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Differential regulation of hepatic cytochrome P450 monooxygenases in streptozotocin-induced diabetic rats

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Abstract

The present investigation was carried out to study the expression of major cytochrome P450 (CYP) isozymes in streptozotocin-induced diabetes with concomitant insulin therapy. Male Sprague-Dawley rats were randomly assigned to untreated control, streptozotocin-induced diabetic, insulin-treated groups and monitored for 4 weeks. Uncontrolled hyperglycemia in the early phase of diabetes resulted in differential regulation of cytochrome P450 isozymes. CYP1B1, CYP1A2, heme oxygenase (HO)-2 proteins and CYP1A2-dependent 7-ethoxyresorufin O-deethylase (EROD) activity were upregulated in the hepatic microsomes of diabetic rats. Insulin therapy ameliorated EROD activity and the expression of CYP1A2, CYP1B1 and HO-2 proteins. In addition, CYP2B1 and 2E1 proteins were markedly induced in the diabetic group. Insulin therapy resulted in complete amelioration of CYP2E1 whereas CYP2B1 protein was partially ameliorated. By contrast, CYP2C11 protein was decreased over 99% in the diabetic group and was partially ameliorated by insulin therapy. These results demonstrate widespread alterations in the expression of CYP isozymes in diabetic rats that are ameliorated by insulin therapy.

Keywords: *Diabetes, oxidative stress, CYP1A2, CYP1B1, CYP2B1, CYP2E1*

Introduction

Cytochrome P450 monooxygenases are a superfamily of heme-thiolate proteins which are involved in the biotransformation of endogenous compounds such as steroids, fatty acids, vitamins, bile acids, leukotriens, thromboxanes and prostaglandins as well as numerous xenobiotics such as drugs, pesticides and environmental pollutants. This enzyme system is a major route by which living organisms can metabolize lipophilic, xenobiotic chemicals into more water-soluble products, thereby facilitating elimination from the body. In contrast to detoxification, the P450 system is also known to convert certain xenobiotics into more toxic products. Numerous chemicals are known to be metabolically activated by these monooxygenases to their atherogenic/or carcinogenic metabolites that covalently bind to cellular macromolecules such as DNA and proteins [reviewed in Ref. 1].

During the oxidation of its substrates, the cytochrome P450 system has also been demonstrated to produce reactive oxygen species (ROS) such as superoxide radical and hydrogen peroxide [2–4]. These ROS produced by the P450 system may serve

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as precursors for the generation of other oxidants. For example, hydrogen peroxide and organic hydroperoxides are known to degrade hemoglobin oxidatively and thus promote the release of iron from the heme chelate [5]. The heme moiety of cytochrome P450 system therefore may serve as an intracellular source of iron capable of catalyzing free radical reactions [6]. Results from several laboratories indicate that iron rich P450 monooxygenases may serve as a source of catalytic iron in various models of tissue injury [7–10]. Furthermore, since the cytochrome P450 monooxygenases are the major drug metabolizing enzymes in the body, changes in these enzymes may cause a rapid elimination of certain drugs whereas the effects of certain drugs would persist for an unreasonable duration.

Recent studies from this laboratory showed that uncontrolled hyperglycemia in the early phase of streptozotocin-induced diabetes in rats is associated with oxidative stress [11,12]. Insulin therapy resulted in significant but incomplete amelioration of hypertension and oxidative stress [11]. More recently we have shown that the activities and protein expressions of major antioxidant enzymes, namely, superoxide dismutase, catalase and glutathione peroxidase were significantly reduced in the livers of diabetic rats compared to the controls [13].

The present study was undertaken to investigate the effect of diabetes and concomitant insulin therapy for 4 weeks on all major classes of CYP isozymes, namely, CYP1B1, 1A2, 2B1, 2E1 and 2C11, in one experiment. Heme oxygenase (HO)-2 was measured as a corollary to understand the mechanism of CYP1A2 and CYP1B1 induction in the diabetic rats. The results demonstrate differential regulation of protein expression of various P450s studied.

