本実験で示した CYP 誘導成分はエーテル画分に多く存在する事実や，*in vivo* における肝重量の増加による CYP 誘導の評価法，ならびに *in vitro* における CYP 阻害による評価法は，今後の検討において役立つと考えられる．

本研究は科研費 (23500999) の助成を受けたものである．

引用文献

Allen, D. O., Ahmed, B., Naseer, K., (1986): Relationships between cyclic AMP levels and lipolysis in fat cells after isoproterenol and forskolin stimulation, *J Pharmacol Exp Ther*, 238(2), 659-64

Bauer, K., Dietersdorfer, F., Sertl, K., Kaik, B., Kaik, G., (1993): Pharmacodynamic effects of inhaled dry powder formulations of fenoterol and colforsin in asthma, *Clin Pharmacol Ther*, 53(1), 76-83

Baumann, G., Felix, S., Sattelberger, U., Klein, G., (1990): Cardiovascular effects of forskolin (HL 362) in patients

with idiopathic congestive cardiomyopathy--a comparative study with dobutamine and sodium nitroprusside, *J Cardiovasc Pharmacol,* 16(1), 93-100

de Souza, N. J., Dohadwalla, A. N., Reden, J., (1983): Forskolin: a labdane diterpenoid with antihypertensive, positive inotropic, platelet aggregation inhibitory, and adenylate cyclase activating properties, *Med Res Rev,* 3(2), 201-19

Ding, X., Staudinger, J. L., (2005): Induction of drug metabolism by forskolin: the role of the pregnane X receptor and the protein kinase a signal transduction pathway, *J Pharmacol Exp Ther,* 312(2), 849-56

Godard, M. P., Johnson, B. A., Richmond, S. R., (2005): Body composition and hormonal adaptations associated with forskolin consumption in overweight and obese men, *Obes Res,* 13(8), 1335-43

Guengerich, F. P., (1995): Influence of nutrients and other dietary materials on cytochrome P-450 enzymes, *Am J Clin Nutr,* 61(3 Suppl), 651S-58S

Inamdar, P. K., Khandelwal, Y., Garkhedkar, M., Rupp, R. H., de Souza, N. J., (1989): Identification of Microbial Transformation Products of 1, 9-Dideoxyforskolin and 7-Deacetyl-1, 9-dideoxyforskolin, *Planta Med,* 55(4), 386-7

Mannel, M., (2004): Drug interactions with St John's wort : mechanisms and clinical implications, *Drug Saf,* 27(11), 773-97

Omura, T., Sato, R., (1964): The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature, *J Biol Chem,* 239, 2370-8

Patten, G. S., Adams, M. J., Dallimore, J. A., Abeywardena, M. Y., (2004): Depressed prostanoid-induced contractility of the gut in spontaneously hypertensive rats (SHR) is not affected by the level of dietary fat, *J Nutr,* 134(11), 2924-9

Reagan-Shaw, S., Nihal, M., Ahmad, N., (2008): Dose translation from animal to human studies revisited, *FASEB J,* 22(3), 659-61

Rendic, S., Di Carlo, F. J., (1997): Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors, *Drug Metab Rev,* 29(1-2), 413-580

Sugiyama, T., Shinozuka, K., Sano, A., Yamada, S., Endoh, K., Yamada, K., Umegaki, K., (2004): [Effects of various ginkgo biloba extracts and proanthocyanidin on hepatic cytochrome P450 activity in rats], *Shokuhin Eiseigaku Zasshi,* 45(6), 295-301

Tompkins, L. M., Wallace, A. D., (2007): Mechanisms of cytochrome P450 induction, *J Biochem Mol Toxicol,* 21(4), 176-81

Umegaki, K., Saito, K., Kubota, Y., Sanada, H., Yamada, K., Shinozuka, K., (2002): Ginkgo biloba extract markedly induces pentoxyresorufin O-dealkylase activity in rats, *Jpn J Pharmacol,* 90(4), 345-51

Umegaki, K., Taki, Y., Endoh, K., Taku, K., Tanabe, H., Shinozuka, K., Sugiyama, T., (2007): Bilobalide in Ginkgo biloba extract is a major substance inducing hepatic CYPs, *J Pharm Pharmacol,* 59(6), 871-7

Virgona, N., Taki, Y., Umegaki, K., (2010): A rapid HPLC with evaporative light scattering method for quantification of forskolin in multi-herbal weight-loss solid oral dosage forms, *Pharmazie,* 65(5), 322-6

Virgona, N., Yokotani, K., Yamazaki, Y., Shimura, F., Chiba, T., Taki, Y., Yamada, S., Shinozuka, K., Murata, M., Umegaki, K., (2011): Coleus forskohlii extract induces hepatic cytochrome P450 enzymes in mice, *Food Chem Toxicol,* 50(3-4), 750-55

Will-Shahab, L., Bauer, S., Kunter, U., Roots, I., Brattstrom, A., (2009): St John's wort extract (Ze 117) does not alter the pharmacokinetics of a low-dose oral contraceptive, *Eur J Clin Pharmacol,* 65(3), 287-94

JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP
Journal of Pharmacy
And Pharmacology
Research Paper

Hepatic cytochrome P450 mediates interaction between warfarin and *Coleus forskohlii* extract *in vivo* and *in vitro*

Kaori Yokotani[a,b], Tsuyoshi Chiba[a], Yoko Sato[a], Yuko Taki[c], Shizuo Yamada[c], Kazumasa Shinozuka[d], Masatsune Murata[b] and Keizo Umegaki[a]

[a]Information Center, National Institute of Health and Nutrition, Shinjuku-ku, [b]Department of Nutrition and Food Science, Ochanomizu University, Bunkyo-ku, Tokyo, [c]Department of Pharmacokinetics and Pharmacodynamics and Global COE Program, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka-shi, Shizuoka and [d]Department of Pharmacology, School of Pharmacy and Pharmacutical Sciences, Mukogawa Women's University, Nishinomiya, Hyogo, Japan

Keywords
Coleus forskohlii; drug–herb interaction; hepatic CYP2C; herbal supplement; warfarin

Correspondence
Keizo Umegaki, Information Center, National Institute of Health and Nutrition, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8636, Japan.
E-mail: umegaki@nih.go.jp

Received March 5, 2012
Accepted June 17, 2012

doi: 10.1111/j.2042-7158.2012.01563.x

Abstract

Objectives This study aimed to determine whether *Coleus forskohlii* extract (CFE) influences the anticoagulant action of warfarin in mice *in vivo* and its relationship with hepatic cytochrome P450 (CYP).

Methods Mice were fed various doses of CFE standardised with 10% forskolin in a normal diet for one week, or in protein diets containing 7% and 20% casein (low and normal) for four weeks. They were then administered with warfarin by gavage on the last two days of the treatment regimen, and blood coagulation parameters, as well as hepatic CYP, were analysed at 18 h after the last dose. Direct interaction between CFE and forskolin with CYP2C was evaluated *in vitro*.

Key findings CFE dose dependently increased hepatic total CYP content and *S*-warfarin 7-hydroxylase activity at a dietary level of ≥0.05%. Warfarin-induced anticoagulation was attenuated by CFE in parallel with CYP induction. The findings were similar in mice fed diets containing CFE and different ratios of protein. CFE directly inhibited CYP2C activity in mouse and human liver microsomes *in vitro*, whereas forskolin was only slightly inhibitory.

Conclusions CFE attenuates the anticoagulant action of warfarin by inducing hepatic CYP2C; thus, caution is required with the combination of warfarin and dietary supplements containing CFE.

