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Effect of Diabetes on Protein Synthesis Rate and Eukaryotic Initiation Factor Activities in the Liver of Virgin and Pregnant Rats

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Abstract

To study the effect of prolonged diabetes on protein synthesis and on the activities of initiation factors eIF-2 and eIF-2B in the liver, female rats were treated with streptozotocin. Some animals were mated and studied on day 20 of pregnancy, whereas others were kept virgin and studied in parallel. The protein synthesis rate was measured with an 'in vitro' cell-free system, and was lower in diabetic pregnant and virgin animals than in pregnant and virgin controls (30–60%). The fetuses of diabetic rats had a lower protein synthesis rate than those from controls, although they always showed a higher protein synthesis rate than their mothers or virgin rats. Protein synthesis rate, RNA concentration, and initiation factor 2 activity were higher in pregnant than in virgin rats. Both activity and level of eIF-2 factor changed in parallel to the protein synthesis rate, although no differences could be detected between control and diabetic animals. The eIF-2B activity in tissue extracts from diabetic virgin rats and fetuses was lower than in extracts from their controls, whereas no differences could be detected between pregnant and virgin control rats nor between pregnant control and pregnant diabetic animals. The percentage of the phosphorylated form of eIF-2 factor, eIF-2(αP), was slightly lower in virgin than in pregnant rats but was unaffected by the diabetic condition, while in diabetic fetuses this parameter was lower than in their corresponding controls. The cyclic adenosine monophosphate dependent protein kinase level was lower in diabetic rats than in controls, whereas no changes in the activity of casein kinase II were found. The isoelectric forms of the β subunit of eIF-2 factor, eIF-2β, were different in the diabetic and the control animals, indicating that insulin deficiency modifies the phosphorylation of specific substrates. Since no differences were detected in RNA or eIF-2 content between control and diabetic rats, translation may, at least partly, be inhibited in the liver by an impairment of peptide chain initiation caused by the decreased eIF-2B activity which nevertheless is independent of eIF-2α phosphorylation.

Key Words

Translation, initiation factors
Diabetes
Pregnancy
Rat liver

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Introduction

The nitrogen imbalance associated with diabetes mellitus is the result of an alteration in protein turnover, such that the rate of protein degradation exceeds the rate of synthesis [reviewed in ref. 1]. Insulin has been shown to correct this imbalance by modifying both pathways, but the mechanism(s) involved are still not completely understood [reviewed in refs. 1 and 2]. The global protein synthesis rate in diabetes may be decreased, depending on (1) the amount of synthetic 'machinery', which includes ribosomes, protein factors, mRNA, and tRNA, or (2) the efficiency by which the available machinery is used to control the rate at which new peptide chains are initiated or elongated [1, 3]. Inhibition of protein synthesis in fast-twitch glycolytic muscles from diabetic rats, such as gastrocnemius and psoas, was due both to loss of tissue RNA and to reduced translational efficiency, resulting from impairment in peptide chain initiation [2, 4, 5]. Protein synthesis is also inhibited in cardiac muscle of diabetic rats, although peptide chain initiation is not impaired [2, 6, 7]. More controversial results have been obtained in liver where both plasma and intracellular proteins are synthesized, and the liver protein synthesis in the diabetic rat has been reported as either unchanged [8, 9] or impaired [10, 11].

The first step in peptide chain initiation is the formation of a ternary complex comprising eukaryotic initiation factor (eIF-2), guanosine 5′-triphosphate (GTP), and initiator methionyl $tRNA_i$ (Met-tRNA), eIF-2 is a necessary prerequisite for the initiation of protein synthesis, since it is required to prime each 40s ribosomal subunit for every translation round. Another initiation factor which is implicated in the process is eukaryotic initiation factor 2B (eIF-2B or guanine nucleotide exchange factor) which catalyses the exchange of eIF-2-bound guanosine 5′-diphosphate (GDP) for free GTP [for reviews see refs. 3, 12, 13]. Since phosphorylation of the α subunit of eIF-2 (eIF-2α) results in both sequestration of eIF-2B into an inactive complex containing eIF-2(αP)·GDP and inhibition of initiation rates, eIF-2(αP) can be considered a potent competitive inhibitor of eIF-2B [reviewed in refs. 14–16]. Moreover, eIF-2B may be involved in modulating translation not only in the presence of changes in eIF-2α phosphorylation but also in their absence [17, 18]. The exchange activity of this factor is acutely stimulated by insulin and growth factors, and it has been shown to be modulated by phosphorylation with several protein kinases included casein kinase II (CKII) and glycogen synthase kinase 3 [19–21].

The aim of this paper was to study the regulation of the inhibition of protein synthesis in the diabetic liver and to examine whether the impairment in liver protein synthesis was produced through regulation of eIF-2B activity. Hyperinsulinemia [22–27] and insulin resistance [28–33] are characteristic features of late gestation, and it is not known how this situation affects protein synthesis. Fetuses from streptozotocin-diabetic rats also became diabetic, since they develop hyperglycemia and decreased circulating insulin levels [28], and we have recently shown polysomal disaggregation in the fetus of the streptozotocin-diabetic pregnant rat which indicates impairment of protein synthesis at the initiation level [34]. The present study was, therefore, extended to determine the effect of streptozotocin diabetes on liver protein synthesis in pregnant rats and their fetuses.