Methods

Animals

Male Sprague-Dawley rats (9-week old) weighing 300–350 g were randomly assigned to the diabetic and normal control groups. Animals assigned to the diabetic group received 65 mg/kg streptozotocin in citrate buffer, pH 4.6 (Sigma Chemical Co., St Louis, MO) via the tail vein. The control group received placebo injection. The diabetic animals were further subdivided into insulin-treated or untreated subgroups. The treated subgroups received ultralente insulin (Eli Lilly Inc., Indianapolis, IN) subcutaneously at an initial dosage of 3 units/100 g once daily. Insulin treatment was begun one day after streptozotocin injection. Insulin dosage was adjusted as needed using twice weekly plasma glucose determinations. Animals were observed for four weeks. Body weights were determined weekly. At the conclusion of the 4-week study period, animals were anesthetized by sodium pentobarbital (100 mg/kg IP) and killed by exsanguination using cardiac puncture. The livers were immediately removed, washed with ice-cold saline, snap frozen in liquid nitrogen and stored at −70°C.

Preparation of microsomal fractions

Liver homogenates (20% w/v) were prepared in 10 mM N-[2-hydroxyethyl]-piperazine-N′-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, containing 320 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 10 μg/ml leupeptin, 2 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (homogenizing buffer) at 0–4°C using a Potter Elvehjem Teflon pestle glass homogenizer. The hepatic cell-free extracts were centrifuged at 2500*g* for 10 min at 4°C. The supernatant fraction thus obtained was spun at 10,000*g* for 10 min at 4°C and then at 105,000*g* for 1 h at 4°C. After washing once, the microsomal pellet was suspended in homogenizing buffer and stored frozen at −70°C. A portion of the microsomes was used for the determination of total protein concentration by using a Bio-Rad kit (Hercules, CA).

Detection of cytochrome P450s and HO-2 proteins by immunoblotting

Microsomal proteins (5 μg each) were electrophoresed in 4–20% Tris-glycine SDS polyacrylamide gels (Novex, San Diego, CA). The separated proteins were transferred onto nitrocellulose membranes (Millipore Corp., Bedford, MA), blocked in 5% dry milk in T-TBS (0.02 M Tris/0.15 M NaCl, pH 7.5 containing 0.1% Tween 20) at room temperature for 3 h, washed 3× with T-TBS and incubated with the primary antibodies (1:2000) for 3 h at room temperature. CYP1B1 antibody was purchased from Gentest Corp (Woburn, MA) whereas all other cytochrome P450 isozymes antibodies were purchased from Oxford Biomedical Research (Oxford, MI). HO-2 antibody was purchased from StressGen Biotech Corp (Victoria, Canada). After washing 5× with T-TBS, the blots were incubated with secondary antibodies (1:2000; anti-rabbit for CYP1B1 and HO-2 and anti-mouse for CYP 1A2, 2E1, 2B1, and 2C11) conjugated with horseradish peroxidase at room temperature for 2 h. After washing 5× with T-TBS, the membranes were developed using ECL reagent (Amersham Life Science Inc.) and subjected to autoluminography. The autoluminographs were scanned with a laser densitometer (Model PD 1211, Molecular Dynamics, Sunnyvale, CA) to determine the relative optical densities of the bands. All the immunoblots were repeated at least 3–4 times and one such representative blot is presented in the present manuscript.

7-Ethoxyresorufin O-deethylase (EROD) activity

CYP1A2-dependent EROD activity was determined spectrophotometrically as described by Prough et al. [14]. Briefly, the assay mixture contained 0.1 mM Tris–HCl buffer, pH 7.8; 10 μM 7-ethoxyresorufin and 20–100 μg microsomal protein in a total volume of 2 ml. Reaction was started by adding 20 μl of 5 mM NADPH. The rate of fluorescence change over time was monitored at 530 nm (excitation) and 585 nm (emission). Resorufin (1 pmol) was added to calibrate each assay. EROD activity is expressed as mean pmol resorufin formed/mg protein/min.

Data analysis

Data are presented as mean ± SEM. Analysis of variance (ANOVA) and post hoc multiple comparison test was used in statistical analysis of the data. *P* values less than 0.05 were considered significant.

Results

Body weights and blood pressure

The untreated diabetic animals exhibited marked hyperglycemia and elevated glycosylated hemoglobin. Daily administration of insulin ameliorated hyperglycemia and lowered glycosylated hemoglobin concentration, although it did not reduce plasma glucose levels to that seen in control animals (Table I). The untreated diabetic animals exhibited a significant weight loss and severe hyperglycemia during the 4-week study period. Insulin therapy prevented diabetes-induced weight loss and facilitated the growth of animals at a moderately lower rate than seen in the control animals (Table I). The untreated diabetic animals also exhibited elevated blood pressure which was partially lowered by insulin therapy [11,12].

