0026-895X/95/030474-06$3.00/0

MOLECULAR PHARMACOLOGY, 47:474–479, 1995.

Insulin Down-regulates Cytochrome P450 2B and 2E Expression at the Post-transcriptional Level in the Rat Hepatoma Cell Line

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Received August 5, 1994; Accepted December 9, 1994

SUMMARY

Cytochromes P450 (P450s) are inducible drug-metabolizing enzymes involved in the metabolism of numerous endogenous and exogenous substrates. The regulation of some of these enzymes during experimental diabetes has been reported, but the direct involvement of insulin and the mechanism of its action remain unclear. The aim of our work was to study the effects of insulin on P450 2B and 2E expression in differentiated Fao hepatoma cells. Exposure of the cells to 0.1 μM insulin caused 60% and 80% decreases in the steady state levels of P450 2B and 2E proteins, respectively, within 24 hr. Before this, a rapid decrease in the corresponding messages was observed. Indeed, 5–6 hr of insulin treatment produced 80 and 50% decreases in P450 2B and 2E mRNA levels, respectively. Nuclear run-on transcription and mRNA turnover studies were performed to determine the mechanism (transcriptional and/or post-transcriptional) by which insulin modulated these mRNA levels. From our results, it can be concluded that insulin down-regulates the expression of P450 2B by shortening the half-life of its mRNA (half-lives of 6.9 hr without insulin and 3.6 hr with insulin), whereas it down-regulates the expression of P450 2E both by weak repression of the transcription rate (−30%) and, in particular, by acceleration of its mRNA turnover (half-lives of 8.5 hr without insulin and 3.3 hr with insulin).

P450s are inducible drug-metabolizing enzymes involved in the metabolism of a wide array of endogenous and exogenous compounds. Alterations of the P450 enzyme composition can affect the metabolism of drugs and steroid hormones, as well as the bioactivation of toxic compounds; consequently, the investigation of P450 enzyme gene regulation is of considerable importance.

To date, the hormonal regulation of the expression of these enzymes remains unclear. Studies on animals made diabetic by chemical treatment suggested that insulin could regulate the expression of microsomal drug metabolism. Indeed, animals with streptozotocin-induced diabetes were more susceptible to the toxicity of chemicals such as carbon tetrachloride and bromobenzene (1). Moreover, hepatic postmitochondrial preparations from these animals were more efficient than preparations from control animals in catalyzing the activation of many chemical procarcinogens, such as nitrosamines and heterocyclic amines (2–4). Insulin-dependent diabetes profoundly modulates the levels of constitutive and inducible forms of P450 (5–9), and these changes are presumably responsible for alterations in the metabolism of various toxins (2, 7, 10). These effects on hepatic P450 expression can be reversed by daily insulin treatment (5, 6, 11, 12). Furthermore, in one model, non-insulin-dependent diabetes did not influence hepatic P450-dependent monooxygenases (13). However, it is unclear whether insulin acts directly or whether the observed changes in P450 expression are secondary to other effects of insulin in diabetic pathology (e.g., impaired secretion of glucagon and growth hormone, hyperglycemia, hyperketonemia, and reductions in plasma testosterone and thyroid hormone levels).

The study of hormonal regulation in mammalian cell cultures offers several advantages, among them the possibility of precisely controlling the cellular environment without the ambiguity of hormonal interactions that may be encountered *in vivo*. Rat cell lines derived from the Reuber H35 hepatoma are particularly convenient for the study of insulin regulation. In contrast to other *in vitro* models, such as hepatocytes in primary culture, they do not require specific insulin supplementation of the culture medium. They offer important advantages for routine use (availability, immortality, and

This work was supported by INSERM, l'Université René Descartes, l'Université Paris-Val de Marne, and l'Association pour la Recherche sur le Cancer (Grant 66–04).

ABBREVIATIONS: P450, cytochrome P450; SDS, sodium dodecyl sulfate; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; PEPCK, phosphoenolpyruvate carboxykinase; IRS, insulin-responsive sequence.

phenotype stability) and possess numerous insulin receptors, by which insulin regulates the expression of several genes (14–17). Moreover, among these rat hepatoma cells, the highly differentiated Fao cells express some of the inducible P450s (18, 19).