Materials and Methods

Experimental Animals

Female Wistar rats from our colony, weighing 180–200 g, were housed in a temperature-controlled room (21–23°C) with 12-hour light-dark cycles and fed a Purina diet (Pamlab, Spain). After an overnight fast, the animals were treated with 40 mg/kg i.v. streptozotocin dissolved in 50 m*M* citrate buffer (pH 4.5); control rats were treated with the same volume of citrate buffer. The animals were refed, and 48 h later a urinary glucose test was performed, and only rats showing glucosuria were included in the study. Part of the animals were treated subcutaneously with bovine ultralente monocomponent insulin (Novo-Nordisk, Denmark) 1.5 IU/day/100 g body weight. After 7 days of treatment, these rats were mated with normal males, and positive pregnancy was estimated by the appearance of sperm in vaginal smears. Insulin treatment was interrupted on day 0 of pregnancy until the 20th day of gestation when the animals were killed. Untreated age-matched nondiabetic pregnant rats and virgin rats receiving the same treatments were studied in parallel. All animals were killed by decapitation. The blood was collected into heparinized tubes, and the plasma was kept at −20°C until processed for glucose determination [35] using Somogyi supernatants [36] and insulin plasma radioimmunoassay [37] with a specific rat kit (Novo Denmark).

Proccesing and Analysis of Samples

Approximately 500 mg of fresh liver was homogenized in buffer H (1:2 w:v for fetuses or 1:2.5 for adults): 50 m*M* Hepes-KOH (pH 7.55), 4 m*M* magnesium acetate, 140 m*M* potassium acetate, 2.5 m*M* dithiothreitol, and 0.32 *M* sucrose. The postmitochondrial supernatant (PMS) was obtained by centrifugation at 11,000 *g* for 10 min and kept at −80°C until processed. The remaining liver was rapidly excised, placed on liquid nitrogen, and stored at −80°C until processed for eIF-2 activity or quantitation. The PMS fraction was used to determine protein and RNA contents by the methods of Bradford [38] and Munro and Fleck [39], respectively, and to measure protein synthesis rate and eIF-2B activity as described below. Amino acid determination was also performed in the PMS fraction using a Beckman 121 MB amino acid analyzer.

The eIF-2 factor was purified from calf brain through a four-step procedure described previously by our laboratory [40]. The purification was performed from the postmicrosomal supernatant, since this fraction contains larger amounts of eIF-2 than the ribosomal salt wash fraction [41]. The factor obtained was 95% pure and free of eIF-2B activity. Pure eIF-2 factor was used to form the eIF-2·[^{3}H]GDP complex for assaying the eIF-2B activity.

Protein synthesis was measured in the PMS fractions according to the method of García et al. [18]. The complete reaction system in a final volume of 50 µl contained: 50 m*M* Hepes-KOH (pH 7.55), 140 m*M* potassium acetate, 2.5 m*M* dithiothreitol, 0.32 *M* sucrose, 1 m*M* adenosine 5′-triphosphate, 0.75 m*M* GTP, 30 m*M* phosphocreatine, 150 µg/ml creatine phosphokinase, 75 µ*M* of all 19 other amino acids apart from leucine, 150 µg of PMS, 50 µ*M* leucine, and 10 µCi of *L*-[4,5-^{3}H]leucine (75 Ci/mmol). 20-µl aliquots (duplicate) were processed after a 45-min incubation at 30°C. The endogenous leucine concentration was measured in all PMS samples, and a significant difference between control and diabetic rats was only found in virgins (1.93 ± 0.21 vs. 2.77 ± 0.17 nmol/mg protein; $p < 0.05$). The corresponding values for control pregnant rats and fetuses (3.80 ± 0.19 and 7.83 ± 0.78 nmol/mg protein, respectively) were significantly higher than those for the virgins.

Frozen liver was homogenized 1:4 (w:v) in 20 m*M* Tris-HCl (pH 7.6) containing 0.2 *M* sucrose, 1 m*M* dithiothreitol, 5 m*M* magnesium acetate, 50 m*M* KCl, 1 m*M* phenylmethyl sulfonyl fluoride, 2 m*M* benzamidine, 100 m*M* sodium fluoride, 20 m*M* β-glycerophosphate, and 20 m*M* sodium molybdate. 4 *M* KCl was added to the postnuclear supernatant at a final concentration of 0.55 *M*, the mixture being stirred for 20 min at 4°C, and the salt wash postmicrosomal supernatant (S-100K) was then prepared by centrifugation at 200,000 *g* for 30 min to minimize phosphatase action. The GTP-dependent binding of eIF-2 factor to Met-tRNA$_i$ was determined as described by Martín et al. [41] in a reaction mixture of 100 µl containing: 20 m*M* Hepes-Tris (pH 7.6) 100 m*M* dithiothreitol, 1 m*M* GTP, 0.5 mg/ml bovine serum albumin, 3 pmol [^{3}H]Met-tRNA, and 30 µg of the S-100K fraction from control and diabetic rats. One unit is defined as the amount of protein that promoted the GTP-dependent binding of 1 pmol of met-tRNA$_i$ to the nitrocellulose filter.

