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Molecular adaptations in islets from neonatal rats reared artificially on a high carbohydrate milk formula

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Abstract

Four day-old rat pups artificially raised on a high carbohydrate (HC) milk formula during their suckling period immediately develop hyperinsulinemia which persists into adulthood despite weaning onto lab chow on day 24. The present study investigates the molecular adaptations in islets isolated from neonatal rats in response to this dietary treatment during their suckling period. There is a significant increase in the level of preproinsulin mRNA and insulin biosynthesis in 12 day-old HC islets compared to islets from age-matched mother-fed (MF) control rats. Pancreatic duodenal homeobox factor-1 (PDX-1) modulates pancreatic ontogeny as well as preproinsulin gene expression in islets from neonatal rats. The mRNA level, DNA binding activity and protein content of PDX-1 are significantly increased in HC islets. The stress-activated protein kinase-2 and phosphatidylinositol 3-kinase have been reported to modulate PDX-1 activity in islets. The mRNA levels of these kinases are increased in HC islets. The mRNA level of upstream stimulatory factor (a modulator of PDX-1 gene expression) is also significantly increased in HC islets. These results indicate that the upregulation of several molecular events, including increases in the gene expression of preproinsulin, transcription factors and kinases may contribute to the chronic hyperinsulinemic state in the HC rats.

Keywords: Nutritional modification; Preproinsulin gene expression; Transcription factors; Kinases; Islets of Langerhans

1. Introduction

An altered nutritional environment during early periods of development (fetal and/or early postnatal) results in adaptations at the cellular and biochemical levels in target tissues to enable survival of the organism during these periods. These early adaptations persist and are expressed later in adulthood even in the absence of the stimulus/stress that initiated them, a process termed ‘metabolic programming’ [1]. Metabolic programming has important implications for adult-onset diseases, as evidence from human epidemiological data and animal studies clearly demonstrate that the quantity and quality of nutrition during critical periods in early development influence the susceptibility of the organism to adult-onset pathological conditions [2–4]. In the rat the ontogeny of the pancreas occurs during the late fetal and early postnatal life [5]. Hence alterations in the availability of nutrients during this period results in compensatory changes in the development and function of the pancreatic islets.

The HC (high carbohydrate) rat model [6,7] allowed to investigate the consequences of an increased carbohydrate intake during the suckling period (56% of the total calories are from carbohydrates in the HC milk formula compared to 8% in rat milk). An immediate response is the onset of hyperinsulinemia, which persists into adulthood even after the withdrawal of the HC formula at the time of weaning [6,7]. The overlap of the critical window of pancreatic organogenesis with that of the high carbohydrate dietary intervention in the immediate postnatal period in this model, induces several cellular and biochemical alterations in islets from neonatal HC rats [8–10]. These include an increase in the number of small sized islets in 12 day-old HC rats [8]. Although smaller in size these islets have a larger area staining immunopositive for insulin [8]. Significant among the biochemical adaptations in neonatal HC islets are the distinct leftward shift in the insulin secretory response to a glucose stimulus, an increase in the low Km hexokinase activity, ability to secrete insulin under stringent Ca^{2+}-deprived conditions, increased response to GLP-1 and ace-

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tylcholine and decreased sensitivity to norepinephrine-induced signals [9,10]. Both 12 day-old and adult HC rats maintain hyperinsulinemia but remain normoglycemic [7,9].

Nutrient-mediated regulation of gene expression is an important mechanism by which an organism adapts to its nutritional environment. For example, several of the DNA/protein interactions on the preproinsulin promoter have been shown to be influenced by glucose [11]. Glucose regulates preproinsulin gene expression via its effects on (i) the mRNA level of preproinsulin and/or the β cell-specific transcription factors or (ii) on the pathways that post-translationally modify the activities of specific transcription factors [12–15]. It is our hypothesis that the change in the nutritional environment from high fat to high carbohydrate in neonatal rats resulting in chronic hyperinsulinemia in the HC rat warrants molecular adaptations at the level of preproinsulin gene transcription. We have, therefore, investigated alterations in gene expression patterns, for example, the gene expression of preproinsulin and pancreatic-duodenal homeobox factor-1 (PDX-1; also known as STF-1, IDX-1, IUF-1 and IPF-1) in islets isolated from 12 day-old HC rats. Our results indicate that the altered insulin secretory pattern observed in HC islets is associated with concomitant changes at the level of transcription of several genes and post translational modifications that enable these HC rats to remain chronically hyperinsulinemic.