The present study was undertaken to investigate the effects of insulin, at a dose routinely added to hepatocyte cultures (0.1 μM) (20), on the expression of P450 2B and 2E in Fao cells. Our results show that insulin directly down-regulates the expression of these two P450s through a post-transcriptional mechanism.

Materials and Methods

Cell culture and treatments. Fao cells are differentiated derivatives of the clonal cell line H4IIEC3, which was derived from the Reuber H35 rat hepatoma (21). They were grown in monolayer culture in modified Ham's F-12 medium containing 5% fetal calf serum. Cells were exposed to insulin (0.1 μM) during the exponential phase of growth. Cultures without any addition were used as controls. This concentration of insulin was previously shown to exert a maximal effect (17, 22).

Microsomal preparations and Western blot analysis. A pellet of cells harvested from five culture dishes (30×10^6 cells) was sonicated and microsomes were prepared by ultracentrifugation (23). Microsomal proteins were electrophoresed on SDS-polyacrylamide gels, according to the method of Laemmli (24), and were electrotransferred to nitrocellulose sheets. These sheets were probed with the following antibodies: monoclonal anti-rat P450 2B1/B2 (25), checked against preparations provided by Dr. F. P. Guengerich (Vanderbilt University, Nashville, TN) (26), and polyclonal anti-rat P450 2E1, provided by Dr. C. S. Yang (Rutgers University, Piscataway, NJ) (27). All antibodies recognized a microsomal protein from Fao cells that migrated as a single band with the same mobility as that recognized in untreated or induced rat liver microsomes. The amount of each P450 was first calculated as arbitrary units (deduced by scanning densitometry) per milligram of microsomal protein and was then expressed as the percentage of the amount of isozyme found in control cells.

Isolation of RNA and RNA blotting analysis. Total RNA from a pellet of cells harvested from five culture dishes (30×10^6 cells) was isolated by the guanidium-HCl method (28). Twenty micrograms of total RNA were subjected to electrophoresis in denaturing formaldehyde/1.2% agarose gels and were transferred to nylon membranes. These membranes were prehybridized and then successively hybridized with several ^{32}P-labeled cDNA probes (to 28 S rRNA, P450 2B mRNA, and P450 2E mRNA). P450 2B and 2E cDNA probes were generously provided by Dr. F. J. Gonzalez (National Cancer Institute, Bethesda, MD). After hybridization, filters were washed twice with 2× standard saline citrate (150 mM sodium chloride, 15 mM sodium citrate, pH 7.0)/0.1% SDS at room temperature and once with 2× standard saline citrate/0.1% SDS at 65° for 30 min and were then processed for autoradiography. Hybridization of the probes for RNA blotting was done according to the method of Maniatis *et al.* (29). The relative intensities of the hybridization signals were determined by scanning with a densitometer (Scanjet II scanner; Hewlett-Packard). For each treatment, the ratio of the amount of specific mRNA to the amount of 28 S rRNA signal was calculated and is expressed with respect to the corresponding ratio found in untreated Fao cells.

Nuclear run-on transcription. Nuclei from Fao cells were isolated at times 0, 30 min, 1 hr, and 2 hr after insulin administration (five plates/treatment). After scraping in phosphate-buffered saline, Fao cells were pelleted and resuspended in 2 ml of lysis solution consisting of 10 mM Tris, pH 7.5, 10 mM sodium chloride, 3 mM magnesium chloride, and 0.5% Nonidet P-40. Nuclei were separated by centrifugation, washed again in the lysis solution, and finally resuspended in a storage buffer consisting of 50 mM Tris, pH 8.0, 5 mM magnesium chloride, 0.1 mM EDTA, and 40% glycerol.

