# 行政院國家科學委員會專題研究計畫成果報告

## 麻醉藥物 Propofol 對於 Streptozotocin 誘導糖尿病倉鼠單氧酵素系統及去氟化代謝能力的影響-試管與活體實驗

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**Abstract**

**Background:** Metabolic activities of cytochrome (cyt.) P450-dependent monooxygenase could be modulated by diabetic state in experimental-diabetic animals. The purpose of this study is to validate the effect of insulin administration to modulate the metabolic activities of cyt. P450's and the defluorination ability to inhalational anesthetics in diabetic animals.

**Methods:** Diabetic state was achieved by intraperitoneal injection of streptozotocin 40 mg/kg once a day for 4 days to golden Syrian hamsters. After stabilization of diabetic state for 6 weeks, a regimen of insulin treatment given subcutaneously was administered. Metabolic activities of cyt. P450's were assessed by reacting with the specific substrates, benzo(a)pyrene, pentoxyresorufin, aniline and erythromycin for metabolic activities of cyt. 1A1, 2B1, 2E1 and 3A4 respectively, in NADPH-generating system within microsomal preparations of diabetic hamsters before and after insulin treatment and were compared with the control group. The ability of defluorination was evaluated by measuring the free fluoride metabolites after incubating the microsomes with enflurane in diabetic and insulin-treated hamsters. Contents of cyt. P450 isozymes were measured before and after insulin treatment by electrophoresis and immunoblotting. Pathological features of hepatocytes in diabetic hamster before and after insulin regimen were evaluated microscopically.

**Results:** The defluorination of enflurane and activity of aniline hydroxylase (cyt. 2E1) was significantly induced by diabetic state ($P < 0.01$). The pentoxyresorufin O-dealkylase (cyt. 2B1) was inhibited nearly 50 % in diabetic hamster liver when compared with control ($P < 0.01$). While the activities of benzo(a)pyrene hydroxylase (cyt. 1A1) and the erythromycin N-demethylase (cyt. 3A4) were basically unaffected by diabetes. Alterations in content of cyt. P450's were parallel to the alterations in enzyme activities. Microscopically, diabetes induced the vacuolization with fatty droplets among the hepatocytes. After treatment by insulin injection, the enzyme activities, protein content and pathologic features returned to the baseline similar to the control.

**Conclusions:** Our data demonstrated that under diabetic state, metabolic activities of cyt. P450's and its extent of defluorination would be polymorphically modulated. After administration of insulin, the activities of cyt. P450's and defluorination of enflurane returned to the baseline as the blood sugar level being normalized. This should remind clinicians of the importance of insulin treatment to the potential drug-to-drug interactions within the diabetic patients.

**Key words:** Diabetes, streptozotocin-induced. Enzymes, cytochrome P450 monooxygenases, defluorination. Insulin. Microsomes, liver, kidney.

## Introduction

Cytochrome (cyt.) P450-dependent monooxygenases are composed of many enzyme families catalyzing the oxidative

metabolism of a variety of substances including endogenous or synthetic organic chemicals such as steroids and drugs.[1] Clinically, isozymes of cytochrome P450's could be modulated (induced or suppressed) in pathological conditions, such as fasting, obesity or diabetes.[2,3] With the knowledge to the diseases and alterations in enzyme activities, efforts have been made to clarify the clinical impacts and potential drug interactions among them. Diabetes, for example, increases the aniline hydroxylation and N-nitrosodiomethylamine demethylation activities within microsomal preparations of hamster, indicating that some P450 isozymes are induced.[4] P450 isozymes such as P450 2A1, 2C6, 2C7, 3A2, 4A2 and 4A3 also increased and other isozymes, P450 1A1 and 2C11 decreased in diabetic state.[4,5] According to our previous study in diabetic model, the major diabetes-inducible cyt. P450 seems to be P450 2E1 which was proved by the increased metabolic activity and the protein amount of P450 2E1.[4] The alterations in hepatic microsomal metabolizing activities after induction of diabetes therefore present as a specific metabolic state in animal entity that affecting its metabolic responses to all the xenobiotics' entry including carcinogens and drugs.

With the above understandings, we try to regulate the altered behaviors of enzymes by correcting the pathology such as controlling the hyperglycemia in diabetes. With this purpose, we design a regimen of insulin treatment based on the previous findings in diabetic hamster model[4] and observe the effect of insulin treatment to the metabolic activities of P450's and defluorination ability to inhalational anesthetics. We describe the present study for quantifying changes in the activities of specific constitutive microsomal forms of cytochrome P450's in the livers and kidneys of hamster with chronic streptozotocin-induced diabetes before and after administration of insulin.