2. Materials and methods

2.1. Materials

Collagenase (Type IV) was from Worthington Biochemicals (Freehold, NJ). TRIzol reagent, murine leukemia virus transcriptase and all the primers were from GIBCO-BRL (Grand Island, NY). PGEM-3Z vector was from Promega (Madison, Wisconsin). [γ-^{32}P] ATP and the chemiluminesence reagents were from NEN-DuPont (Boston, MA). L- [4,5-^{3}H]-Leucine was from ICN (Costa Mesa, CA). Stress-activated protein kinase (SAPK) assay kit and Protein A Sepharose were from Upstate Biotechnology (Lake Placid, NY). Anti-human insulin antibody was from Linco Research (St. Charles, MO). Protein assay reagent was from Bio-Rad (Hercules, CA). Minimal essential medium and all other reagent grade chemicals were from Sigma (St. Louis, MO). The antibody to PDX-1 was a kind gift from Dr. J. F. Habener (Massachusetts General Hospital, Boston, MA).

2.2. Animal protocol

All procedures were performed with the approval of the Institutional Animal Care and Use Committee. Timed pregnant Sprague-Dawley rats were purchased from Zivic Miller Laboratories (Zelienople, PA) and were fed Purina rat chow and water *ad libitum.* Newborns from different litters were pooled and 12 pups were assigned to each mother. On postnatal day 4 intragastric cannulas were placed in the pups under light anesthesia for the experimental group. These pups were raised artificially on a high carbohydrate (polycose) milk formula (HC) as described previously [7]. Rat pups in groups of 12 reared by the mother (mother-fed, MF group) served as control. The HC rats were individually housed in Styrofoam cups floating on a temperature controlled water bath at 37°C, and fed at the rate of 0.45 Kcal/g body weight/day. The caloric contribution of the macronutrients in the HC formula was 24% protein, 56% carbohydrate and 20% fat and in rat milk it is 24% protein, 8% carbohydrate and 68% fat. On day 12 the rats were killed and the pancreas removed for the isolation of islets.

In order to eliminate the possibility that the artificial rearing procedure *per se* does not cause hyperinsulinemia in rats, we have artificially reared rats on a high-fat (HF) milk formula [6,7,16]. The macronutrient composition of the HF formula is similar to that of rat milk [6]. Our earlier results indicate that the rats raised on the HF formula do not develop hyperinsulinemia and maintained metabolic profiles similar to that observed in MF control rats [6,7,16].

2.3. Isolation of islets

Pancreatic islets of Langerhans were isolated from 12 day-old rat pups using collagenase digestion essentially as described previously [17]. Briefly, the animals were killed by decapitation and the pancreas pooled from two rats were digested with collagenase (Worthington, CLS-IV) in a 37°C waterbath with vigorous agitation for 10–12 min. After separation by sedimentation and washing, islets were manually picked under a stereomicroscope and used for experiments requiring fresh islets or washed in phosphate buffered saline (PBS) and stored at −80°C until further use.

2.4. Insulin biosynthesis

Newly synthesized insulin labeled using L-[4,5-^{3}H]-leucine was determined by an immunoprecipitation method as described by Halban et al. [18] using an anti-insulin antibody except for the incubation time which was reduced to 1 hr. Briefly, freshly isolated islets (~100) were washed three times in wash buffer [Krebs-Ringer bicarbonate (KRB)-Hepes buffer containing 5 mg/ml bovine serum albumin (BSA)] and transferred to 0.5 ml of Eagle's minimal essential medium lacking leucine and containing 8.3 mM glucose, 5 mg/ml BSA and 25 μCi of L-[4–5 ^{3}H]-leucine (specific radioactivity 63 Ci/mmole) and incubated for 1 hr at 37°C under 5% CO_2. The islets were washed three times in KRB-Hepes buffer containing 5 mg/ml BSA and transferred to 100 μl of 0.2 M glycine containing 2.5 mg/ml BSA, pH 8.8 (glycine-BSA buffer) and sonicated for 15 sec 2X. The sonicate was centrifuged (30,000g) for 30 min at 4°C. The supernatant was used for subsequent analyses. Insulin was precipitated from 20 μl of the supernatant using the standard insulin antibody and Protein A-Sepharose. Du-

Table 1
Sequences of PCR primers and PCR conditions for the analysis of specific mRNAs.

mRNA	GenBank accession no.	Primer sequences (5′–3′)	PCR reaction conditions				Size of PCR product	
			Den.	Ann.	Ext.	Cyc.	cDNA (bp)	Competitor (bp)
Insulin	J00747	TGCCCAGGCTTTTGTCAAACAGCACCTT CTCCAGTGCCAAGGTCTGAA	95	65	72	25	187	165
PDX-1	S67435	CCACACAGCTCTACAAGGACC CGTTGTCCCGCTACTACGTTTC	95	62	72	28	614	529
USF-1	AF026476	CAAGTACGTCTTCCGAAC CTTGTGGTGACATCATC	95	45	72	30	362	306
SAPK-2	U91847	ATTCAACGATGTGTACC GTTCATCTTCGGCATCTG	95	46	72	28	514	417
PI3K	AB009636	CACTGATAACTCGGCACAAC CTCGACATGGAGGTTGTAGC	95	58	72	30	377	322

plicate incubations were performed for all animals and correction for non-specific binding was done using a non-immune serum instead of the insulin antibody. The results are expressed as incorporation of radioactivity in immunoprecipitates of insulin.