Nuclei ($10–20 \times 10^6$/tube) were incubated for 30 min at 30° in a buffer containing 100 mM Tris·HCl, pH 7.9, 50 mM sodium chloride, 0.4 mM EDTA, 1.2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 350 mM ammonium sulfate, 4 mM manganese chloride, 10 mM creatine phosphate, 10 μg/ml creatine phosphokinase, 2 mM CTP, 2 mM GTP, 2 mM ATP, 500 units/ml RNasin, and 1 mCi/ml [α-^{32}P]UTP (3000 Ci/mmol; Amersham). After labeling, the preparation was successively treated with 10 μg/ml DNase (RNase-free) for 15 min at 37°, 40 μg/ml proteinase K for 15 min at 37°, and 100 μg/ml proteinase K and 1% SDS for 30 min at 37°. RNA was isolated by sequential guanidium chloride extraction and sodium acetate/ethanol precipitation. An aliquot of radioactively labeled RNA was counted in a β-scintillation counter. Labeled RNA (15×10^6 cpm/blot) was incubated for 65–70 hr at 42° with a nylon membrane on which 5 μg of various cDNA probes (pUC, calmodulin, P450 2B, P450 2E, and c-*fos*) had been previously blotted. The blots were then washed, treated with RNase A, and exposed to X-ray film. The hybridization signals were quantitated by densitometric scanning (Scanjet II scanner; Hewlett-Packard). The relative rates of transcription were calculated by dividing values obtained for P450 and c-*fos* by those obtained for calmodulin, after subtraction of the value obtained for pUC DNA, and are expressed as the percentage of the corresponding ratio found in untreated Fao cells.

mRNA half-life determinations. mRNA turnover was assessed at different times after treatment of cells with the transcription inhibitor DRB (30). Fao cells were treated with insulin (0.1 μM) or left untreated for 3 or 5 hr and were then treated with 100 μM DRB. Total RNA was isolated 0, 1, 2, 3, 4, 6, 14, and 24 hr after DRB treatment. At that dose, DRB treatment for 24 hr did not affect the number or viability of Fao cells. The amount of mRNA at time 0 (the time of addition of DRB) in each group (control and insulin treated) was assigned a value of 100%, and all other values for each treatment group at different time points were expressed as percentages of the time 0 value. First-order decay rate constants were derived and used to calculate half-life values.

Results

The expression of three P450 proteins in Fao cells was studied using Western blot analysis (Fig. 1). P450 2B and 2E were readily detected in a microsomal preparation of these cells (Fig. 1A, *lanes 1* and *5*), in agreement with our previous observations (19). In the case of P450 3A, only a very faint band was revealed by this assay (data not shown). Exposure of the cells to 0.1 μM insulin for 24 hr caused 60 and 80% decreases in the amounts of P450 2B and 2E protein, respectively (Fig. 1). In contrast, the amount of P450 3A remained constant (data not shown). The mechanism of insulin action was further investigated in the case of P450 2B and 2E. The amount of mRNA was evaluated by RNA blotting analysis (Fig. 2). The P450 2B probe detected a message of about 2.1 kilobases and the P450 2E probe a message of 1.8 kilobases. A 5-hr insulin treatment decreased the amount of P450 2B mRNA by 80% (Fig. 2A). Similarly, insulin elicited a 50% decrease in the amount of P450 2E mRNA (Fig. 2A). A time course study of the effect of insulin showed that down-regulation of P450 mRNAs started at 3 hr and was almost maximal at 5 hr of treatment (Fig. 2B). These data show that the effect of insulin is pretranslational.

To determine whether down-regulation of P450 2B and 2E messages caused by insulin was the result of repressed transcription of these genes and/or accelerated turnover of the P450 2B and 2E mRNA, nuclear run-on transcription and