Effect of diabetes and insulin therapy on CYP1A2 protein and EROD activity

CYP1A2 protein was significantly induced in the hepatic microsomes of diabetic rats and was ameliorated by insulin therapy (Figure 1, upper panel). Likewise the EROD activity was also significantly induced in diabetic rats (Figure 1, lower panel) and was decreased to the control levels after treatment with insulin. CYP1B1 protein was induced by 4.5-fold in the diabetic rats as compared to the untreated controls and was partially ameliorated by insulin therapy (Figure 2).

Effect of diabetes and insulin therapy on CYP2B1, 2E1 and 2C11 proteins

CYP2B1 protein was induced 9-fold in the diabetic group ($p < 0.01$) and insulin therapy partially ameliorated the expression of this protein (Figure 3). CYP2E1 protein was induced 8-fold in the diabetic group ($p < 0.01$) and insulin therapy resulted in almost complete amelioration of this protein (Figure 4). By contrast, CYP2C11 (Figure 5) protein was decreased by over 99% in the diabetic group compared to the controls ($p < 0.01$) and was partially ameliorated by insulin therapy (Figure 5).

Effect of diabetes and insulin therapy on HO-2 protein expression

Treatment of the rats with streptozotocin caused a 2.4-fold induction of HO-2 protein in the hepatic microsomes of the rats ($p < 0.01$). Insulin therapy partially ameliorated the enzyme protein (Figure 6).

Discussion

The cytochrome P450 family 1 consists of three isozymes, cytochrome P450 (CYP) 1A1, 1A2 and 1B1 [15]. In general, CYP1A1 is not expressed in normal adult tissues but can be induced several fold by polycyclic or halogenated hydrocarbons [1], oxidized tryptophan [16–18] and hyperoxia [19–21]. CYP1A2, which is constitutively expressed in the liver, is primarily involved in oxidative metabolism of xenobiotics and is capable of metabolically activating numerous procarcinogens including aflatoxin B1,

Table I. Body weights and plasma concentration of glucose and glycosylated hemoglobin.

Parameter measured	Group		
	CTL	DM	DM + I
Body weights (g)			
Week 0	302 ± 14	326 ± 7	303 ± 5
Week 4	404 ± 34	296 ± 14⋆	345 ± 7
Plasma glucose†	106 ± 5	525 ± 21‡	180 ± 6⋆
Glycosylated hemoglobin (%)	6.6 ± 0.8	12.0 ± 0.4‡	8.0 ± 0.5

Data are mean ± SEM.
⋆ $p < 0.05$ versus CTL group.
† Average of plasma glucose levels measured twice weekly at 10:00 AM.
‡ $p < 0.005$ versus all other groups.