Introduction

The use of herbal supplements has increased worldwide.[1] Such dietary supplements are perceived as safe because the ingredients are natural and they have been used for centuries in oriental cultures. However, adverse effects have been associated with herbal supplements as their use has increased. The causes of these adverse effects include contamination with pharmaceutical agents or toxic substances by poor manufacturing practices or by adulteration,[2,3] allergic reactions, and interactions with prescribed drugs.[4,5]

Among the causes of adverse effects, drug–herb interactions cause the most concern because consumers of herbal supplements often take prescribed drugs concomitantly[2,4,5] and health professionals might be unaware of possible interactions.[6,7] In addition, a decrease in efficacy or an increase in the adverse effects of prescribed drugs might interfere with appropriate medical care and have a fatal outcome. Interactions between some herbal ingredients, such as St John's wort[8] and ginkgo biloba,[9] have been documented, but those for other herbal ingredients remain unknown.

Weight-loss supplements are popular, but they can cause health problems.[10] *Coleus forskohlii* is a popular herbal ingredient for commercial weight-loss dietary supplements. *C. forskohlii* is native to India,[11] where it has been used for centuries in Ayurvedic medicine to treat various diseases of the cardiovascular, respiratory, gastrointestinal and central nervous systems.[12] Extracts of *C. forskohlii* (CFE) roots contain the diterpene forskolin, which increases cAMP concentrations via the activation of adenylate cyclase, resulting in various therapeutic effects against asthma and idiopathic congestive cardiomyopathy.[13,14] Theoretically, an increase in cAMP induced by forskolin will enhance lipolysis leading to elevated fat degradation and physiological fat utilisation, and thus promote fat and weight loss. In fact, forskolin increases both cAMP accumulation and lipolysis in fat cells,[15,16] and

CFE standardised with forskolin reduces fat accumulation in ovariectomised rats[17] and induces favorable effects on body fat in overweight women and obese men.[18,19]

We previously showed that feeding mice with a diet containing CFE (standardised with 10% forskolin) obviously dose- and time-dependently induced hepatic cytochrome P450 (CYP) enzymes.[20] Significant induction of the hepatic CYP content and CYP2C activity was evident at an intake dose of 0.05%; the CFE dose of 60 mg/kg body weight in mice corresponded to about 5 mg/kg body weight of a human equivalent dose when calculated using the body surface normalisation method.[21] Forskolin had little effect on CYP enzyme induction, indicating that an unknown factor is involved. These findings suggests that CFE interacts with prescribed drugs. However, whether CFE actually interacts with drugs *in vivo* remains unclear.

The oral anticoagulant, warfarin, interacts with various foods and drugs,[22,23] resulting in serious adverse events such as bleeding and thrombus. Warfarin generally comprises a racemic mixture of the two active enantiomers, *R*- and *S*-warfarin. The latter has powerful anticoagulant action[24,25] and is metabolised by the CYP2C subfamily of enzymes,[26] which CFE induces in mice.[20] Warfarin binds exclusively to albumin in the blood, and an increase in unbound warfarin due to a decrease in albumin enhances the anticoagulant action.[24] Plasma albumin is likely to decrease in individuals on a diet and weight-loss supplements containing CFE. We found that feeding rats with a low-protein diet induced hepatic CYP and decreased plasma albumin.[27] These changes in plasma albumin and CYP induction caused by the low-protein diet counteracted the influence of warfarin on anticoagulation.

This study evaluates the interaction of CFE with warfarin in mice *in vivo* in terms of hepatic CYP induction and the effect of a low-protein diet. We also examined the direct interaction between CFE and CYP2C enzymes in mouse and human liver microsomes *in vitro*. Our results clearly showed that CFE interacted with warfarin and attenuated the anticoagulant action of warfarin *in vivo*, and that CYP2C enzyme induction was involved in the mechanism of the interaction.

Materials and Methods

Materials

Powdered CFE standardised with 10% forskolin was prepared as follows. Dried roots of *C. forskohlii*, obtained from Bangalore in southern India, were crushed and supercritically extracted under CO_2 gas. The forskolin-rich extract (20–30%) was mixed with dextrin to a forskolin concentration of 10%. These processes were outsourced to Tokiwa Phytochemical Co. Ltd (Chiba, Japan). The CFE comprised: water, 5.6%; protein, 0.3%; lipids, 22.7%; ash, 2.2% and carbohydrates, 69.2%.

The components of the AIN93G semi-purified diet were purchased from Oriental Yeast Co. Ltd (Tokyo, Japan) and included cornstarch, vitamin-free casein, cellulose, mineral mixture (AIN93G) and vitamin mixture (AIN93G). The composition of the AIN93G semi-purified diet has been described by Reeves.[28] We analysed CYP2C enzymes using *S*-warfarin, 7-hydroxywarfarin, 7-ethoxycoumarin and diclofenac purchased from Sigma-Aldrich Inc. (St Louis, MO, USA), and NADPH from Oriental Yeast Co Ltd. The P450-Glo CYP2C9 Screening System (Luciferin-H) and NADPH regeneration systems were obtained from Promega Co. (Madison, WI, USA). Human liver microsomes pooled from 50 donors were obtained from Life Technologies Co. (Carlsbad, CA, USA). Reagents for blood coagulation tests were obtained from Sysmex Co. (Kobe, Japan). Forskolin and 1,9-dideoxyforskolin were obtained from Sigma-Aldrich Inc. and all other reagents were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan).

Animal experiments

In experiment 1, male ICR mice, four weeks old (CLEA Japan, Inc., Tokyo, Japan), were housed at a constant temperature (23 ± 1°C) under a 12-h light–dark cycle in polypropylene cages. After acclimatisation for one week, the mice were fed the AIN93G semi-purified diet and CFE (0, 0.01, 0.05 and 0.15%) for one week.

In experiment 2, a study on the effects of dietary protein, mice were fed for four weeks with a diet containing either 7% (low) or 20% (normal) casein protein based on the AIN93G semi-purified formula with CFE (0, 0.05 and 0.15%). Table 1 shows the composition of the two diets.

In both experiment 1 and 2, each group consisted of six mice. The mice were administered by intragastric gavage with warfarin racemate (1.0 and 0.25 mg/kg in experiments 1 and 2) dissolved in 0.5% carboxymethylcellulose or vehicle for the last two days of the treatment regimen. In experiment 2,

Table 1 Composition of experimental diets (g/kg)

Ingredient	Dietary protein level	
	Low-protein diet (7%)	Normal-protein diet (20%)
Vitamin-free casein	70	200
Corn starch	641.5	529.5
Cellulose	50	50
Sucrose	120	100
L-Cystine	1.05	3.00
Choline bitartate	2.5	2.5
Soybean oil (no additives)	70	70
Vitamin mixture (AIN93G)	10	10
Mineral mixture (AIN93G)	35	35
Tertiary butylhydroquinone	0.014	0.014

we selected a low dose of warfarin to detect slight changes that might be induced by diets containing different ratios of protein. The mice were anaesthetised with pentobarbital and killed at 18 h after the final administration of warfarin according to a report by Sato *et al.*[29] Blood was collected from the caudal vena cava into tubes containing 3.2% sodium citrate (1 : 9 dilution) for analysis of blood coagulation, and into other tubes for serum preparation. The livers were immediately removed, weighed, snap frozen with dry ice and stored at −80°C.

All procedures were in accordance with the National Institute of Health and Nutrition guidelines for the Care and Use of Laboratory Animals, and approved by the ethical committee in the National Institute of Health and Nutrition (No. 1011, May 10th, 2010).

Analytical methods

High-performance liquid chromatography analysis of Coleus forskohlii extract

The CFE used in this study was characterised by HPLC equipped with UV detection (at 210 nm) and evaporative light scattering detection (ELSD). The HPLC conditions and sample preparation were basically according to a validated HPLC method described elsewhere.[30] Briefly, CFE sample extracted with acetonitrile was injected into HPLC-UV-ELSD. HPLC apparatus was a Shimadzu HPLC-VP system (Shimadzu Corporation, Kyoto, Japan). The sample was applied to an L-column ODS, 4.6 × 250 mm, 5μm particle size (Chemical Inspection & Testing Institute, Tokyo, Japan) at 35°C and eluted with a linear gradient of water (A) and acetonitrile (B). The gradient protocol was 0–12.0 min, 50–80% B; 12.1–39.0 min, 100% B at a flow rate of 1.2 ml/min. Forskolin and 1,9-dideoxyforskolin in the sample were quantified by HPLC-ELSD. The actual content of forskolin and 1,9-dideoxyforskolin was 10.37 g/100 g and 1.21 g/100 g, respectively.