## Materials and Methods

### *Animals and preparations*

The study was approved by the National Science Council of Taiwan, R.O.C. (NSC 87-2314-B-038-053, 1997-8). Male Syrian golden hamsters, 6-8 weeks old, weighing 80-100 g, were purchased from the Animal Center of the College of Medicine (National Taiwan University, Taipei, Taiwan). They were housed and stayed for stabilization in a photo-controlled environment with 12 h light period for at least 1 week before treatment. To induce diabetes, freshly prepared streptozotocin in 0.05 M citrate buffer, pH 4.5, at a dose of 40 mg/kg body weight was injected intraperitoneally once a day for 4 days.[6] The hamsters of control group were injected normal saline as vehicle. To assure the diabetic state of hamster, urinary glucose was measured every other day and determined by a commercially available enzymic kit (Sigma, St Louis, MO, USA). Animals excreted > 5% glucose in the urine for 6 weeks after last injection were considered stable as chronic diabetic. The first group of hamsters as diabetic control was now separated from the second group as diabetic with insulin treatment. After 6 weeks, insulin zinc suspension (Lente Insulin, Lilly, USA) was administered twice a day subcutaneously to control the hyperglycemic state of diabetic hamsters in experimental group. The dosage of insulin was started from 2 to 14 units/g body weight with incremental increases of 2 units per dose in every other day until the blood sugar became normal. Blood sugar was monitored by measuring the sugar level within the urine as well as by the intermittent blood sampling from the retrobulbar venous plexus. The whole course for treatment was around 2 weeks before stabilization of blood sugar. Animals were sacrificed by capitation after another 2 weeks for stabilization of blood sugar, liver and kidney tissues were removed, rinsed and homogenized in an ice-chilled 1.15 % KCl (w/v) solution. After differential centrifugation, washed microsomes were prepared from homogenized tissues as described by Alvares and Mannering.[7] Microsomes from the kidneys were pooled from every 4 animals and frozen at -70°C until pellets were resuspended in potassium phosphate buffer 0.1 M at pH 7.4 for assay.

Microsomal protein was assayed by the method of Lowry using bovine serum albumin as standard.[8]

***Defluorination assay***

Defluorination of enflurane was assessed by reacting of enflurane with liver and renal microsomes of control, diabetic animals with or without insulin treatment in the NADPH-generating system. The microsomal incubation mixture contained protein 5 mg/ml, NADPH 2 mM and 2 µl of enflurane in 2 ml, and Tris HCl buffer 100 mM (pH = 7.4) was incubated at 37 C for 30 min and stopped on ice. Defluorination of enflurane was assessed by measuring fluoride metabolites using an Orion fluoride-specific combined electrode (Boston, MA, USA).[9] Standard curves of fluoride concentrations were calibrated using freshly prepared free standard fluoride solutions.

***Monooxygenase assays***

Microsomes from diabetic animals were compared with the control and insulin-treated group and were assessed in the NADPH-generating system. Monooxygenases were assayed by reacting with specific marker substrates for each enzyme system. Aniline hydroxylation, indicating the activity of cytochrome P450 2E1, was determined by measuring the formation of end product *p*-aminophenol from aniline.[10] The incubation system contained glucose-6-phosphate dehydrogenase 2.8 iu/ml, glucose-6-phosphate 1 mM and NADP 0.1 mM in Tris buffer 0.075 mM with aniline hydrochloride 0.5 mM. Microsomes were incubated with various concentrations of propofol in the 37°C water bath for 20 min. The reaction was stopped by 20% trichloroacetic acid solution. After centrifugation, the supernatant was mixed with $Na_2CO_3$ and phenol solution to extract the product, which could be measured spectrophotometrically at 630 nm. Erythromycin demethylation, indicating the activity of cytochrome P450 3A4, was determined by measuring the formation of formaldehyde using Nash's reagent.[11] Incubation of microsomes contain 7.5 mM semicarbazide HCl, glucose-6-phosphate 4 mM, NADP 0.4 mM, glucose-6-phosphate dehydrogenase 0.4 iu/ml and erythromycin 12 mM in $KH_2PO_4$-$K_2HPO_4$ buffer 0.02 mM (pH 7.4). The reagent was incubated in 37°C water bath for 15 min and reaction was stopped by 1.24 mM $ZnSO_4 \cdot 7H_2O$. After neutralization and centrifugation, the supernatant reacted with Nash's reagent and the product was detected by UV/VIS spectrophotometry at wavelength of 412 nm using formaldehyde as standard.[11]