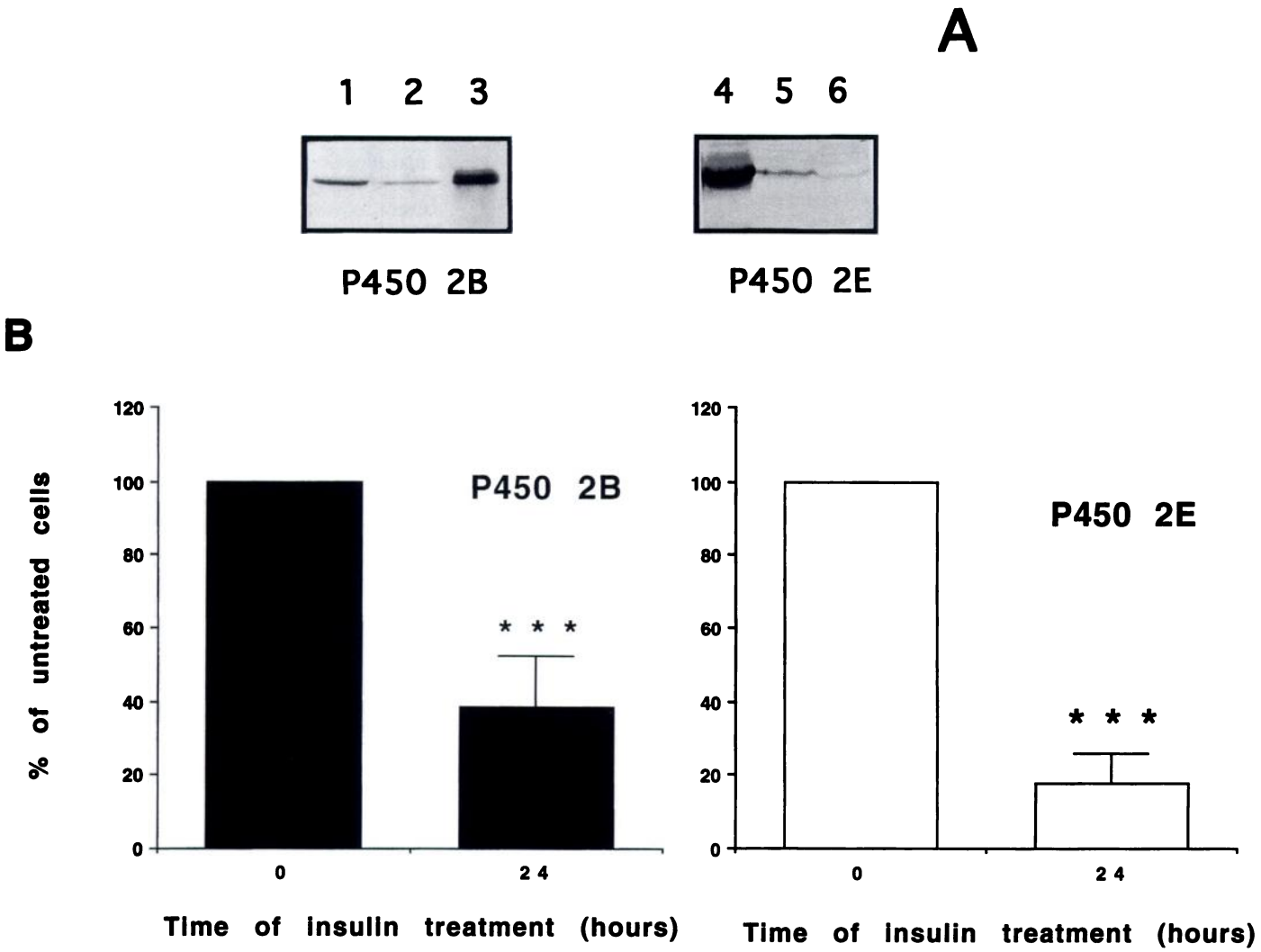

Fig. 1. A, Western blot of microsomes from Fao cells and rat liver, probed with a monoclonal anti-rat P450 2B antibody (*lanes 1-3*) and a polyclonal anti-rat P450 2E antibody (*lanes 4-6*). *Lanes 1* and *5*, microsomes from untreated Fao cells (20 μg); *lanes 2* and *6*, microsomes from 24-hr insulin-treated Fao cells (20 μg); *lane 3*, microsomes from phenobarbital-induced rat liver (0.5 μg); *lane 4*, microsomes from untreated rat liver (15 μg). B, Effects of 24-hr insulin treatment on the relative amounts of P450 2B (■) and 2E (□) protein. *Bars*, mean ± standard error of three or four separate experiments. ***, $p < 0.001$.