Analysis of cytochrome P450 content and activity

The liver was rinsed with 0.9% (w/v) NaCl, homogenised in 50 mmol/l Tris-HCl buffer (pH 7.4) containing 0.25 mol/l sucrose and separated by centrifugation at 10 000*g* at 4°C for 30 min. The supernatant was clarified by centrifugation at 105 000*g* at 4°C for 60 min and used as microsomes to determine the CYP levels. The total CYP content and the activity of the CYP2C subtype enzyme as *S*-warfarin 7-hydroxylase were determined as described.[31] We investigated CYP2C-specific inhibition (experiment 3) using the 6'deoxyluciferin (Luciferin-H) provided in the P450-Glo assay (Promega), with untreated microsomes from mouse and human livers as enzyme sources and diclofenac as a positive inhibitor, according to the manufacturer's instructions. Luminescent signals from the reaction were measured by luminometry (GloMax96 Microplate Luminometer; Promega), and the inhibitory activity of CFE, forskolin or diclofenac on CYP2C enzyme was determined as ratio (%) of treatment with vehicle. CYP2C activity was measured using various concentrations of CFE and Luciferin-H substrate to construct Dixon plots. Activity was expressed as relative light units (RLU)/mg protein/min.

Other analyses

Plasma samples were immediately centrifuged at 4320*g* at 4°C for 10 min. Coagulation parameters (prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombotest Owren (TTO)) were measured using an automated blood coagulation analyser (CA-50; Sysmex) according to the manufacturer's protocol. PT and TTO are indicators of the extrinsic and common pathways of the coagulation cascade, respectively, and are used to monitor warfarin therapy. On the other hand, APTT is an indicator of both the intrinsic and common pathways of the coagulation cascade. Protein concentrations were determined using BCA protein assay kits (Pierce, Rockford, IL, USA). Serum albumin was determined using the A/G *B*-test Wako (Wako Pure Chemical Industries).

Statistical analyses

Data are presented as means and standard error (SE) for individual groups and were statistically analysed using one-way (experiment 1) and two-way (experiment 2) analysis of variance with Tukey's multiple comparison test or Student's *t*-test when two groups were compared. Differences at $P < 0.05$ were considered significant. All statistical analyses were performed using Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

High-performance liquid chromatography profile of *Coleus forskohlii* extract

CFE is a natural plant product and the components may vary due to the extraction and preparation methods. Thus, to characterise the profile of CFE sample used in this study, the sample was injected into HPLC-UV-ELSD. Many peaks were observed in the chromatogram of HPLC-UV detection, while four peaks were observed in the chromatogram of HPLC-ELSD (Figure 1). The content of forskolin and 1,9-dideoxyforskolin in the CFE sample was 10.37% and 1.21%, respectively.

Interaction between *Coleus forskohlii* extract and warfarin in blood coagulation (experiment 1)

One week of dietary CFE dose dependently increased the total hepatic CYP content and activity of *S*-warfarin

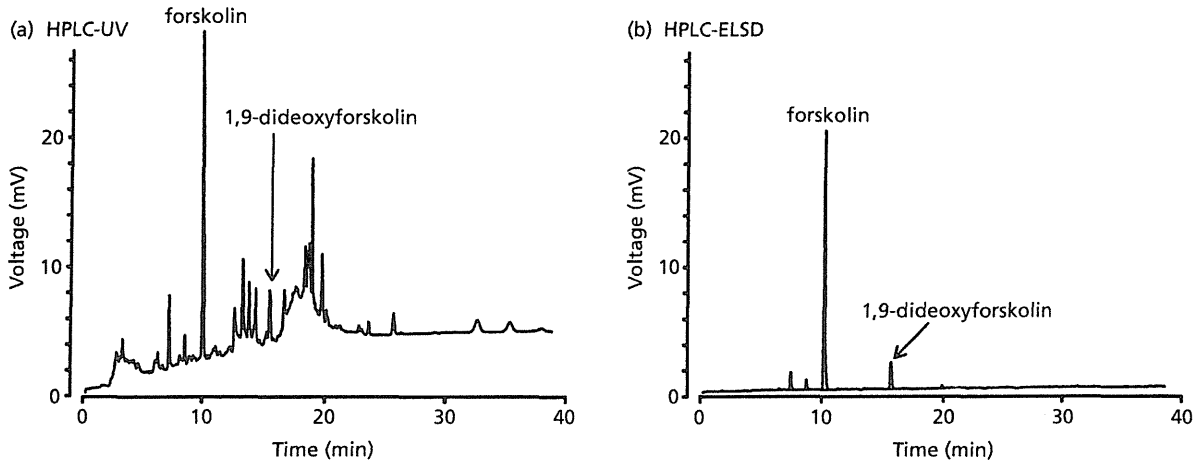

Figure 1 Typical HPLC chromatograms of the *Coleus forskohlii* extract (CFE) used in this study. CFE sample extracted with acetonitrile was injected into HPLC with UV detection (at 210 nm) and evaporative light scattering detection (ELSD). Details of conditions are given in Methods.

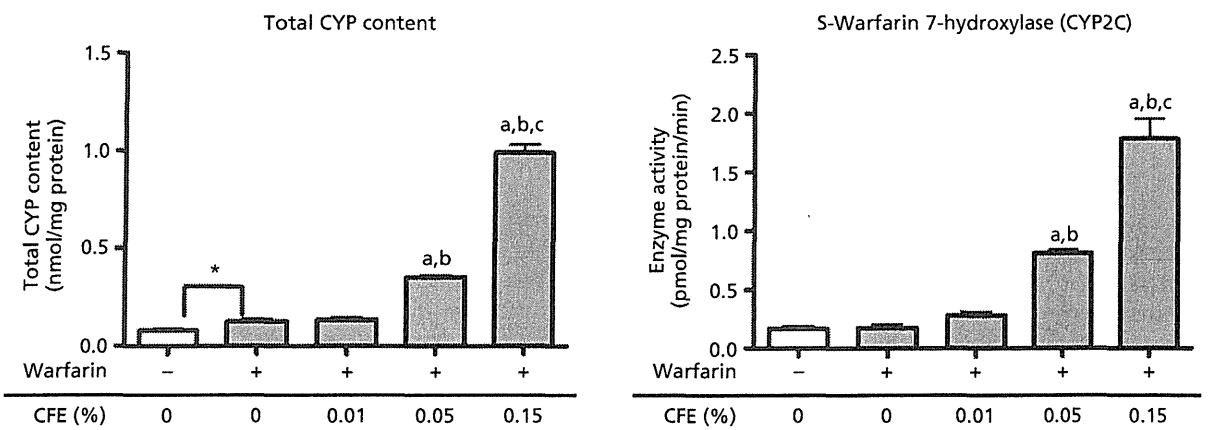

Figure 2 Changes in total cytochrome P450 content and *S*-warfarin 7-hydroxylase in livers of mice administered with various doses of *Coleus forskohlii* extract (CFE) and warfarin (experiment 1). Values are expressed as means and SE, $n = 6$. $^{*}P < 0.05$, compared with CFE (0%) without warfarin. $^{a}P < 0.05$, compared with CFE (0%) with warfarin. $^{b}P < 0.05$, compared with CFE (0.01%) with warfarin. $^{c}P < 0.05$, compared with CFE (0.05%) with warfarin.

7-hydroxylase, a CYP2C enzyme. Significant induction was evident at dietary CFE doses above 0.05%, which corresponded to a dose of 72 mg/kg body weight (Figure 2). Liver weight significantly increased at a CFE dose of 0.15% (Table 2). Warfarin alone (1 mg/kg) for the last two days of the treatment regimen slightly increased the total CYP content, but did not induce *S*-warfarin 7-hydroxylase activity. The anticoagulant effect of warfarin, evaluated by blood coagulation parameters (PT, APTT and TTO), was dose-dependently attenuated by CFE (Figure 3), which corresponded with the induction of CYP enzymes.