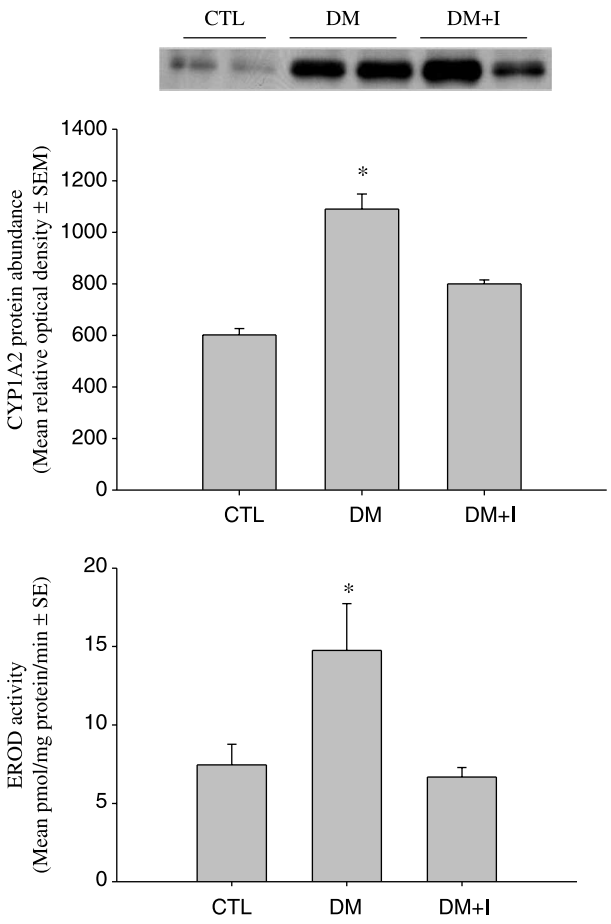

Figure 1. Representative Western blot and group data depicting the expression of CYP1A2 protein (upper panel) in the hepatic microsomes (5 μg protein each) of normal control rats (CTL, $n = 6$), untreated diabetic rats (DM, $n = 6$) or diabetic rats treated once daily with ultralente insulin (DM + I, $n = 5$). $\star P < 0.001$ versus CTL group. EROD activity (lower panel) in the hepatic microsomes of normal control rats (CTL, $n = 6$), untreated diabetic rats (DM, $n = 6$) or diabetic rats treated once daily with ultralente insulin (DM + I, $n = 5$). $\star P < 0.001$ versus CTL group.

arylamines, heterocyclic amine food mutagens, and polycyclic aromatic hydrocarbons (PAHs) carcinogens and atherogens. CYP1B1, like CYP1A1, metabolizes numerous carcinogens like PAHs [22]. Several arylamines have also been shown to be metabolized by human CYP1B1 expressed in yeast, and it was proposed that CYP1B1 may play an important role in the extrahepatic metabolism of these and other compounds [22].

In animal models, PAHs, inducers of CYP1A1/1A2, are known to act as initiators and/or accelerators of plaque development [23]. Additionally, estrogens, 17β-estradiol and estrone, are metabolized by CYP1A2/1A1 resulting in the formation of the 2- and 4-catecol estrogens and 16α-hydroxylation [24]. Unless detoxified, catecol estrogens may be oxidized to electrophilic metabolites, catecol estrogen quinines, that can react with DNA to form depurinating and stable products. These adducts, particularly depurinating adducts, can

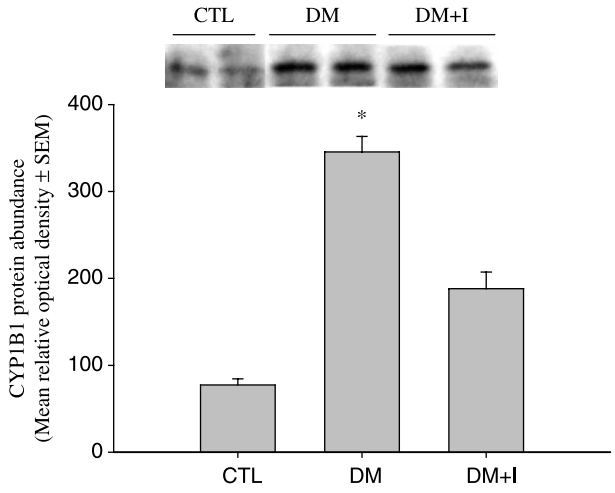

Figure 2. Representative Western blot and group data depicting the expression of CYP 1B1 protein in the hepatic microsomes (5 μg protein each) of normal control rats (CTL, $n = 6$), untreated diabetic rats (DM, $n = 6$) or diabetic rats treated once daily with ultralente insulin (DM + I, $n = 5$). $\star P < 0.001$ versus CTL group.

lead to oncogenic mutations and may subsequently initiate many human cancers [25]. CYP1A2 has also been suggested to play a critical role in mammalian neonatal survival [26]. Recently it has been reported that the low inducibility genotype for *CYP1A2* is associated with an increased risk of myocardial infarction. This effect was independent of smoking status and suggests that a substrate of CYP1A2 that is detoxified rather than activated may play a role in coronary heart disease [27].