mRNA turnover studies were performed. Nuclei from Fao cells exposed to insulin for various times were incubated with [α-^{32}P]UTP, after which labeled P450 2B and 2E run-on transcripts were quantitated (Fig. 3). Calmodulin, rather than actin or glyceraldehyde-3-phosphate dehydrogenase, was used to correct for loading variability. Indeed, insulin treatment does not significantly change the calmodulin transcription rate, whereas it modifies that of actin and glyceraldehyde-3-phosphate dehydrogenase (9, 15). After 2 hr of insulin treatment, the transcription rate of P450 2B was unchanged, whereas that of P450 2E was about 70% of the initial rate (Fig. 3A). However, initially a small increase in P450 2B and 2E transcription was observed within 30 min of insulin addition. As shown in Fig. 3B, this effect of insulin was transient and transcription rates of P450 2B and 2E returned to basal levels (P450 2B) or slightly below basal levels (P450 2E) within 1–2 hr. In a control experiment, c-*fos* gene transcription was increased 2.5–3-fold by a 30-min insulin treatment (data not shown), as shown previously (16).

To ascertain whether insulin affected the half-life of the P450 2B and 2E mRNAs, the rate of turnover of these messages was determined by addition of an inhibitor of transcription (DRB) to cells that had been preincubated for 3 hr in the presence or absence of insulin. Preliminary experiments revealed that actinomycin D was particularly toxic to Fao cells, preventing its use in mRNA half-life determinations. The half-life of the P450 2B messages was shortened from 6.9 hr to 3.6 hr and that of the P450 2E messages from 8.5 hr to 3.3 hr after exposure of Fao cells to insulin (Fig. 4). A similar effect on mRNA half-life was observed when insulin pretreatment lasted for 5 hr (data not shown).

We conclude that, in Fao cells, insulin down-regulates the expression of P450 2B and 2E mainly by shortening the half-lives of their mRNAs. In the case of P450 2E, we cannot eliminate the possibility of a contribution of weak repression of the transcription rate.

Discussion

It is now well accepted that insulin-dependent diabetes induces modifications in P450 composition. These modifications may result either from a direct action of insulin or,