Effect of dietary protein on interaction between warfarin and *Coleus forskohlii* extract (experiment 2)

Various doses of CFE in the 7% or 20% casein diets for four weeks dose-dependently induced hepatic total CYP content and the activity of *S*-warfarin 7-hydroxylase in mice. The overall induction was higher than that in experiment 1. This would be due to the longer period of CFE administration (Figure 4). We selected a low dose of warfarin to detect slight changes that might be induced by diets containing different ratios of protein. Warfarin (0.25 mg/kg) administration for

Table 2 Changes in body weight, liver weight and food intake of mice administered various doses of *Coleus forskohlii* extract (CFE) and warfarin (experiment 1)

CFE treatment	0%	0%	0.01%	0.05%	0.15%
Warfarin treatment	–	+	+	+	+
Final body weight (g)	34.4 ± 0.9	34.1 ± 0.7	33.7 ± 0.6	34.2 ± 0.7	35.0 ± 0.5
Liver weight (%/body weight)	5.84 ± 0.10	5.72 ± 0.18	5.23 ± 0.18	5.88 ± 0.18	6.87 ± 0.17[a,b,c]
Average dairy food intake (g)	4.9 ± 0.07	4.9 ± 0.06	4.9 ± 0.05	4.9 ± 0.10	4.9 ± 0.09

Values are expressed as means ± SE, $n = 6$. *$P < 0.05$, compared with CFE (0%) without warfarin. [a]$P < 0.05$, compared with CFE (0%) with warfarin. [b]$P < 0.05$, compared with CFE (0.01%) with warfarin. [c]$P < 0.05$, compared with CFE (0.05%) with warfarin.

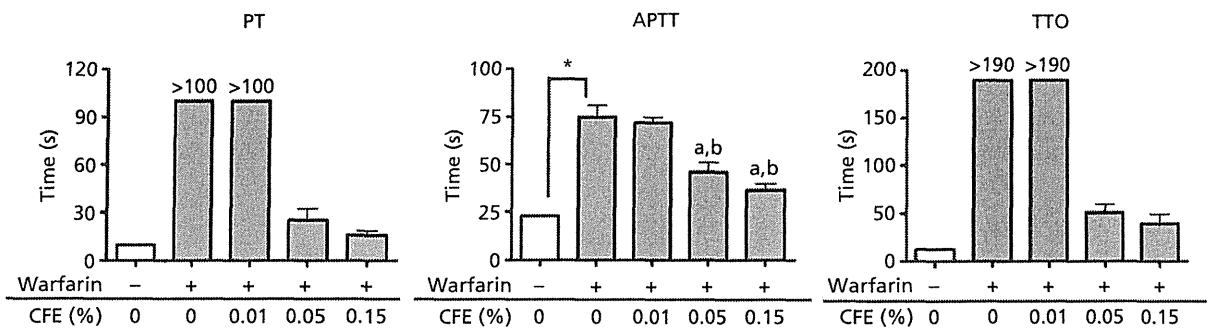

Figure 3 Changes in warfarin-induced blood coagulation parameters in mice administered with various doses of *Coleus forskohlii* extract (CFE) and warfarin (experiment 1). Coagulation parameters are prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombotest Owren (TTO). Values are expressed as means and SE, $n = 6$. *$P < 0.05$, compared with CFE (0%) without warfarin. [a]$P < 0.05$, compared with CFE (0%) with warfarin. [b]$P < 0.05$, compared with CFE (0.01%) with warfarin.

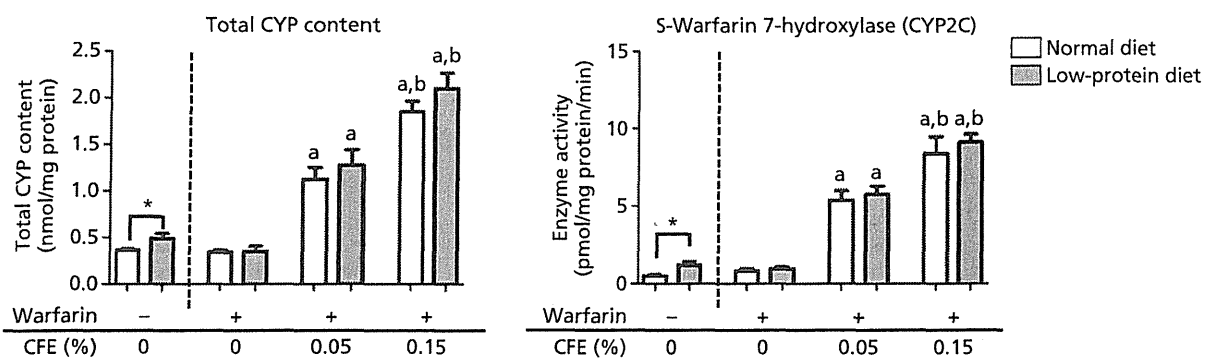

Figure 4 Changes in total cytochrome P450 content and *S*-warfarin 7-hydroxylase in livers of mice administered with various doses of *Coleus forskohlii* extract (CFE) in low (7%) or normal (20%) protein diets and warfarin (experiment 2). Values are expressed as means and SE, $n = 6$. *$P < 0.05$, compared with CFE (0%) without warfarin. [a]$P < 0.05$, compared with CFE (0%) with warfarin. [b]$P < 0.05$, compared with CFE (0.01%) with warfarin.

the last two days of the regimen did not affect such changes. The amount of dietary protein affected the groups that were not treated with warfarin; the effect was obvious in the group administered with the low, compared with the normal, protein diet. However, the effect was less clear in the groups treated with warfarin and CFE. Body weight was lower in the group fed the low, rather than the normal, protein diet (Table 3), but the plasma albumin concentration did not differ between the two groups at any time. The magnitude of warfarin-induced blood coagulation parameters decreased in the groups administered with CFE, but the effect of dietary protein was unclear (Figure 5). The decrease in blood anticoagulation parameters corresponded to the increase in CYP enzyme induction.

Table 3 Changes in body weight, liver weight, serum albumin concentration and food intake of mice administered with various doses of *Coleus forskohlii* extract (CFE) in low (7%) or normal (20%) protein diet and warfarin (experiment 2)

	Normal protein diet (20% casein)				Low-protein diet (7% casein)			
Warfarin treatment	–	+	+	+	–	+	+	+
CFE	0%	0%	0.05%	0.15%	0%	0%	0.05%	0.15%
Final body weight (g)	41.4 ± 1.5	39.4 ± 1.7	41.4 ± 1.3	41.1 ± 1.2	37.1 ± 1.2	35.7 ± 1.2	37.4 ± 1.3	37.9 ± 1.4
Liver weight (%/body weight)	3.86 ± 0.14	3.91 ± 0.11	5.71 ± 0.21[a]	8.01 ± 0.48[a,b]	3.73 ± 0.22	4.38 ± 0.31	4.99 ± 0.05	5.95 ± 0.14[a,b]
Serum albumin (Concentration, g/dl)	3.26 ± 0.16	3.12 ± 0.21	3.13 ± 0.19	3.06 ± 0.24	3.48 ± 0.15	3.62 ± 0.12	3.11 ± 0.07	2.96 ± 0.18
Average dairy Food intake (g)	4.8 ± 0.13	4.7 ± 0.11	4.6 ± 0.06	4.6 ± 0.10	4.8 ± 0.04	4.7 ± 0.06	4.8 ± 0.06	4.6 ± 0.09

Values are expressed as means ± SE, $n = 6$. [a]$P < 0.05$, compared with (0%) with warfarin in the same protein diet. [b]$P < 0.05$, compared with CFE (0.05%) with warfarin in the same protein diet.