The eIF-2B activity was measured by its capacity to exchange eIF-2-bound [^{3}H]GDP with free GTP in the PMS fraction from control and diabetic livers [18]. A binary complex of pure eIF-2 and [^{3}H]GDP was formed (10 min, 30°C) in a mixture containing 20 m*M* Hepes-Tris (pH 7.6), 100 m*M* KCl, 1 m*M* dithiothreitol, 0.5 mg/ml bovine serum albumin, [^{3}H]GDP (0.8 µmol/l, 12.4 Ci/mmol), and eIF-2 (95% pure). The eIF-2B assays were carried out at 30°C in a

Table 1. General parameters

Animals	Control	Diabetic
Body weight, g		
Virgin	228 ± 3^{a}	$203 \pm 10^{a,*}$
Pregnant	331 ± 8^{b}	$290 \pm 14^{b,*}$
Fetus	3.82 ± 0.12^{c}	$3.05 \pm 0.16^{c,**}$
Liver weight, g		
Virgin	8.56 ± 0.36^{a}	9.18 ± 0.34^{a}
Pregnant	12.80 ± 0.45^{b}	$15.58 \pm 0.95^{b,*}$
Fetus	0.23 ± 0.01^{c}	$0.20 \pm 0.02^{c,*}$
Plasma glucose, mg/dl plasma		
Virgin	118 ± 1^{a}	$652 \pm 45^{a,***}$
Pregnant	88 ± 3^{b}	$509 \pm 21^{b,***}$
Fetus	21 ± 2^{c}	$337 \pm 11^{c,***}$
Plasma insulin, μU/ml plasma		
Virgin	28.1 ± 3.7^{a}	$10.9 \pm 1.3^{a,***}$
Pregnant	41.7 ± 5.4^{b}	$14.7 \pm 2.0^{a,***}$
Fetus	120 ± 23^{c}	$38.3 \pm 4.6^{c,***}$

The rats were sacrified, and tissues were immediately excised, weighed, and frozen in liquid nitrogen until further processing. Glucose and insulin were measured in plasma samples as described in Materials and Methods. The values represent the mean ± SEM from 10–11 different animals per group. Asterisks represent significant differences between control and diabetic animals: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Comparison between virgin and pregnant rats and their corresponding fetuses was also made, and different superscript letters for the group values indicate statistically significant differences between them (at least $p < 0.05$).

final volume of 205 μl containing 20 m*M* Hepes-Tris (pH 7.6), 1 m*M* dithiothreitol, 100 m*M* KCl, 0.5 mg/ml bovine serum albumin, 1.5 m*M* magnesium chloride, fetal (25 μg) or adult (100 μg) PMS, 780 μ*M* GDP, and 1.1–1.8 pmol eIF-2·[^{3}H]GDP.

Protein kinase assays were performed according to previously described procedures [42], and the final volume of 50 μl contained 20 m*M* Tris-HCl (pH 7.5), 5 m*M* magnesium chloride, 5 m*M* sodium fluoride, 2 mg/ml phosvitin or 1 mg/ml histone, [γ^{32}P]adenosine 5′-triphosphate (100 μ*M*, 0.25 μCi), and 25 μg S-100K fractions in the absence or presence of 5 μg/ml heparin and 10 μ*M* cyclic adenosine monophosphate. The CKII activity was measured by using phosvitin as the exogenous substrate, since it has been proven to be the better substrate than casein for this kinase [43]. CKII and cyclic adenosine monophosphate dependent protein kinase (PKA) activities were considered the difference between the activity in the absence and presence of the inhibitor or modulator, respectively. One unit is defined as the amount of enzyme that catalyzes the incorporation of 1 pmol of $^{32}P_i$ during a 10-min incubation.

The S-100K supernatant from the liver of control and diabetic rats was resolved in horizontal isoelectric focusing (IEF) slab gels and analyzed by protein immunoblot and densitometric scanning as described by Martín et al. [41].

Statistical analysis of the data was performed by Student's t test.

Results

Table 1 shows some characteristics of the experimental animals. Diabetic rats had a lower body weight than controls, and the difference was significant for both virgin and pregnant rats as well as for the fetuses. The liver weight was higher in pregnant than in virgin rats, and the diabetic condition increased this parameter in both groups, although the difference was only significant in the diabetic pregnant rats. The fetal liver weight was lower in diabetic than in control rats. The glucose levels in plasma were lower in pregnant rats than in virgins and also lower in the fetuses than in their mothers; diabetes enhanced this parameter in the three groups (table 1). The plasma insulin levels were higher in pregnant than in virgin rats and much higher in the fetuses; they decreased very markedly with diabetes in all groups (table 1).

Protein and RNA contents were determined in the homogenates from control and diabetic animals. As shown in table 2, no changes in protein concentration were found between pregnant and virgin rats nor between

control and diabetic rats, whereas the protein concentration in fetuses was significantly lower than in adult animals. The RNA concentration in the homogenates was similar under most of the conditions studied, although it was higher in control pregnant than in virgin rats and significantly lower in diabetic pregnant than in control pregnant rats (table 2).