Pentoxyresorufin dealkylation, indicating the activity of cytochrome 2B, was assayed by the method of Lubet.[12] The microsomal suspension, 0.5 mg/ml, reacted in 0.05 mM Tris buffer, with 0.025 mM $MgCl_2$, 1.8 mg/ml bovine serum albumin, 1.45 µM NADPH, and 10.6 µM pentoxyresorufin. The system was incubated in 37°C water bath for 10 min and stopped by adding methanol. After centrifugation, the metabolite in supernatant was detected by fluorescence spectrophotometer using rhodamine B as standard. Benzo(a)pyrene hydroxylation for aryl hydrocarbon hydroxylase activity, indicating the activity of cytochrome P450 1A, was determined by measuring the formation of phenolic metabolites by the method of Nebert and Gelboin.[13] In dark room, microsomes 2 mM were incubated in phosphate buffer containing NADPH 1.05 mM, $MgCl_2$ 2.9 mM, $KH_2PO_4$-$K_2HPO_4$ buffer 0.08 mM (pH 7.4) and bovine serum albumin 0.2 mg/ml. The generation system was incubated at 37°C for 10 min with the substrate, benzo(a)pyrene 1 mM, and stopped by adding acetone. The fluorescent metabolites were extracted sequentially by n-hexane and NaOH and measured by spectrofluorimetry.[13]

*Electrophoresis and Immunoblotting*

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was conducted using the discontinuous system of Laemmli.[14] The slab gel, 1.5 mm thick, contained 3 and 7.5 % acylamide (w/v) in the stacking and separation gels, respectively. Following electrophoresis, the gel was stained for protein using Coomassie blue. Transfer of microsomal proteins from the

slab gel to nitrocellulose membrane was carried out following the method of Towbin et al.[15] Immunodetection of P450 2E1, 2B1 and 1A1 was carried out by reacting with the rabbit and mouse monoclonal and polyclonal antibodies against human P450's and was analyzed by densitometry.[16-18]

*Microscopic analysis*

The liver tissues from diabetic and insulin-treated hamsters were freshly fixed by 7 % of formalin. The tissues were sliced and stained by H.E. staining which was then examined via 200 to 400-fold of magnification under light microscope (Olympus, Japan) for the existence of pathology.

Unless otherwise stated, all results are presented as mean ± standard deviation (SD). Data were analyzed using one-way and two-way analyses of variance and significant differences before and after insulin treatment were identified by the repeated measurements of ANOVA. $P < 0.05$ was considered statistically significant.

## Results

The diabetic state of hamsters was achieved by intermittent intraperitoneal injection of streptozotocin with successful rate of 62 % (37/60) and relatively low mortality (17 %, 10/60). Diabetic state in hamsters caused significant increases of tissue/body weight ratio in both liver and kidney tissues that were shown in table 1 ($P < 0.05$). After the treatment of insulin through subcutaneous injection, the ratio was dramatically decreased and showing no difference to the control group. Quantitative analyses of microsomal protein content, total amount of cytochrome P 450 protein and coenzyme, NADPH-cyt. P-450 *c* reductase all demonstrated no significant differences in either induction of diabetic state or after insulin treatment (Table 1).

Metabolic activities of cytochrome P-450 isozymes were assessed among the control, diabetic group and diabetic animals with insulin treatment by reacting with specific substrates in microsomal preparations. The streptozotocin-induced diabetes caused significant increases of aniline hydroxylase (cyt. 2E1) activities in both liver (113 %) and kidney (83 %) ($P < 0.01$). After the treatment of insulin, 2E1 activity declined to the baseline without significant difference to the control groups (Table 2). The benzo(a)pyrene hydroxylase (cyt. 1A1) activities decreased slightly in liver (5 %) but significantly in kidney tissues (31 %, $P < 0.05$). After administration of insulin, their activities returned to normal and close to the control groups (Table 2). The activities of erythromycin N-demethylase (cyt. 3A4) showed no statistically significant differences after induction of diabetes or insulin treatment in both liver and kidney tissues. The pentoxyresorufin O-dealkylase (cyt. 2B1) activities were significantly decreased in both liver (37 %) and kidney (60 %) after the induction of diabetes ($P < 0.01$). Insulin treatment caused the enzyme activities normalized back to the level of control groups (Table 2). Defluorination ability was assessed by reacting the microsomes with enflurane in NADPH-generating system. The defluorination of enflurane was significantly increased within liver (90 %) and kidney tissues (57 %) in animals with diabetic state when compared with control groups ($P < 0.01$). After the administration of insulin, the extents of defluorination declined close to the control groups (Table 2).