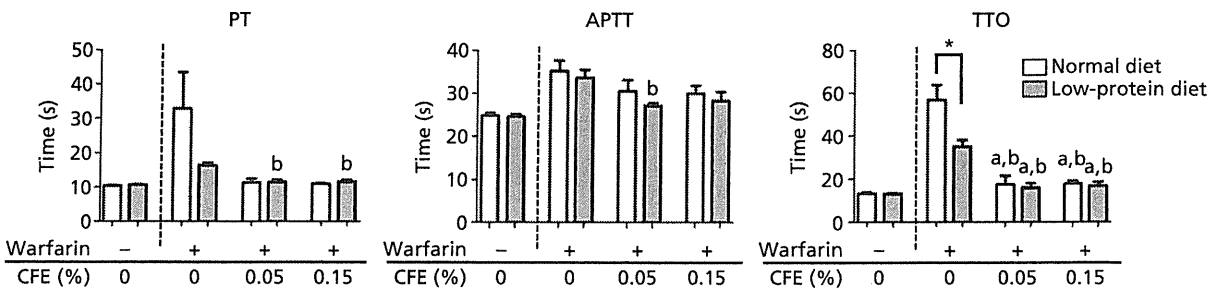

Figure 5 Changes in warfarin-induced blood coagulation parameters in mice administered with various doses of *Coleus forskohlii* extract (CFE) in low (7%) or normal (20%) protein diets and warfarin (experiment 2). Coagulation parameters are as described in the legend to Figure 2. Values are expressed as means and SE, $n = 6$. *$P < 0.05$, compared with CFE (0%) without warfarin. [a]$P < 0.05$, compared with CFE (0%) with warfarin. [b]$P < 0.05$, compared with CFE (0.01%) with warfarin.

Direct interaction of *Coleus forskohlii* extract on CYP2C enzyme in human and mouse microsomes *in vitro* (experiment 3)

We compared the direct interaction of CFE with CYP2C enzymes between mouse and human liver microsomes. We found that CFE dose-dependently inhibited CYP2C activity in all microsomes, with a 50% inhibitory concentration (IC_{50}) value of 7 and 9 μg/ml for mice and humans, respectively (Figure 6). Under these conditions, the IC_{50} of diclofenac, which is a positive control that commonly serves as a probe for CYP2C9 assays in humans, was similar between the mouse and human microsomes, at 8 and 10 μg/ml, respectively. The inhibitory effect of CFE on mouse CYP2C was characterised in a Dixon plot (Figure 7), which showed an approximate Ki value of 7.5 μg/ml. To clarify the contribution of forskolin on the inhibitory effect, 25 μg/ml of CFE and 2.5 μg/ml of forskolin, which was equivalent to the amount in the CFE, were added to the CYP2C assay. Pure forskolin was slightly inhibitory, whereas CFE was more so in both mouse and human liver microsomes. The values (% of control activity, means ± SE of four determinations) for forskolin vs CFE

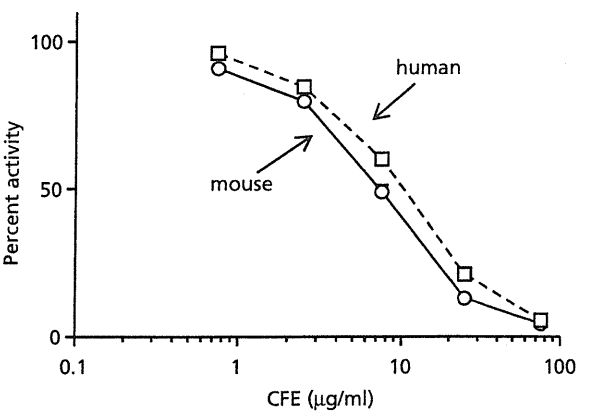

Figure 6 Inhibitory effect of *Coleus forskohlii* extract (CFE) on CYP2C activity of mouse and human liver microsomes (experiment 3). CYP2C activity was determined in presence of 0.75–75 μg/ml CFE. Values are expressed as means and SE of four determinations.

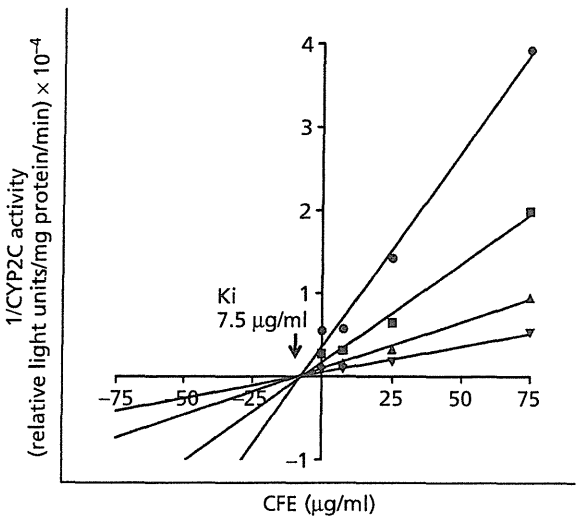

Figure 7 Effect of *Coleus forskohlii* extract (CFE) on CYP2C activity in mouse liver microsomes *in vitro* (experiment 3). Dixon plot shows CYP2C activity measured in presence of 7.5–75 μg/mL of CFE in mouse liver microsomes *in vitro*. Loaded 6'deoxyluciferin (Luciferin-H) concentrations: ●, 50 μM; ■, 100 μM; ▲, 200 μM; ▼, 400 μM. Points represent means of three determinations.

in mouse and human microsomes were 75 ± 1.3 vs 12 ± 1.0 and 92 ± 3.3 vs 17 ± 0.3 (both $P < 0.01$), respectively.

Discussion

CFE contains forskolin, which activates adenylate cyclase and thus induces various pharmacological effects. Currently, CFE standardised with 10% forskolin is a popular herbal ingredient of dietary weight-loss supplements. Here, we showed that CFE attenuated the anticoagulant action of warfarin *in vivo*, and that hepatic total CYP and *S*-warfarin 7-hydroxylase induced by CFE, but not by forskolin, were involved in the mechanism of action. Experiment 1 showed that warfarin interacted with CFE at doses of approximately 70 mg/kg body weight for one week. This dose in mice corresponds to about 6 mg/kg in humans calculated using the body surface normalisation method.[21] Notably, the calculated human dose was within the range of CFE intake from commercial dietary weight-loss supplements, suggesting that the intake of warfarin together with dietary supplements containing CFE increases the risk of thrombus formation.

Consumers of dietary weight-loss supplements containing CFE might also be on extreme diets, which could result in their having a low-protein nutritional status. Warfarin binds exclusively to albumin in the blood, and an increase in unbound warfarin resulting from a decrease in albumin might enhance the anticoagulant action of CFE.[24] On the other hand, we found that feeding a low-protein diet to rats increases hepatic CYP levels,[27] which might attenuate the anticoagulant action of warfarin. Thus, precisely how CFE interacts with warfarin under conditions of a low-protein diet should be determined. Our results showed that various doses of CFE administered over a period of four weeks dose-dependently diminished the anticoagulant action of warfarin in mice fed diets containing low and normal amounts of protein. The diminished anticoagulant action of warfarin corresponded to the induction of total CYP and *S*-warfarin7-hydroxylase activity, which were slightly higher in the mice fed the low-protein diet. The effect of this diet on the interaction between CFE and warfarin and the effect on anticoagulation was less clear, perhaps because of the following. The induction of CYP by four weeks of CFE administration was so obvious that it obscured the effect of the low-protein diet. Furthermore, plasma albumin was notably unaffected by the low-protein diet under our experimental conditions.