The rate of protein synthesis was determined in liver PMS fractions from all animals. As shown in figure 1A, when expressed per unit protein, the protein synthesis appeared significantly lower in the diabetic livers of the three groups of animals studied than in their respective controls. As shown in figure 1B, a similar inhibitory effect of protein synthesis in diabetics was found when the data are expressed relative to RNA. In spite of this effect, the protein synthesis was always higher in pregnant than in virgin rats and also higher in fetuses than in adult animals (fig. 1).

To determine whether the changes in the protein synthesis rates could be related to changes in initiation factor activities, the eIF-2B activity was also measured in the PMS fraction. To measure the eIF-2B activity by the GDP exchange assay, the conditions for the different samples were first settled. Different amounts of PMS protein from the liver of each experimental animal were studied in the exchange reaction over time, as shown in the representative experiment in figure 2. It appears that the eIF-2B activity is much higher in fetuses than in adult animals, and the activity of the factor is proportional to the amount of PMS assayed: 25–100 μg in fetuses or 100–200 μg in adult rats. The eIF-2B activity was then measured in 25 μg of fetal or 100 μg of adult PMS after an incubation period of 2.5 min. As shown in figure 3, a significant decrease in the activity of the factor was found in the diabetic livers from virgin rats and fetuses as compared with controls, whereas the differences obtained from the pregnant rats did not reach significance levels. Although the eIF-2B activity appeared much higher in the fetuses than in adult rats, pregnant and virgin rats showed a similar activity level (fig. 3).

Table 2. Protein and RNA contents in the livers of control and diabetic rats

Animals	Control	Diabetic
Protein, mg/g tissue		
Virgin	257.7 ± 10.6[a]	269.1 ± 12.2[a]
Pregnant	233.5 ± 12.5[a]	231.1 ± 10.0[a]
Fetus	92.1 ± 14.0[c]	84.7 ± 9.4[c]
RNA, mg/g tissue		
Virgin	6.57 ± 0.44[a]	5.47 ± 0.38[a]
Pregnant	9.45 ± 0.53[b]	7.40 ± 0.53[b,*]
Fetus	6.32 ± 0.67[a]	6.43 ± 0.30[a]

Protein and RNA content were determined in the homogenates from the livers of control and diabetic rats as described in Materials and Methods. The values represent the mean ± SEM from 4–10 different animals per group. Statistical analysis as in table 1.

The eIF-2 activity was measured by its capacity to form a ternary complex with GTP and Met-tRNA$_i$, and the results are shown in table 3. The eIF-2 activity was significantly higher in the fetuses than in pregnant or virgin rats and slightly higher in pregnant than in virgin rats. Nevertheless, no difference between control and diabetic animals was found in any of the groups studied.

The levels of eIF-2α and eIF-2(αP) were determined in the S-100K fraction from liver tissue. As shown in table 3, no differences in the levels of eIF-2α+(αP) or in the degree of phosphorylation of eIF-2(αP) were found between control and diabetic adult animals, while a decrease in the amount of eIF-2(αP) in the diabetic fetus was observed as compared