By the densitometer, we assessed the exact protein content of each cytochrome P450 isozyme by the specific binding with the polyclonal or monoclonal antibodies through the method of immunoblotting. Among the enzymes, cyt. 3A4 showed no significant alterations in the assessment of metabolic activities within the diabetic state and insulin treatment. So, we measured the protein density for the rest of enzymes, cyt. 2E1, 1A and 2B. Analyzed by densitometry, the protein amount of each enzyme could be specifically quantitated by its immunological reactivity. The diabetic condition produced marked increases in the protein densities of cyt. 2E1 in both hepatic and renal microsomes (Table 3). After insulin treatment, the protein densities decreased and being similar to the control group. The

hepatic and renal P450 1A in diabetic hamsters were 3.5-fold and 2.6-fold increased respectively when compared with the control groups. They became only modestly increased after administration of insulin. The densities of cyt. 2B demonstrated decreases in both liver and kidney microsomes after the induction of diabetic state. They shifted to almost normal levels after insulin treatment (Table 3).

Microscopically, vacuolization among the hepatocytes surrounded by numerous fat droplets that were corresponding with the phenomenon of fatty liver induced by diabetes (Fig. 1A). After the treatment of insulin, the vacuolization and fat droplets disappeared and returned to the normal architecture of hepatocytes in periportal regions (Fig. 1B).

## Discussion

Diabetes increases the aniline hydroxylation activity of hepatic and renal microsomes indicating that the major diabetes-inducible cytochrome P450 seems to be cyt. P450 2E1, which is the isozyme also, induced by acetone, ethanol or starvation in our previous studies.[4,19,20] Interestingly, volatile anesthetics such as enflurane, isoflurane and sevoflurane were considered metabolized majorly by cyt. P450 2E1.[21,22] Not only hepatic but also renal metabolic products of free fluoride compounds due to the breakdown of inhalational anesthetics by the *in situ* activities of cyt. P450 2E1 have been found to be closely related to the clinical nephrotoxicity.[23] The actual interactions between the metabolic substrates, such as inhalational anesthetics and altered enzyme activities in pathological state (diabetes) before and after treatment (insulin injection) are the primary concerns in this study.

In the present study, we try to speculate the impact of diabetes and insulin treatment onto the cytochrome P450 enzyme system through different aspects of investigation. We assessed the followings such as (1) the microsomal contents for cyt. P450 and *c* reductase using spectral analysis (2) the metabolic activities for the cyt. P450 isozymes by measuring specific substrate reactivity (3) the protein content of each enzyme using specific antibodies in immunoblot (4) the pathological alterations under microscopic evaluation. The tissue/body weight ratio increased in diabetic hamsters, esp. in liver, which was compatible with the pathological findings of fatty degeneration (increased fat droplets) noted microscopically. The alterations in the metabolic activities of P450 isozymes seemed to be polymorphic. According to this study and our previous data, cyt. 2E1 increased, cyt. 1A1 and 2B1 decreased, and cyt. 3A4 unchanged in the diabetic state.[4] The balance of the polymorphic alterations of activities and protein amount among various isozymes accounts for the reason why the amounts of microsomal protein and total cyt. P450 content was basically unchanged after the induction of diabetes.