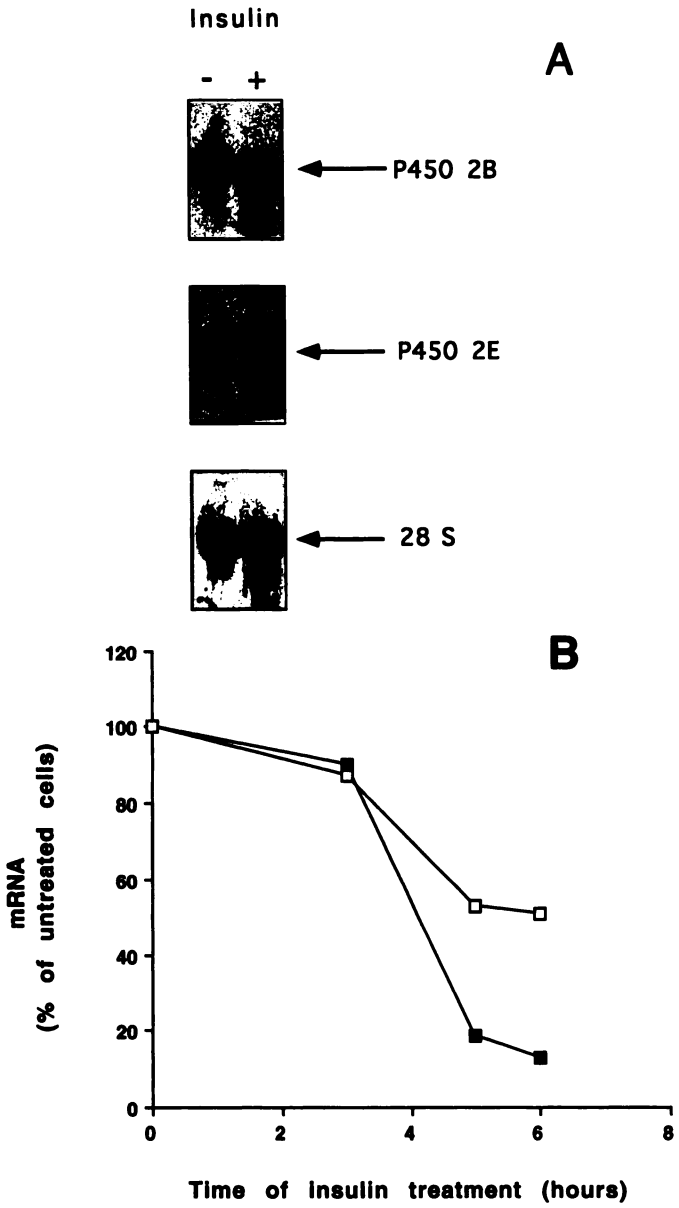

Fig. 2. A, RNA blotting analysis of total RNA (about 20 μg) prepared from Fao cells treated or not with insulin (0.1 μM) for 5 hr. Nitrocellulose sheets were successively hybridized with ^{32}P-labeled cDNA probes coding for P450 2B mRNA, P450 2E mRNA, and 28 S rRNA. B, Time course of the effects of insulin on the relative amounts of P450 2B (■) and 2E (□) mRNA. Fao cells were treated for the indicated times, after which total RNA was isolated. RNA (20 μg) was assayed for P450 2B and 2E message abundance by RNA blotting. The data points at times 2 and 6 hr for P450 2B and 2 and 5 hr for P450 2E are the average of two independent determinations, which differed by <15%. Means ± standard deviations were 19 ± 12 (three experiments) at time 5 hr for P450 2B and 51 ± 6.8 (three experiments) at time 6 hr for P450 2E.

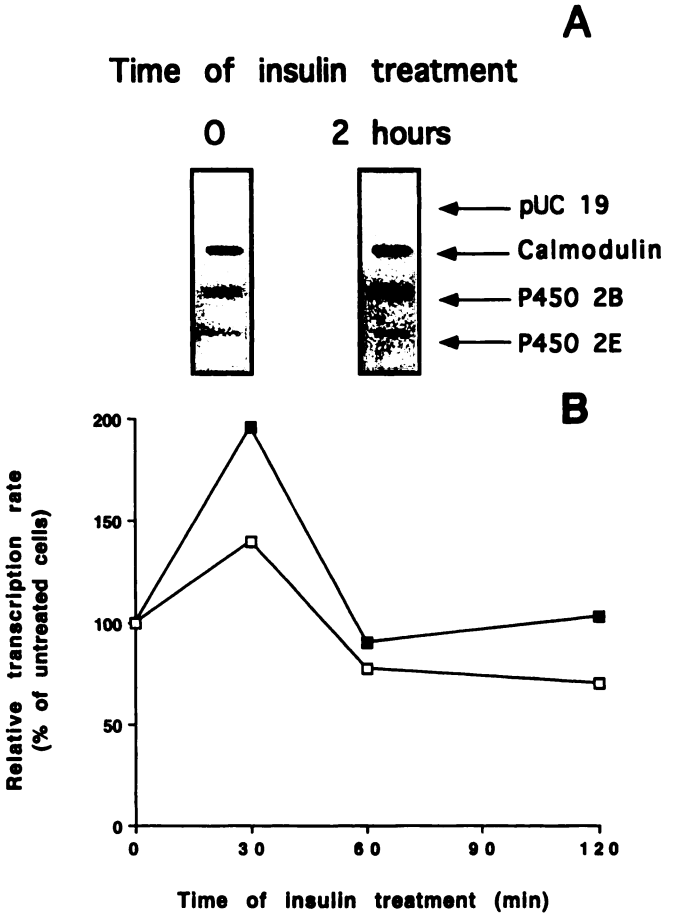

Fig. 3. A, Run-on transcription of P450 2B and 2E genes. Fao cells were treated with insulin (0.1 μM) for 2 hr. Nuclei were isolated and the transcription was measured using a nuclear run-on assay in which the isolated nuclei were incubated in the presence of [^{32}P]UTP. The labeled RNA was hybridized with pUC, calmodulin, P450 2B, and P450 2E cDNAs that were immobilized on nitrocellulose sheets. B, Run-on transcription of the P450 2B (■) and 2E (□) genes as a function of time after addition of insulin. The data presented are from two independent experiments.