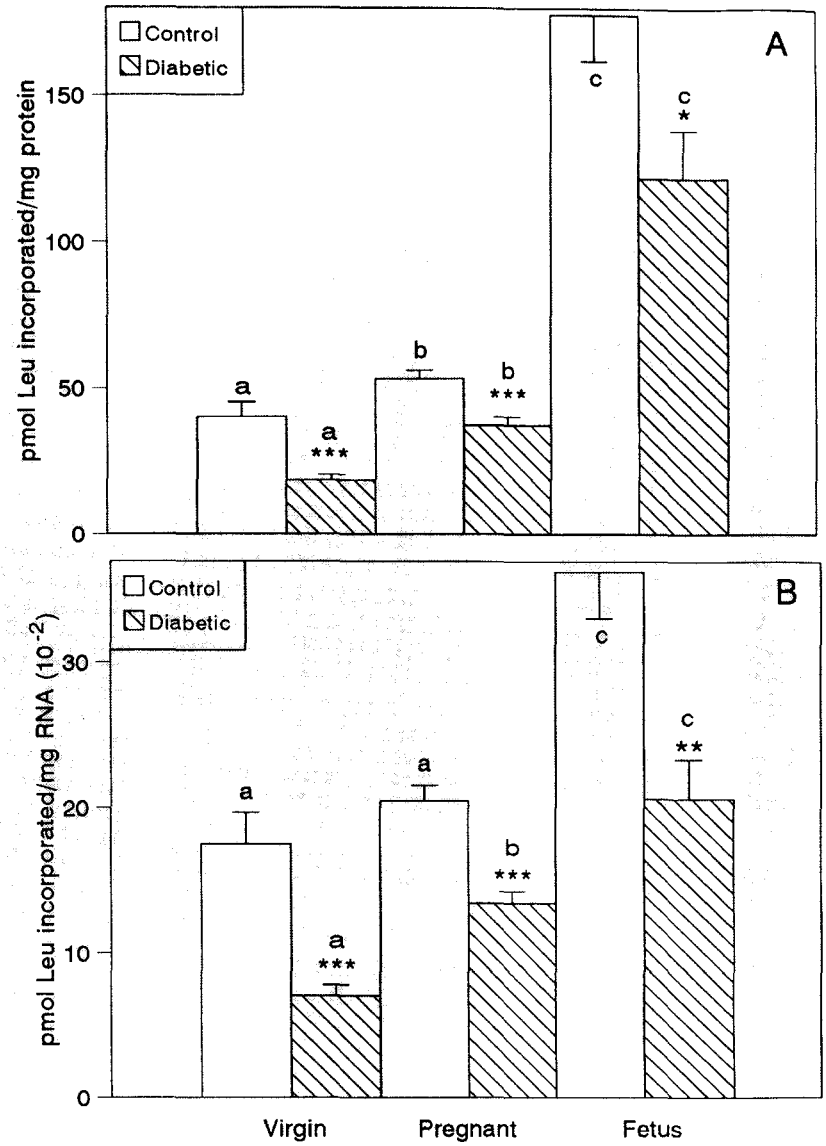

Fig. 1. Rate of protein synthesis 'in vitro' in livers of control and diabetic rats. The rate of protein synthesis was determined 'in vitro' as described in Materials and Methods in PMS fractions from liver after a 45-min incubation. Samples were processed in duplicate, and the values represent the mean ± SEM of 6–9 animals for each group. **A** Amount of Leu incorporated per milligram PMS protein. **B** Amount of Leu incorporated per milligram PMS RNA. The asterisks represent significant differences between control and diabetic animals: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Comparisons between virgin and pregnant rats and their corresponding fetuses were also made, and different superscript letters for the group values indicate statistically significant differences between them (at least $p < 0.05$).

with controls. The eIF-2 levels parallel the values found for its activity and were significantly higher in the fetus than in adult rats and slightly higher in pregnant than in virgin rats.

Both CKII and PKA activities were measured in S-100K fractions from the livers of control and diabetic animals. As shown in table 4, no differences between control and diabetic animals were detected, but both total CK (phosvitin) and CKII activities appeared higher in pregnant rats than in virgins, and the activities in the fetuses were even higher than in their respective mothers. The kinase activity remaining in the presence of heparin was attributed to casein kinase I or other protein kinases phosphorylating acidic substrates. The PKA activity was significantly lower in diabetic virgin rats than in their controls, whereas only a slight but not significant decrease was observed in the pregnant rats and their fetuses. The PKA activity was lower

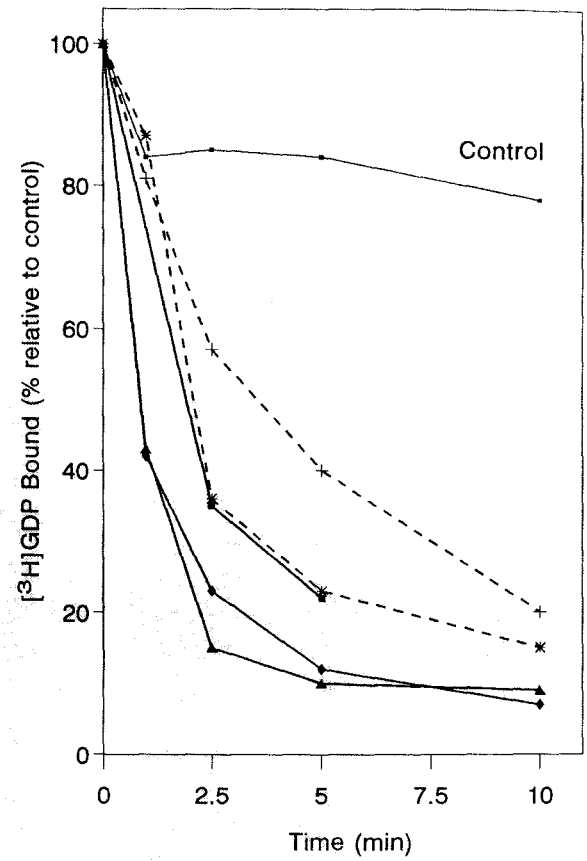

Fig. 2. Stimulation of the exchange of [^{3}H]GDP for unlabeled GDP on eIF-2 by eIF-2B from adult and fetal liver. Guanine nucleotide exchange assays were performed by measuring the loss of radioactivity from preformed eIF-2·[^{3}H]GDP binary complex (1.1–1.8 pmols) as described in Materials and Methods. The radioactivity remaining bound after each incubation time was determined by retention on nitrocellulose filters and is expressed as percentage relative to control. Different amounts of PMS from fetal (——) or adult (– – –) liver were used as a source of eIF-2B activity: + = 100 μg adult liver; * = 200 μg adult liver; ■ = 50 μg fetal liver; ◆ = 100 μg fetal liver; ▲ = 200 μg fetal liver.