The *CYP1A1, 1A2* and *1B1* genes have been demonstrated to be under the regulatory control of the aryl hydrocarbon receptor (AhR), a member of the basic helix-loop-helix (bHLH) family of transcription factors. Following ligand binding, the cytosolic

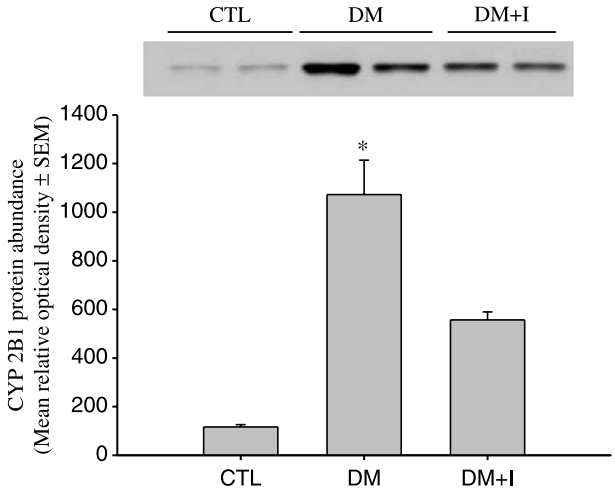

Figure 3. Representative Western blot and group data depicting the expression of CYP 2B1 protein in the hepatic microsomes (5 μg protein each) of normal control rats (CTL, $n = 6$), untreated diabetic rats (DM, $n = 6$) or diabetic rats treated once daily with ultralente insulin (DM + I, $n = 5$). $\star P < 0.001$ versus CTL group.

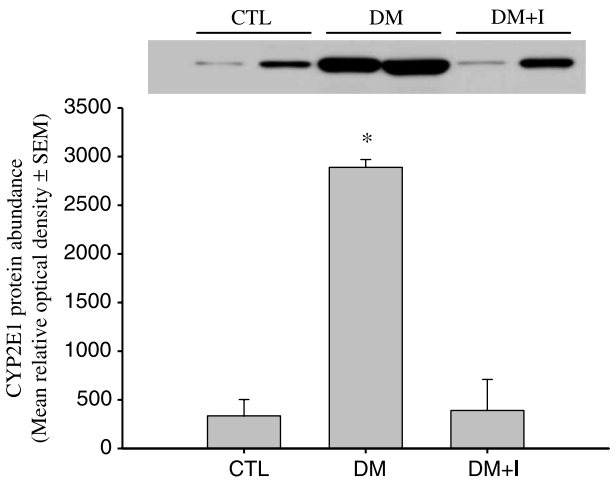

Figure 4. Representative Western blot and group data depicting the expression of CYP 2E1 protein in the hepatic microsomes (5 μg protein each) of normal control rats (CTL, $n = 6$), untreated diabetic rats (DM, $n = 6$) or diabetic rats treated once daily with ultralente insulin (DM + I, $n = 5$). $\star P < 0.001$ versus CTL group.

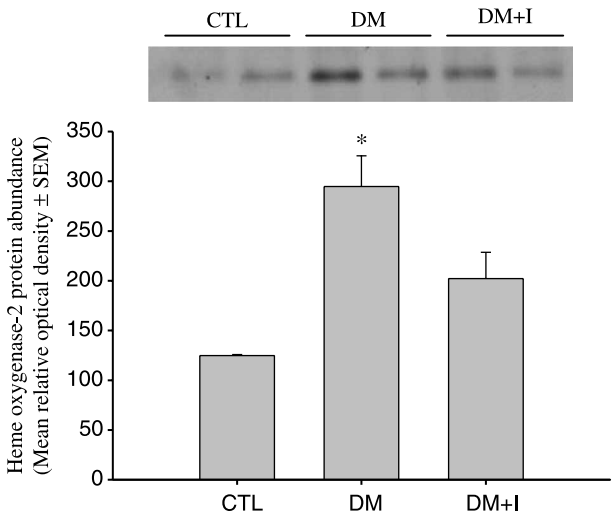

Figure 6. Representative Western blot and group data depicting the expression of HO-2 protein in the hepatic microsomes (5 μg protein each) of normal control rats (CTL, $n = 6$), untreated diabetic rats (DM, $n = 6$) or diabetic rats treated once daily with ultralente insulin (DM + I, $n = 5$). $\star P < 0.001$ versus CTL group.

ligand-AhR complex undergoes transformation, during which it dissociates from two molecules of 90 kD heat shock protein (HSP90) and at least one additional protein, it translocates into the nucleus, and following its association with at least one nuclear bHLH protein, Ah Receptor Nuclear Translocator (ARNT), it is converted into its high-affinity DNA binding form [28]. The binding of the transformed heteromeric AhR/ARNT complex to its specific DNA recognition site, the xenobiotic (dioxin) responsive elements, leads to chromatin and nucleosome disruption, increased promoter accessibility, and increased rates of transcription of *CYP1A1*/*1A2* genes. Regulatory sequences responsible for AhR-regulated gene transcription have been identified in the 5′ flanking region of both the *CYP1A1* [28] and *CYP1B1* [29] genes. Although the molecular mechanism(s) that control the expression of CYP1A2 have not been studied as well as that of CYP1A1, it has been suggested that CYP1A2 is regulated through AhR-specific and promoter-specific elements [30].