indirectly, from other changes induced by diabetes. Several hormonal and metabolic consequences of diabetes, such as hyperketonemia (12) and a reduction in circulating growth hormone levels (6), have indeed been suspected to at least partially mediate these effects. However, the contribution of these modifications has been questioned. For instance, the P450 2E family is induced in both sexes of diabetic rats (31), whereas growth hormone secretion is impaired only in male rats (8); furthermore, growth hormone administration to male rats failed to reverse the effect of diabetes (8). On the other hand, it was suggested that the induction of P450 2E1 in fasting rats, as in diabetic rats, was due to an increase in circulating ketone bodies; however, fasting conditions do not produce ketone levels sufficient to account for the induction observed (12). Our work establishes that insulin is able to directly control P450 2B and 2E expression, decreasing the levels of the respective mRNA and subsequently the protein levels. Our aim was then to determine the mechanism (transcriptional and/or post-transcriptional) by which insulin modulated these mRNA levels.

Numerous studies, reviewed by O'Brien and Granner (32), show that insulin can regulate the transcription of several genes, either negatively (PEPCK, aspartate aminotransferase, and insulin-like growth factor-binding protein-1) or positively (c-*fos* and α-amylase). An IRS of 10 base pairs (TGGTGTTTTG) in the PEPCK promoter was shown to mediate part of the insulin response (33), and sequences with similarities to this IRS were present in other genes regulated by insulin (32). It is interesting to note that a sequence with a match of eight of 10 base pairs with the PEPCK IRS was identified in the P450 2E promoter (34) between bases −1434 and −1425 (TGAGGTTTTG). The presence of such a se-