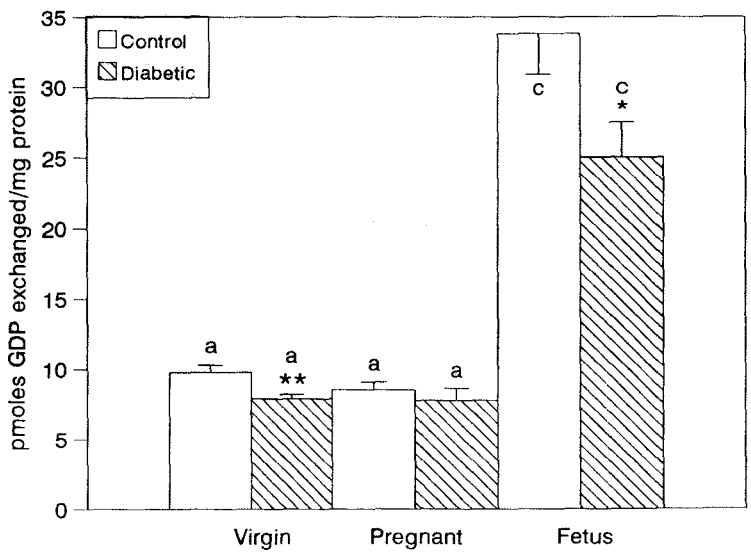

Fig. 3. eIF-2B activity in the liver from control and diabetic rats. The eIF-2B activity was measured in the PMS fraction (fetal 25 μg or adult 100 μg) by the GDP exchange assay described in Materials and Methods and in figure 2. The amount of binary complex eIF-2·[^{3}H]GDP was the same for all experiments: 1.1–1.8 pmol. The time of exchange reaction was 2.5 min, and each of the analyses was performed in duplicate. The values represent the mean ± SEM of 6–8 animals for each group. Statistical analysis as in figure 1.

Table 3. Initiation factor 2 activity and levels in the livers from control and diabetic rats

Animals	Control			Diabetic		
	activity U/mg protein	levels AU/μg protein		activity U/mg protein	levels AU/μg protein	
		eIF-2(αP)	EIF-2α+(αP)		eIF-2(αP)	eIF-2α+(αP)
Virgin	0.99±0.10[a]	158±78[a] (10)	1,544±53[a]	0.86±0.09[a]	164±37[a] (12)	1,318±102[a]
Pregnant	1.46±0.25[a]	310±50[a] (18)	1,697±326[a]	1.12±0.19[a]	326±67[a] (23)	1,406±194[a]
Fetus	2.39±0.39[c]	462±12[c] (18)	2,506±177[c]	2.26±0.23[c]	212±177[a] (10)	2,039±273[c]

The eIF-2 activity was measured in salt-washed supernatants by its capacity to form a ternary complex as described in Materials and Methods, and the results are expressed as units per milligram protein. The values represent the mean ± SEM from 6–8 different animals per group. The levels of total eIF-2, eIF-2α+(αP), and the phosphorylated eIF-2α, eIF-2(αP), were determined by protein immunoblot analysis and densitometric scanning with a monoclonal antibody to eIF-2α in S-100K samples subjected to IEF gels (0.75 mm thick, 5% acrylamide, 0.125% N,N′-methylene bisacrylamide, 8.5 *M* urea, and 2% ampholines at pH 4–6) as described in Materials and Methods. The results are given as arbitrary units (AU) per microgram protein. The values represent the mean ± SEM from 3–4 different animals. Percentage of eIF-2(αP) in total eIF-2α+(αP) in parentheses. Statistical analysis as in table 1.

in the pregnant rats and fetuses than in virgin rats. The PKA activity obtained in the absence of the nucleotide, which represents the phosphorylation of basic substrates by other kinase activities, decreased in diabetic animals as compared with control animals.

To study the possible modification of specific substrates in diabetic animals, S-100K fractions of liver tissue from control and diabetic rats were subjected to IEF, and the different isoelectric bands recognized by the anti-β serum were quantified. This study was only performed in virgin rats, since they appeared to be the most affected by the diabetic condition. As shown in table 5, up to three bands with different isoelectric points were detected, the most basic form being β_1 (5.98) and the most acidic form being β_3 (5.65); differences in both their levels and proportional distribution between control and diabetic animals were found.

Discussion

Protein synthesis in diabetes has been studied in several animal tissues, mostly muscle, where profound changes in protein synthesis machinery have been shown after a short-term diabetes (2 days) [2]. Results with tissue liver are scarce and controversial, and 1 week or more was required after the induction of diabetes before any change in liver polysome profiles could be detected [34, 44]. In the present work, protein synthesis was studied in the livers of rats with prolonged experimental diabetes (27 days). The cell-free system utilized here to measure protein synthesis was initially used for liver and brain tissue [18, 45, 46] and allows the specific activity of the precursor to be controlled, since synthesis occurs in the presence of a high concentration of exogenous amino acids and accurately reflects changes occurring 'in vivo' [18, 46].

The present results show that during late pregnancy protein synthesis is enhanced in

Table 4. Protein kinase activities in control and diabetic rats

Animals	Control		Diabetic	
	phosvitin	CKII	phosvitin	CKII
Virgin	435±26[a]	242±26[a]	461±33[a]	272±36[a]
Pregnant	1,464±163[b]	750±120[b]	1,128±147[b]	662±130[b]
Fetus	2,118±166[c]	1,015±62[b]	2,031±83[c]	978±35[b]
	histone	PKA	histone	PKA
Virgin	954±183[a]	1,568±68[a]	380±37[a,*]	971±98[a,**]
Pregnant	1,079±74[a]	815±99[b]	547±109[a,***]	605±112[a]
Fetus	954±69[a]	1,070±158[b]	698±69[c]	961±158[a]

Two different protein kinase activities (CKII and PKA) were measured in the S-100K fraction as described in Materials and Methods. The results are expressed as units per milligram protein and represent the mean ± SEM from 4–8 animals per group. Statistical analysis as in table 1.