The CYP2C subfamily is associated with *S*-warfarin metabolism in humans and mice.[32] However, whether or not the pathways of CFE interaction with warfarin are the same in humans and mice *in vivo* remained unclear. We therefore evaluated the direct interaction between CFE and forskolin on CYP2C enzyme using the CYP2C9 Screening System in mouse and human liver microsomes *in vitro*. The results showed that CFE inhibits CYP2C activity in human and mouse microsomes to a similar extent, whereas forskolin only slightly inhibited the activity. The inhibitory potency of forskolin was considerably lower than that of CFE containing 10% forskolin. This finding was consistent with those of our previous in-vivo mouse study,[20] which showed that an unidentified substance in CFE induces CYP enzymes *in vivo* and inhibits them *in vitro*. These findings imply that the unidentified substance is a substrate of the CYP2C enzyme. The IC_{50} of diclofenac, a substrate for human CYP2C9, and of CFE were comparable between human and mouse liver microsomes in this study. This observation indicates that CFE interacts with other drugs, such as warfarin, that are metabolised by CYP2C9 in humans. We characterised CFE interaction with CYP2C in mouse liver microsomes using Dixon plots. The Ki value was 7.5 μg/ml and the inhibition was non-competitive. However, to conclude the type of inhibition would be difficult at present because CFE has many components, including forskolin, which might be involved in the inhibitory reaction in liver microsomes. If the substance associated with CYP induction *in vivo* and inhibition *in vitro* could be identified and isolated, the Ki value would be lower and the CFE mode of action would be clearer.

Although CFE is generally standardised with 10% forskolin as an active substance, the contribution of forskolin to the interaction of CFE with warfarin was negligible in this study and in our previous study.[20] Accordingly, the substance causing CYP induction must be identified and eliminated from CFE preparations for their safe inclusion in dietary

weight-loss supplements. There are many substances in the CFE, and it is unknown whether the inducer of CYP2C enzyme is a single component at present. Ding and Staudinger reported that forskolin and 1,9-dideoxyforskoiln, a nonadenylate cylcase-activating analogue, induced *CYP3A* gene expression through Pregnane X receptor (PXR) in cultured hepatocytes.[33] The CFE materials used in the present study contained 1.21% of 1,9-dideoxyforskolin. Therefore, 1,9-dideoxyforskolin may be a candidate involved in *CYP2C* induction. However, the intestinal absorption of 1,9-dideoxyforskoiln as well as forskolin is unknown. The use of weight-loss supplements seem to be higher in women than in men, and it is important to clarify whether sex difference exists in the induction of CYP by CFE intake. The assay system used in this study would be helpful to identify the active substances and in future detailed study.

Theoretically, CFE is thought to induce bleeding when taken with antiplatelet drugs, because the forskolin in CFE inhibits platelet functions.[34,35] The findings of our previous study[20] seemed to contradict this theory, since CFE induced both CYP3A and CYP2C, which catalyses 50% and 20% of prescribed drugs, respectively.[36,37] The induction of CYP by CFE attenuated the effect of warfarin in the present study, and would also diminish the effects of antiplatelet drugs, thereby resulting in an increased risk of thrombus formation. Healthcare professionals should not blindly accept existing information, but should observe and communicate with patients who are receiving warfarin, antiplatelet drugs or other drugs metabolised by CYP2C and CYP3A while consuming dietary weight-loss supplements containing CFE.

Conclusions

Coleus forskohlii extract induced CYP2C and diminished the anticoagulant property of warfarin in mice *in vivo*. We also showed that CFE inhibited CYP2C from mouse and human liver microsomes *in vitro*, whereas forskolin did not. The substance involved in CYP induction *in vivo* and inhibition *in vitro* remains undefined.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This study was financially supported in part by a Grant-in-Aid from the Food Safety Commission, Japan (No. 0807), and by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (JSPS) (No. 23500999).

References

1. Gershwin ME *et al.* Public safety and dietary supplementation. *Ann N Y Acad Sci* 2010; 1190: 104–117.
2. Bent S, Ko R. Commonly used herbal medicines in the United States: a review. *Am J Med* 2004; 116: 478–485.
3. van Breemen RB *et al.* Ensuring the safety of botanical dietary supplements. *Am J Clin Nutr* 2008; 87: 509S–513S.
4. Izzo AA, Ernst E. Interactions between herbal medicines and prescribed drugs: an updated systematic review. *Drugs* 2009; 69: 1777–1798.
5. Ulbricht C *et al.* Clinical evidence of herb-drug interactions: a systematic review by the natural standard research collaboration. *Curr Drug Metab* 2008; 9: 1063–1120.
6. Sleath B *et al.* Ethnicity and physician-older patient communication about alternative therapies. *J Altern Complement Med* 2001; 7: 329–335.
7. Giveon SM *et al.* Are people who use 'natural drugs' aware of their potentially harmful side effects and reporting to family physician? *Patient Educ Couns* 2004; 53: 5–11.
8. Zhou SF, Lai X. An update on clinical drug interactions with the herbal antidepressant St. John's wort. *Curr Drug Metab* 2008; 9: 394–409.
9. Uchida S *et al.* Effects of Ginkgo biloba extract on pharmacokinetics and pharmacodynamics of tolbutamide and midazolam in healthy volunteers. *J Clin Pharmacol* 2006; 46: 1290–1298.
10. Pittler MH *et al.* Adverse events of herbal food supplements for body weight reduction: systematic review. *Obes Rev* 2005; 6: 93–111.
11. Bhat SV *et al.* Structures and stereochemistry of new labdane diterpenoids from Coleus forskohlii briq. *Tetrahedron Lett* 1977; 19: 1669–1672.
12. Ammon HP, Muller AB. Forskolin: from an ayurvedic remedy to a modern agent. *Planta Med* 1985; 51: 473–477.
13. Bauer K *et al.* Pharmacodynamic effects of inhaled dry powder formulations of fenoterol and colforsin in asthma. *Clin Pharmacol Ther* 1993; 53: 76–83.
14. Baumann G *et al.* Cardiovascular effects of forskolin (HL 362) in patients with idiopathic congestive cardiomyopathy–a comparative study with dobutamine and sodium nitroprusside. *J Cardiovasc Pharmacol* 1990; 16: 93–100.
15. Allen DO *et al.* Relationships between cyclic AMP levels and lipolysis in fat cells after isoproterenol and forskolin stimulation. *J Pharmacol Exp Ther* 1986; 238: 659–664.
16. Okuda H *et al.* Relationship between cyclic AMP production and lipolysis induced by forskolin in rat fat cells. *J Lipid Res* 1992; 33: 225–231.
17. Han LK *et al.* Effects of Coleus forskohlii on fat storage in ovariectomized rats. *Yakugaku Zasshi* 2005; 125: 449–453.