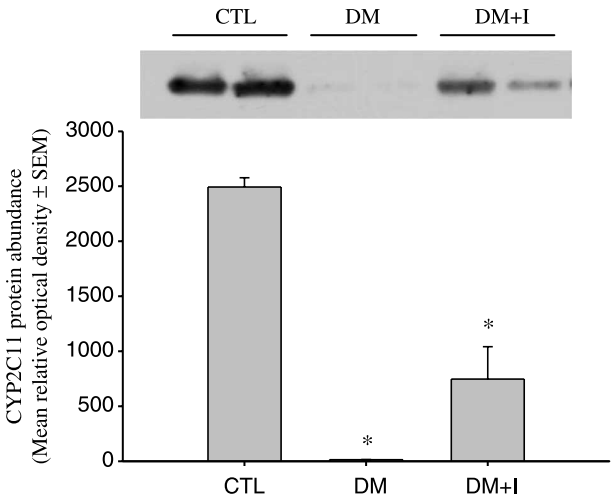

Figure 5. Representative Western blot and group data depicting the expression of CYP 2C11 protein in the hepatic microsomes (5 μg protein each) of normal control rats (CTL, $n = 6$), untreated diabetic rats (DM, $n = 6$) or diabetic rats treated with once daily ultralente insulin (DM + I, $n = 5$). $\star P < 0.001$ versus CTL group.

Our results demonstrate that HO-2 protein is significantly increased in the hepatic microsomes of diabetic rats. HO is the rate-limiting enzyme in the catabolism of heme to biliverdin. Biliverdin reductase catalyzes the conversion of biliverdin to bilirubin, which may then be conjugated by uridine diphosphate-glucuronosyltransferase (UDPGT) before biliary excretion [31]. Two isoforms of HO have been characterized. HO-1, the stress-induced isoform, has also been classified as heat-shock protein 32 K [32]. By contrast, the constitutive isoform, HO-2, is the major isoform present under physiological conditions. Increases in HO activity play a role in attenuating the overall production of ROS thereby protecting the tissues against oxidative stress [32,33].

The exact mechanism(s) for the induction of CYP 1A2/1B1 proteins observed herein could not be discerned in the present study. Even so, it is tempting to speculate as following: Humans with Crigler-Najjar syndrome and the corresponding Gunn rat animal model experience severe hyperbilirubinemia due to congenital defect in the UDPGT gene responsible for bilirubin conjugation [34]. Administration of inducers of CYP1A1/1A2 to Gunn rats markedly lower plasma bilirubin levels [35] and a PAH-inducible bilirubin degradation pathway in rat liver microsomes is

inhibited by an antibody that recognizes CYP1A1/1A2 [36]. Consistent with the possibility that bilirubin may be a substrate for CYP1A1/1A2 and cause substrate-mediated transcriptional regulation of the CYP1A1/1A2 genes, the congenitally jaundiced Gunn rat has been shown to be hyperbilirubinemic and exhibits an increased level of CYP1A1/1A2 expression [37]. Bilirubin-induced CYP1A1 gene transcription in mouse heaptoma cells occurs through direct interaction with the AhR [38]. Additionally, both bilirubin and biliverdin have been demonstrated to be AhR ligands and activate AhR-dependent CYP1A1/1A2 gene expression [39]. Furthermore, increased production of bilirubin in streptozotocin-treated diabetic rats is ameliorated by insulin treatment [40].