Table 5. Quantitation of the β subunit of eIF-2 in the liver from control and diabetic rats

Isoelectric forms of eIF-2β	Control		Diabetic	
	AU	%	AU	%
β_1	3,335±1,019	5.8	11,498±5,224	25.3
β_2	30,782±5,392	53.9	25,559±5,150	56.3
β_3	23,001±6,259	40.3	8,380±4,508	18.4
β_{1+2+3}	57,118±11,800		45,437±10,188	

The isoelectric forms of eIF-2β were determined by IEF, protein immunoblot analysis, and densitometric scanning with an immunoaffinity-purified polyclonal antibody to eIF-2β in S-100K samples from virgin rats. Conditions of IEF as in table 3 with 2% ampholines at ranges 5–7 and 3–10 (4:1, v:v).

the liver. Streptozotocin diabetes decreases protein synthesis of both pregnant and nonpregnant animals, and although these differences could be related to the hyperinsulinemia found in the control pregnant rats as compared with virgin animals, this condition did not occur in the diabetic animals in which plasma insulin values did not differ between pregnant and nonpregnant groups. Although it was unexpected, the different rate of protein synthesis found between pregnant and virgin rats could be related to the augmented levels

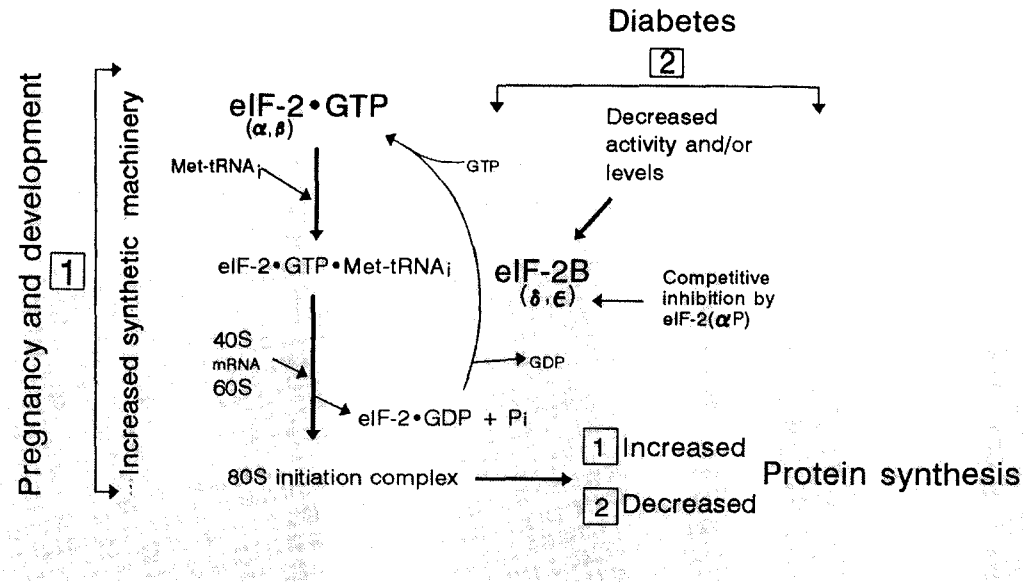

Fig. 4. Schematic presentation of the role of the different factors studied. Only the subunits which are susceptible to phosphorylation are shown. [Based on ref. 53.].

of RNA and eIF-2 activity found in the former group, an effect that was unaffected by the diabetic condition of the animals.

The protein synthesis rate is inhibited in the liver of diabetic virgin and pregnant rats. Since no diminution in the liver protein content or even an augmented liver weight was seen in the diabetic animals, these results would imply a decrease in protein degradation. Although the anticatabolic role of insulin is supported by several studies [2], the normal nutritional state of the diabetic animal as well as a chronical diabetic situation versus an acute diabetes have been shown to reduce the rate of protein breakdown in diabetic animals even to subnormal values [2, 7], and both situations concur in our model of diabetes.

As was expected, the protein synthesis rate appeared greatly enhanced in fetuses, but this effect was significantly lower in those from diabetic mothers. This concurs well with the high level of initiation factor 2, with the eIF-2B activity found in the liver of fetuses from control mothers, and with the decrease observed in the diabetic fetuses and could also be related to the intense hyperinsulinemia found in the fetuses from control mothers which is significantly lower in diabetic fetuses. These effects are a direct consequence of the diabetic condition of the fetuses (or their mothers) rather than a direct action of the drug, since this was given to their mothers several days prior to conception.

Both eIF-2 activity and levels measured in experimental control animals paralleled the changes observed in the protein synthesis rate and were slightly higher in pregnant than in virgin rats and appeared greatly enhanced in fetuses. Nevertheless, neither of these parameters seems to be responsible for the inhibition of protein synthesis observed in the diabetic animals (fig. 4).