18. Henderson S *et al.* Effects of coleus forskohlii supplementation on body composition and hematological profiles in mildly overweight women. *J Int Soc Sports Nutr* 2005; 2: 54–62.
19. Godard MP *et al.* Body composition and hormonal adaptations associated with forskolin consumption in overweight and obese men. *Obes Res* 2005; 13: 1335–1343.
20. Virgona N *et al.* Coleus forskohlii extract induces hepatic cytochrome P450 enzymes in mice. *Food Chem Toxicol* 2011; 50: 750–755.
21. Reagan-Shaw S *et al.* Dose translation from animal to human studies revisited. *FASEB J* 2008; 22: 659–661.
22. Holbrook AM *et al.* Systematic overview of warfarin and its drug and food interactions. *Arch Intern Med* 2005; 165: 1095–1106.
23. Fugh-Berman A, Ernst E. Herb-drug interactions: review and assessment of report reliability. *Br J Clin Pharmacol* 2001; 52: 587–595.
24. Palareti G, Legnani C. Warfarin withdrawal. Pharmacokinetic-pharmacodynamic considerations. *Clin Pharmacokinet* 1996; 30: 300–313.
25. Taki Y *et al.* Ginkgo biloba extract attenuates warfarin-mediated anticoagulation through induction of hepatic cytochrome P450 enzymes by bilobalide in mice. *Phytomedicine* 2011; 19: 177–182.
26. Kumar V *et al.* CYP2C9 inhibition: impact of probe selection and pharmacogenetics on in vitro inhibition profiles. *Drug Metab Dispos* 2006; 34: 1966–1975.
27. Taki Y *et al.* Effects of Ginkgo biloba extract on the pharmacokinetics and pharmacodynamics of tolbutamide in protein-restricted rats. *J Pharm Pharmacol* 2011; 63: 1238–1243.
28. Reeves PG *et al.* AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 1993; 123: 1939–1951.
29. Sato K *et al.* Comparison of the anticoagulant and antithrombotic effects of YM-75466, a novel orally-active factor Xa inhibitor, and warfarin in mice. *Jpn J Pharmacol* 1998; 78: 191–197.
30. Virgona N *et al.* A rapid HPLC with evaporative light scattering method for quantification of forskolin in multi-herbal weight-loss solid oral dosage forms. *Pharmazie* 2010; 65: 322–326.
31. Umegaki K *et al.* Ginkgo biloba extract markedly induces pentoxyresorufin O-dealkylase activity in rats. *Jpn J Pharmacol* 2002; 90: 345–351.
32. Martignoni M, ed. *Species and Strain Differences in Drug Metabolism in Liver and Intestine*, University of Groningen, 2006.
33. Ding X, Staudinger JL. Induction of drug metabolism by forskolin: the role of the pregnane X receptor and the protein kinase a signal transduction pathway. *J Pharmacol Exp Ther* 2005; 312: 849–856.
34. Christenson JT *et al.* The effect of forskolin on blood flow, platelet metabolism, aggregation and ATP release. *Vasa* 1995; 24: 56–61.
35. Agarwal KC *et al.* Significance of plasma adenosine in the antiplatelet activity of forskolin: potentiation by dipyridamole and dilazep. *Thromb Haemost* 1989; 61: 106–110.
36. Tompkins LM, Wallace AD. Mechanisms of cytochrome P450 induction. *J Biochem Mol Toxicol* 2007; 21: 176–181.
37. Rendic S, Di Carlo FJ. Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metab Rev* 1997; 29: 413–580.

J Nutr Sci Vitaminol, 59, 37–44, 2013

Influence of Dietary Macronutrients on Induction of Hepatic Drug Metabolizing Enzymes by *Coleus forskohlii* Extract in Mice

Kaori YOKOTANI[1,2], Tsuyoshi CHIBA[1], Yoko SATO[1], Tomoko NAKANISHI[1], Masatsune MURATA[2] and Keizo UMEGAKI[1,*]

[1] *Information Center, National Institute of Health and Nutrition, 1–23–1 Toyama, Shinjuku-ku, Tokyo 162–8636, Japan*

[2] *Department of Nutrition and Food Science, Ochanomizu University, 2–1–1 Otsuka, Bunkyo-ku, Tokyo 112–8610, Japan*

(Received August 27, 2012)

Summary From studies in mice, we have reported that *Coleus forskohlii* extract (CFE), a popular herbal weight-loss ingredient, markedly induced hepatic drug metabolizing enzymes, especially cytochrome P450 (CYP), and interacted with co-administered drugs. This study was designed to examine how the induction of drug metabolizing enzymes by CFE was influenced by different levels of macronutrients in the diet. Mice were fed a non-purified diet or semi-purified diet with and without CFE (0.3–0.5%) for 14–18 d, and changes in the ratio of liver weight to body weight, an indicator of hepatic CYP induction, and hepatic drug metabolizing enzymes were analyzed. The ratio of liver weight to body weight, content and activities of CYPs, and activity of glutathione *S*-transferase were higher in a semi-purified standard diet (AIN93G formula) group than in high sucrose (62.9%) and high fat (29.9%) diet groups. Different levels of protein (7%, 20%, and 33%) in the diets did not influence CFE-induced CYP induction or increase the ratio of liver weight to body weight. The effect of CFE on the ratio of liver weight to body weight was higher with a semi-purified diet than with a non-purified diet, and was similar between dietary administration and intragastric gavage when the CFE dose and the diet were the same. There was a positive correlation between CFE-induced CYP induction and the content of starch in the diets, suggesting that dietary starch potentiates CFE-induced CYP induction in mice. The mechanism of enhanced CYP induction remains unclear.

Key Words *Coleus forskohlii*, cytochrome P450, macronutrients, administration route, dietary starch

Coleus forskohlii extract (CFE) is a popular herbal ingredient for commercial weight-loss dietary supplements (*1*). *C. forskohlii* is native to India (*2*), where it has been used for centuries in Ayurvedic medicine to treat various diseases of the cardiovascular, respiratory, gastrointestinal and central nervous systems (*3*). CFE contains the diterpene forskolin as in Fig. 1, which increases cAMP concentrations via the activation of adenylate cyclase, resulting in various therapeutic effects against asthma and idiopathic congestive cardiomyopathy (*4*, *5*). Theoretically, an increase in cAMP induced by forskolin will enhance lipolysis leading to elevated fat degradation and physiological fat utilization, and thus promote fat and weight loss. It has been shown that forskolin increases both cAMP accumulation and lipolysis in fat cells (*6*, *7*), and CFE standardized with forskolin reduces fat accumulation in ovariectomised rats (*8*) and induces favorable effects on body fat in overweight women and obese men (*9*, *10*).

Currently, drug–herb interactions are becoming a source of serious concern in relation to adverse effects, because consumers of herbal supplements often take prescribed drugs concomitantly (*11–13*) and health professionals might be unaware of possible interactions (*14*, *15*). A decrease in efficacy or an increase in the adverse effects of prescribed drugs might interfere with appropriate medical care and have a fatal outcome. Drugs are metabolized by the Phase I and Phase II enzymes; the former is catalyzed by cytochrome P450 (CYP) enzymes, and the latter is catalyzed conjugation enzymes such as glutathione *S*-transferase (GST) and UDP-glucuronosyltransferase (*16*). Interactions between some herbal ingredients, such as St John's wort (*17*) and ginkgo biloba (*18*), have been documented and shown to be mediated by CYPs, but those for other herbal ingredients remain unknown. We previously showed that feeding mice a diet containing CFE (standardized with 10% forskolin) dose- and time-dependently induced hepatic CYPs and GST enzymes (*19*). Significant induction of the hepatic CYP content and CYP2C activity was evident at an intake dose of 0.05%; the CFE dose was 60 mg/kg body weight in mice and corresponded to about 5 mg/kg body weight of a human equivalent dose when calculated using the body surface normalization method (*20*). We also reported the interaction

*To whom correspondence should be addressed.
E-mail: umegaki@nih.go.jp

of warfarin and CFE in mice in vivo, where CFE attenuated the anticoagulant action of warfarin via induction of hepatic CYPs, especially CYP2C, which is involved in active (*S*)-warfarin metabolism (*21*). Furthermore, we showed that CFE induced CYPs in vivo and directly inhibited CYP2C activity in vitro as well. In both in vivo and in vitro studies, the effect of forskolin, a biologically active marker, was negligible, indicating the contribution of unknown substances in the CFE (*19*, *22*).

Users of weight loss supplements may have an extreme meal with different macronutrient compositions. There are 4 popular weight loss diets: Atkins (very low in carbohydrate), Zone (low in carbohydrate), Ornish (very high in carbohydrate), and LEARN (Life style, Exercise, Attitude, Relationships and Nutrition) (*23*). These differences in dietary macronutrients may influence drug-metabolizing enzymes (*24*). Rats with protein-calorie malnutrition decreased hepatic CYP levels (CYP1A2, 2C11, 2E1 and 3A1/2) (*25*), and rats fed a high-sucrose diet exhibited decreased hepatic content of CYP1A1, CYP3A2 and GST activity (*26*). It has also been shown that a diet deficient in carbohydrate remarkably enhanced liver mixed-function oxidase activity and the metabolism of carbon tetrachloride in rats (*27*). Based on these findings, it is important to determine how dietary macronutrients influence CFE-induced hepatic CYP induction.