The results obtained in the present study demonstrate a marked induction of the CYP2B1 and CYP2E1 proteins in the diabetic group and insulin therapy ameliorated CYP2E1 protein. CYP2B1 protein was partially ameliorated by insulin therapy although there was no significant difference between the expression of this protein in the untreated controls and the insulin treated group. CYP2E1 catalyzes the oxidation of numerous xenobiotics including, acetaminophen, benzene, carbon tetrachloride, ethanol, *N*-nitrosodimethylaime and certain nitrosamines which are widely used as food additives [41]. Therefore, the bioactivation of these types of protoxicants by CYP2E1 places particular emphasis on this isoform in human health. Furthermore, Knoop and Tierney [42] have reported that after CYP2E1 is induced by low doses of ethanol, the toxicity of many toxic or procarcinogenic substances is potentiated. The CYP2E1 protein is increased by treatment of the rats with acetone, ethanol, pyrazol and other compounds through a substrate-induced protein stabilization [43]. Furthermore, due to its existence predominantly in high spin form, CYP2E1 also reduces dioxygen to reactive oxyradicals such as superoxide anion and hydrogen peroxide, which act as initiators of membrane lipid peroxidation [44–46]. Therefore, because of the ability of CYP2E1 to generate ROS and the known toxicity of these ROS, CYP2E1 plays a key role in the pathogenesis of liver injury. In streptozotocin-induced diabetes, tissue-specific alterations in CYP2E1 activity have been reported due to the increased production of ketone bodies [47–49]. The results obtained in the present study on the induction of CYP2E1 protein in the diabetes group are consistent with those observed by Raza et al. [49] except that the magnitude of the induction of this protein observed in the present study is much higher than that seen by Raza et al. [49].

Hepatic CYP2B isozymes are induced by numerous compounds such as barbiturates, pesticides, acetone, isosafrole and pregnenolone-16-α-carbonitrile [50]. Induction of CYP2B has been suggested to play a role in tumor promotion and increased hepatocarcinogenesis, probably due to their role in the activation of a number of procarcinogens such as aminoanthracene, benzo[a]pyerene and certain tobacco-specific nitrosamines [50]. CYP2B isozymes also metabolize numerous clinically important drugs such as diazepam, buproprion and chemotherapeutic pro-drugs such as cyclophosphamide [51,52]. Induction of hepatic CYP2B1 in diabetes could alter plasma drug levels. Therefore, in uncontrolled diabetes, this could lead to reduction in the efficacy of drugs metabolized to inactive compounds. In contrast, pro-drugs could be metabolized to their active metabolites more quickly, leading to toxicity. CYP2B1 has also been reported to metabolize cocaine to a toxic metabolite [53], thus an additive or synergistic increases in liver damage may occur when cocaine is used by diabetic individuals. In fact, inhibition of CYP2B isozymes protects against cocaine-mediated hepatotoxicity in rats [53]. Furthermore, CYP2B1 has also been reported to play an important role in puromycin-induced nephrotic syndrome by serving as a site for the generation of ROS and a significant source of catalytic iron [54].

The most striking observation in the present study was an almost complete disappearance of CYP2C11 protein in the hepatic microsomes of diabetic rats which was partially ameliorated by insulin. The constitutively expressed CYP2C11 is subjected to regulatory influences such as age, sex and tissue-specific factors [1]. This male specific isozyme, representing over 50% of the total P450 in male rat liver [55], catalyzes the 2α and 16 α hydroxylation of testosterone and also metabolizes sildenafil [56]. This isozyme is not expressed in immature rats but is induced at puberty in males but not in females. The developmental pattern of CYP2C11 is imprinted by exposure to androgen during the neonatal period and is ultimately regulated by the pulsatile pattern of pituitary growth hormone secretion that is characteristic of adult male rats [57]. It has been shown that the effects of diabetes and castration are similar and that insulin stimulates the synthesis and release of testosterone and thus indirectly maintains the male pattern of hepatic metabolism [58]. It has been proposed that the two hormones, insulin and testosterone, act through a common mediator, growth hormone, to exert their influence on the liver [58].

The results presented in the present communication suggest that insulin can regulate the expression of microsomal cytochrome P450 monooxygenases. However, it is not possible to precisely discern whether insulin acts directly or the observed changes in these monooxygenases are secondary to other effects of insulin in diabetes mellitus, namely, hyperglycemia, reactive species of oxygen, advanced glycation end products, impaired secretion of glucagons and growth hormone, hyperketonemia, and

reduction in plasma testosterone and thyroid hormone levels or streptozotocin itself.

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