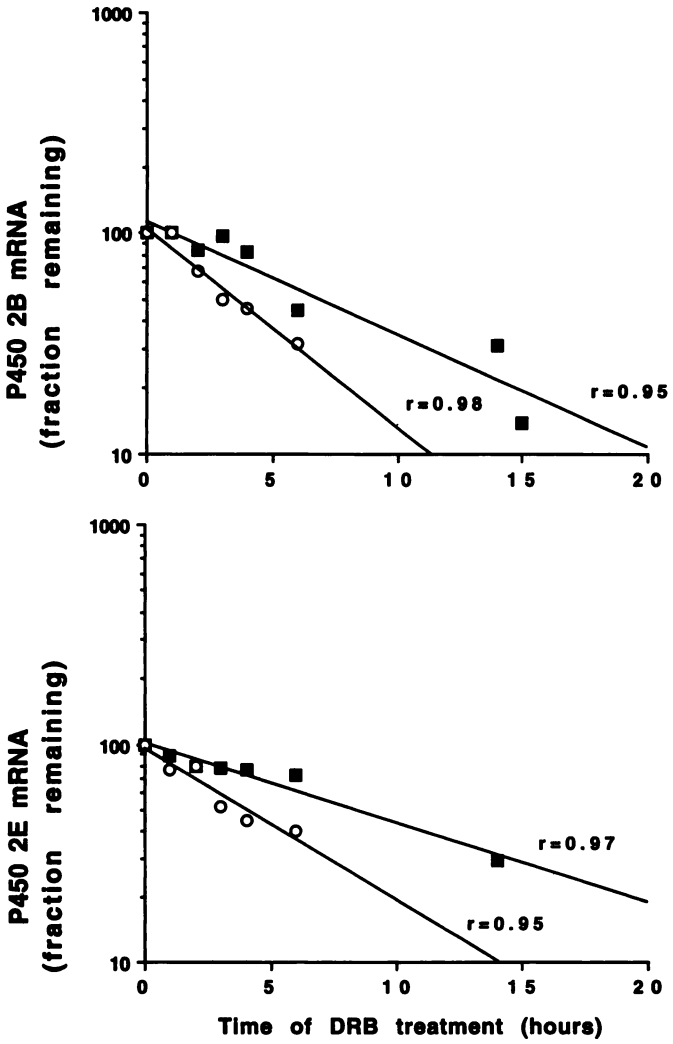

Fig. 4. Effects of insulin on the turnover rate of P450 2B and 2E mRNA. Fao cells were treated with 0.1 μM insulin (○) or left untreated (■) for 3 hr, at which time cells were treated with 100 μM DRB (time 0); total RNA was then isolated at the indicated times. P450 2B and 2E mRNA abundance was determined by Northern blot analysis, and results were quantitated by densitometry and normalized with respect to time 0 values. The first-order decay rate constants were derived and used to calculate half-life values. The results were combined from two independent experiments.

quence in a promoter does not prove that it is functional (17); however, it is possible that this sequence may mediate the weak transcriptional repression of P450 2E by insulin that we observed.

The main conclusion of the present study is that insulin accelerates the rate of P450 2B and 2E mRNA turnover. Thus, in Fao cells, the primary effect of insulin is post-transcriptional. Most of the effects of insulin are transcriptional (32); however, such a mechanism of insulin action has already been reported. Flores-Riveros *et al.* (35) showed that insulin down-regulates the expression of glucose transporter 4 both by repressing transcription of the gene and by accelerating mRNA turnover. Even in the case of the PEPCK gene, where the insulin effect is mainly transcriptional, there have been some reports suggesting an effect on mRNA turnover (36). However, these conclusions have recently been questioned (37). Nevertheless, it is clear that one effect of insulin is regulation of mRNA turnover. In some cases (such as for P450 2B), it is, in Fao cells, the only mechanism of insulin action.

Current evidence suggests that the rate of transcription is the major point of regulation of most hepatic P450 genes. However, recent studies have shown that hepatic P450s are also controlled by post-transcriptional mechanisms (38–40). To our knowledge, until now there have been no published reports of half-life determinations for P450 2B and 2E messages in rats. The half-lives of P450 2B and 2E proteins are known. P450 2B is half-degraded within 19 hr (41), whereas P450 2E1 is degraded via a biphasic pathway consisting of both rapid and slow components, with approximate half-lives of 7 hr and 37 hr, respectively (42). A slight dissociation was observed between the down-regulation of the proteins and that of their corresponding mRNAs. In the case of P450 2B, there is a 60% decrease in the amount of protein and an up to 80% decrease in the amount of mRNA. This is probably due to the longer half-life of the protein. In the case of P450 2E, the decrease is greater for the protein. The degradation of this protein displays a rapid component, with a half-life of 7 hr (42); however, direct effects of insulin or of metabolic changes elicited by this hormone on the protein cannot be excluded.

Our findings show that insulin is capable of shortening the half-lives of P450 2B and 2E messages. This is in good agreement with observations made in diabetic rats, where mRNA stabilization was suggested to be the mechanism of P4502E1 induction (12, 38). This stabilization could be due to the absence of insulin in diabetic rats. Recent work on mRNA decay showed that the sequence elements regulating it are found throughout the message (43). Our results raise the questions of whether insulin acts directly on one or several of these sequences and whether the mechanisms interfering with mRNA destabilization are the same for the two messages.

It is well known that there is a rapid decline in P450 content in cultured hepatocytes during the first 24 hr of culture *in vitro* (44). Our data suggest one possible mechanism for this decline. Indeed, insulin is routinely added to the culture medium, at the dose used in this study, to maintain differentiation of hepatocytes. It is possible that insulin may be at least partly responsible for this observed P450 loss.

In conclusion, our observations provide additional evidence for the role of insulin in the regulation of hepatic metabolism in rats, by influencing P450 2B and 2E expression. It would be interesting to determine whether insulin affects the expression of other P450s, particularly in humans.

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