In this study in mice, we examined how induction of drug metabolizing enzymes, especially CYPs, was influenced by CFE with regard to route of administration and dietary conditions that differ in macronutrient compositions. In our previous study, CYP induction by CFE was well correlated with an increase in the ratio of liver weight to body weight (*22*). Therefore, we measured CYP content and activities in the liver as well as the ratio of liver weight to body weight as a reliable indicator of CYP induction. The present study in mice had two benefits: one was to clarify dietary conditions that can minimize possible drug–CFE interactions via CYP induction, and the second was to establish experimental diet conditions that can readily be used to seek unknown substances in CFE that induce CYPs in vivo.

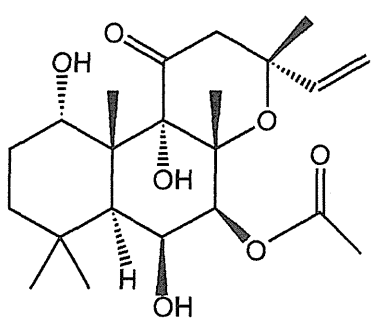

Fig. 1. Chemical structure of forskolin.

MATERIALS AND METHODS

Materials. Powdered CFE standardized with 10% forskolin was prepared as follows. Dried roots of *C. forskohlii* obtained from Bangalore in southern India were crushed and supercritically extracted under CO_2 gas. The forskolin-rich extract (20–30%) was mixed with dextrin to a forskolin concentration of 10%. These processes were performed by Tokiwa Phytochemical Co. Ltd. (Chiba, Japan). CFE comprised: water, 5.6%; protein, 0.3%; lipids, 22.7%; ash, 2.2%; and carbohydrates, 69.2%. For CYP enzyme assays, resorufin, pentoxyresorufin, (*S*)-warfarin, 7-hydroxywarfarin, 7-ethoxycoumarin, testosterone, 6β-hydroxytestosterone, and corticosterone, and glutathione were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). NADPH was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Experimental diets. Non-purified commercial rodent diet (CE-2) was supplied by CLEA Japan, Inc. (Tokyo, Japan). The non-purified diet comprised: water, 88 g/kg diet; crude protein, 252 g/kg diet; crude lipids, 44 g/kg diet; total ash, 70 g/kg diet; crude fiber, 44 g/kg diet and soluble non-nitrogenous matter, 502 g/kg diet according to the manufacturer's information. A semi-purified standard diet was prepared based on the composition of the AIN93G formula of Reeves et al. (*28*). Various semi-purified diets with different compositions of macronutrient were prepared as shown in Table 1. The high-starch diet that differed only in the source of the carbohydrate and the high-fat diet were isonitrogenous per kilocalo-

Table 1. Composition of semi-purified experimental diets (g/kg diet).

Ingredient	Standard	High sucrose	High fat	Low protein	High protein
Cornstarch	529.486	0	205.082	641.436	417.536
Sucrose	100	629.486	126	120	80
Casein	200	200	250	70	330
Soybean oil	70	70	292	70	70
Cellulose	50	50	63	50	50
Vitamin mixture (AIN93G)	10	10	13	10	10
Mineral mixture (AIN93G)	35	35	44	35	35
L-Cystine	3	3	3.76	1.05	4.95
Choline hydrogen tartrate	2.5	2.5	3.14	2.5	2.5
Tertiary butylhydroquinone	0.014	0.014	0.018	0.014	0.014

Table 2. Body weight and liver weight of mice fed a non-purified diet or semi-purified standard diet with and without *C. forskohlii* extract (CFE).

Diet	Non-purified		Semi-purified (standard)	
CFE treatment	−	+	−	+
Average daily food intake (g)	5.8±0.21	5.4±0.18 [0.93]	5.0±0.15[a]	4.8±0.11 [0.97][b]
Calculated CFE dose (mg/kg body weight)	0	845±26.0	0	732±17.6[b]
Final body weight (g)	31.4±0.42	30.3±0.30 [0.96]	33.1±0.84[b]	32.9±0.45 [0.99][b]
Liver weight (g)	1.44±0.05	2.08±0.05 [1.4][a]	1.43±0.05[b]	2.63±0.20 [1.8][a,b,c]
(%/body weight)	4.58±0.11	6.87±0.10 [1.5][a]	4.31±0.11[b]	7.99±0.57 [1.9][a,c]

Mice were fed a non-purified diet or semi-purified diet with and without 0.5% *C. forskohlii* extract (CFE) for 2 wk.
Values are expressed as mean and SE (n=5). Number in brackets indicates the increase in the ratio for its respective diet group without CFE.
[a] Significant difference from non-purified diet without CFE at $p<0.05$.
[b] Significant difference from non-purified diet with CFE at $p<0.05$.
[c] Significant difference from semi-purified diet without CFE at $p<0.05$.

rie. Low, normal, and high protein diets were prepared as isoenergetic by adjusting the proportion of carbohydrate in the diets. The ingredients for the semi-purified diets were purchased from Oriental Yeast Co., Ltd.

Animal experiments. Male 4-wk-old ICR mice (CLEA Japan, Inc.) were housed at a constant temperature (23±1°C) with a 12-h light-dark cycle in polypropylene cages. After acclimation for 1 wk, the mice were divided into treatment groups (5–6 mice per group) and were administered CFE as follows.

In a comparison of non-purified diet and semi-purified standard diet, CFE was added at a concentration of 0.5% (w/w) to each diet, and given to mice ad libitum for 2 wk. In a comparison of the route of administration, mice were either fed a semi-purified standard diet with 0.5% CFE or given it daily by intragastric gavage of CFE dissolved in 0.5% (w/v) carboxymethylcellulose for 2 wk. In this administration route study, the daily dose of CFE was adjusted to 750 mg/kg body weight. In studies of the effect of macronutrients (i.e., starch, fat and protein), the CFE dose was reduced to 0.3% (w/w) in the semi-purified diets and the treatment term was set at 18 d, because the dietary effects were thought to need a longer period at this CFE dose. In the study, food intake in each group was adjusted to keep a similar intake dose of CFE. At the end of each treatment, mice were anesthetized with pentobarbital and killed. Their livers were removed immediately, weighted, snap frozen with dry ice and stored at −80°C until analysis.

All procedures were in accordance with National Institute of Health and Nutrition Guidelines for the Care and Use of Laboratory Animals and were approved by the Ethical Committee in the same institute.

Analytical methods

Analysis of drug-metabolizing enzymes: The liver was rinsed with 0.9% (w/v) NaCl, homogenized in 50 mmol/L Tris-HCl buffer (pH 7.4) containing 0.25 mol/L sucrose, and separated by centrifugation at 10,000 ×g at 4°C for 30 min. The supernatant was centrifuged at 105,000 ×g at 4°C for 60 min to prepare microsomal and cytosol fractions. The total CYP content and the activities of various CYP enzymes were determined using the microsomal fraction, and glutathione *S*-transferase (GST) activity was determined using cytosol fraction, as described previously (*29*). The subtypes of CYP enzymes examined and the corresponding CYPs were pentoxyresorufin *O*-dealkylase, CYP2B; (*S*)-warfarin 7-hydroxylase, CYP2C; and testosterone 6β-hydroxylase, CYP3A. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA).

Statistical analyses. Data are presented as means and standard error (SE) for individual groups and were analyzed statistically using one-way ANOVA (in the non-purified diet versus semi-purified diet and CFE administration route studies) and two-way ANOVA (in the macronutrient studies) with Tukey's multiple comparison test. Differences at $p<0.05$ were considered significant. All statistical analyses were performed using Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Dietary treatment of CFE with non-purified and purified diets and by different administration routes

Mice were fed a 0.5% CFE in a non-purified commercial rodent diet (CE-2) or semi-purified standard diet for 2 wk. In the semi-purified diet groups, food intake was lower, but body weight was higher compared with the non-purified diet groups (Table 2). This discrepancy might be due to high bioavailability of ingredients in the semi-purified diet compared with crude natural ingredients used in the non-purified diet. In the CFE-treated groups, liver weight in the semi-purified diet group was higher, but the increase in the ratio of liver weight to body weight did not differ between the two CFE groups, which could be caused by the low dose of CFE in the semi-purified diet group. When mice were fed the same semi-purified standard diet, increases in liver weight and