The mechanism of P450's induction is a topic of substantial current interest. Recent results have pointed to a possible role of acetone for enzyme induction.[24] Acetone is also the key intermediate for the gluconeogenic pathway, which may be an important source of glucose during starvation.[20] Based on the fact that cyt. P450 2E1 is induced by diabetes and fasting, both ketogenic conditions as well as by the treatment with acetone, a ketone body, might suggest the common pathway for the induction of cyt. P450 isozymes.[20,24,25] Other studies indicated that the induction of cyt. P450 2E1 is accompanied by an elevation of its mRNA. Recent results suggested that the elevation of P450 mRNA in diabetes was probably due to mRNA stabilization and that acetone intermediate might retard the rate of degradation of P450's.[26] Both of these mechanisms might contribute the alterations of cyt. P450's during the diabetic state. From the above data, we also noticed that differences existed among the changes of various cyt. P450 isozymes during the induction of the diabetic state. The discrepancy between the inductive properties of different cyt. P450 isozymes indicated that the diabetes might affect the cyt. P450 enzymes in an isozyme-specific and tissue-specific manner.[1,24]

Our study provides a stable diabetic model for studying the effect of insulin to cyt. P450 activities and defluorination ability to inhalational anesthetics. The results of present investigations demonstrate that insulin exerts a reverse effect on the diabetes-mediated modulations of cyt. P450 isozymes. Various mechanisms could possibly account for the action of insulin on hepatic and renal cyt. P450's. Treatment of chemical-induced diabetes with insulin could restore the hepatic and renal levels of isozymes to normal directions due to the pretranslational modifications in molecular level.[24,27] The mRNA could be significantly modulated (normalized) after insulin treatment so that it would modify the following steps of the protein synthesis for the cyt. P450 isozymes.[24,27] Also, cross relation with the growth hormone and insulin in diabetic state has recently been postulated. Studies in hypophysectomized rats indicated that the alteration in serum growth hormone level also could cause the enhancement of hepatic contents of P450's in diabetic and fasting conditions.[27] However, the treatment of hypophysectomized rats with insulin had no effect, and treatment of diabetic rats with growth hormone or a suppressing agent of somatostatin showed trivial effects on P450's.[27] These results suggest that insulin does not act directly as a substitute of growth hormone, but exerts its effect indirectly through the normalization of a growth hormone-mediated process in diabetic animals.[27]

By our present data, defluorination of enflurane was enhanced by the diabetic state in the liver and kidney. The extent of enhancement in defluorination was less than the extent of increase in cyt. 2E1 activity within the diabetic animals. This supported the data of Kharasch's that cyt. 2E1 might be the major but not the only enzyme involved in the metabolism of defluorination reaction of inhalational anesthetics.[21] What's the clinical meaning to measure defluorination activity and other metabolic reactions within the diabetic kidney microsomes? Mazze et al. investigated the nephrotoxicity caused by methoxyflurane in rat's model and demonstrated that the serum fluoride metabolites decomposed by liver might be the causative factors for the renal toxicity.[28,29] Kharasch proposed the discrepancy that methoxyflurane could cause more significant nephrotoxicity than sevoflurane with similar serum fluoride concentrations after using these two anesthetics.[23] Data showed that methoxyflurane was metabolized more extensively than sevoflurane within renal microsomes.[23] He postulated that intrarenal fluoride produced by kidney rather than liver cyt. P450's might be playing the major role in the cause of nephrotoxicity.[23] So, renal P450's activity as well as it's defluorination ability become more and more important in assessing the nephrotoxicity caused by drug metabolism. In the present data, diabetic state enhanced the extent of defluorination of enflurane and the administration of insulin reversed the effect. Functionally, we could imply that diabetes might increase the risk for nephrotoxicity due to the enhancement of fluorinated metabolites production and the insulin treatment could reverse the effect and play a protective role for the nephrotoxicity, although the *in vivo* effect still needs further investigation.

The cyt. 2B1, is the major inducible enzyme cross-reacted with anesthetics such as phenobarbitals.[1] By our data, it was inhibited during diabetes and could reversed by insulin. Diabetes showed no effect on to benzo(a)pyrene hydroxylation (cyt. 1A1) and erythromycin N-demethylation (cyt. 3A4). The function of cyt. 1A1 is that it is the major metabolic enzyme in liver and extrahepatic tissues handling the exogenous carcinogens.[1] While cyt. 3A4 was noted to be one of the important enzymes involved in the metabolizing the narcotics and benzodiazepines such as alfentanil and midazolam.[30,31] By our data, neither diabetic state nor insulin treatment would cause the interference with carcinogens, narcotics or benzodiazepines in drug metabolizing level.

In conclusion, drug metabolizing enzyme activities and defluorination ability was significantly modulated by the diabetic state

and could be successfully reversed by the administration of insulin. This phenomenon and therapeutic strategy could remind clinicians about the importance of potential drug-to-drug interactions in clinical diabetic patients.

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附件：封面格式

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