The eIF-2B activity is clearly inhibited in the liver of diabetic virgins and fetuses, while no significant differences were found in diabetic mothers as compared with control ani-

mals. The lack of effect of diabetes on the eIF-2B activity in the pregnant rat is quite surprising, but even more so is the fact that the activity of the factor is not enhanced in the pregnant rat as compared with the virgin. The liver from pregnant rats, in accordance with its significant increase in size, resembles a growing tissue where multiple factors would be responsible for the increased protein synthesis rate. The eIF-2B activity is the exception when compared with the other stimulated parameters such as insulin levels, eIF-2 factor activity and levels, eIF-2(αP) levels and CKII activity which are highly enhanced in the fetus and are also increased, although less so, in the pregnant rats. The percentage of eIF-2(αP) which is required to block eIF-2 recycling and consequently to inhibit the initiation step depends on the eIF-2/eIF-2B ratio in a given cell. In reticulocyte lysate this ratio is known to be about 7:1, and a level of eIF-2(αP) of about 25–30% can completely block initiation [16]. Unfortunately we have not been able to measure the eIF-2B levels in the liver, but the increased percentage of eIF-2(αP) found in the pregnant rat, which is even higher in the diabetic condition, could explain the decreased activity of eIF-2B factor in these animals. Since insulin could decrease eIF-2α phosphorylation [47], the possibility exists that maternal insulin resistance during late gestation impedes the manifestation of the potential effect of insulin on eIF-2(αP) levels, but the confirmation of this hypothesis requires further study. Besides, a direct effect on eIF-2B activity and/or levels produced during gestation cannot be excluded.

While the diabetic condition produces an inhibition of the eIF-2B activity in virgins and fetuses of about 20%, the inhibition in total protein synthesis is almost 50% in the virgin rat; so it is probable that the differences would be due to the inhibition of albumin synthesis which is impaired by a decrease in its mRNA levels [1]. This last mechanism might be practically the only one operating in pregnant rats, since the eIF-2B activity is not inhibited. Another potential regulatory mechanism involved in the control of translation initiation by insulin has recently been revealed and implies the phosphorylation of 4E-BP1 and 4E-BP2, two proteins, the binding of which to eIF-4E regulates the 5′-cap function of mRNA [48]. The contribution of this mechanism, therefore, cannot be ruled out.

eIF-2(αP) is a competitive inhibitor of eIF-2B in the nucleotide exchange reaction [49], but our results prove that the decrease in eIF-2B activity in the liver is not due to the phosphorylation of eIF-2, since there is no increase in the level of eIF-2(αP) between control and diabetic animals. These results are in complete agreement with those of Karinch et al. [17] and Welsh and Proud [50] found in gastrocnemius muscle from diabetic animals where no changes in the phosphorylation status of eIF-2α, or even a decrease like the one found here in the diabetic fetus, are reported.

Insulin acutely activates eIF-2B in fibroblasts by a mechanism that is independent of alterations in the phophorylation of eIF-2α, and a direct modulation of eIF-2B activity through phosphorylation of its ε subunit has been proposed [50]. Since the CKII activity has been shown to be modulated by insulin [51], and this kinase phosphorylates the ε subunit of eIF-2B, increasing its activity 'in vitro' [20], the CKII activity was measured in control and diabetic animals, but no difference between them was found. It has been described that the K_m for casein or phosvitin of CKII from diabetic rats is two-fold lower than that of control animals [52]. Since the CKII activity was measured at saturating concentrations of phosvitin, alterations in the phosphorylation of specific substrates such as eIF-2B factor could exist even in the absence of differences in the activity of CKII. Moreover,

our results do not exclude the possibility that other kinases, such as casein kinase I or glycogen synthase kinase 3, might phosphorylate eIF-2B and modulate its activity [19, 53] (fig. 4).

The eIF-2β subunit is a specific substrate for CKII, protein kinase C, and also PKA [40], and although the actual role of the β subunit in translational initiation has not been established, it is believed to be required for maximal interaction of eIF-2 with eIF-2B [54]. eIF-2β is present in crude extracts from eukaryotic cells in three to four isoelectric forms due to phosphorylation and/or other covalent modification [55]. The levels of two out of the three isoelectric forms of eIF-2β detected in the present work are modified in the diabetic animals as compared with controls. The changes in the isoelectric points of the different forms might be due to modulation of the phosphorylation status by the corresponding kinases, such as CKII or PKA, or even phosphatases, since a decreased protein phosphatase 1 activity has been described in the liver of alloxan-induced diabetic rats [56].

The results presented here, therefore, show that protein synthesis is inhibited in the liver after prolonged diabetes. This inhibition is accompanied by a decrease in eIF-2B activity, without changes in the levels of RNA or eIF-2 factor. Interestingly, the decreased eIF-2B activity also seems to be responsible for the inhibition of protein synthesis observed in the liver of the fetuses from diabetic mothers, whereas maternal insulin resistance could be responsible for the lack of response to this inhibitory mechanism in the diabetic pregnant rats.

These results resemble those described for fast-twitch glycolytic muscles, such as gastrocnemius and psoas, where the diabetes-induced impairment in peptide chain initiation has been related to an inhibition of eIF-2B activity [17, 50]. Differences in the phosphorylation state of eIF-2α are not responsible for the inhibition of eIF-2B activity; instead, a direct modulation of eIF-2B activity by the availability of insulin and/or changes in the levels of the factor may be responsible for the inhibition of initiation.

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