2.5. RNA isolation and cDNA synthesis

Total RNA was isolated from islets obtained from 12 day-old HC and age-matched MF control rats using the TRIzol reagent-phenol-chloroform procedure. cDNA was prepared using 6 μg total islet RNA and 20 pmol Random Hexamers in a 30 μl solution containing 50 mM Tris-HCl, pH 8.4, 75 mM KCl, 4 mM $MgCl_2$, 10 mM dithiothreitol, 0.125 mM of each dNTP, and 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL). After incubation for 1 hr at 42°C, the reaction mixture was heated to 70°C for 15 min to inactive the reverse transcriptase. The cDNA was stored at −20°C.

The levels of specific mRNAs were determined using a semi-quantitative polymerase chain reaction (PCR)-based assay as described previously [19]. A known amount of synthetic competitor DNA was added to each reaction. A semiquantitative RT-PCR assay [20] in which a same amount of competitor template added to each reaction was used to compare the levels of specific mRNAs in islets from HC and MF rats. This internal standard was amplified using the same primers as the experimental cDNA target and was designed to generate a PCR product that was easily distinguished from the cDNA target because of its difference in size. All competitor DNAs for measuring the mRNAs for preproinsulin, upstream stimulatory factor-1 (USF-1), phosphatidylinositol 3-kinase (PI3-kinase) and stress-activated protein kinase-2 (SAPK-2) were prepared by introducing a small internal deletion into the cloned cDNA using a PCR-based mutagenesis procedure [21]. PDX-1 competitor DNA was prepared by digesting the cloned PDX-1 cDNA with Apa 1 and Mlu 1, filled in with Klenow-DNA-polymerase and religated. All competitor DNAs were cloned into pGEM-3Z vector (Promega). PCR reactions were carried out in a 50 μl volume containing cDNA, competitor DNA, dNTPs, 10 pmol of a pair of oligonucletide primers (Table 1), 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, and 1 U of Taq DNA polymerase. The PCR products were separated by electrophoresis in a 2% agarose gel, and analyzed by Bio-Rad Gel Doc 1000 and Molecular Analyst Software for quantitative analysis and normalized using competitor controls. The results are expressed as fold change in HC animals compared to age-matched MF controls.

2.6. Preparation of nuclear extracts

Nuclear extracts were prepared using a modification of the method of Schreiber et al. [22]. Islets were centrifuged for 10s in a microcentrifuge and resuspended in 400 μl of 10 mM Hepes, pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonylfluoride (PMSF), 10 mM NaF, 10 mM sodium molybdate, 10 mM β-glycerophosphate, 10 mM sodiumvanadate, and 10 mM p-nitrophenylphosphate. Islets were allowed to swell on ice for 15 min before adding 25 μl of 10% (v/v) Nonidet P-40. The islets were then vortexed for 15s and centrifuged for 30s in a microcentrifuge. The pellet, which was enriched in nuclei, was resuspended in 60 μl of 20 mM Hepes, pH 7.9, containing 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatinA, 0.1mM p-aminobenzoic acid, 10 μg/ml aprotinin, 5% (v/v) glycerol, 10 mM NaF, 10 mM sodium molybdate, 10 mM β-glycerophosphate, 10 mM sodiumvanadate, and 10 mM p-nitrophenyl phosphate. Nuclear extracts were then centrifuged for 2 min at 4°C in a microcentrifuge. The supernatant was collected, aliquoted into small volumes, and stored at −70°C.

2.7. Mobility shift assays

Electrophoretic mobility gel shift assays (EMSA) were performed using the oligonucleotide probe of the A3 PDX-1

binding site, which corresponds to sequence from positions –206 to −227 bp of the rat insulin I or II gene promoter. Nuclear extracts (4 μg of protein) were incubated with the ^{32}P-labeled probe for 20 min at room temperature in binding buffer containing 10 mM Tris/HCl (pH 7.5), 50 mM KCl, 2 μg of poly [d (I.C)], 5 mM DTT, 1mM EDTA and 5% (v/v) glycerol. For competition experiments, the nuclear extract was incubated with 100-fold excess of unlabeled oligonucleotide for 20 min at room temperature before addition of the probe. Gel loading buffer was added and the mixtures were loaded on to a non-denaturing 4.5% (w/v) polyacrylamide gel (acrylamide/bisacrylamide, 29:1) run in 0.5 X TBE (50 mM Tris, 50 mM boric acid and 1 mM EDTA), at 10–15 v/cm for 2.5 h. The gel was dried and subjected to autoradiography.

2.8. SAPK-2 assay

The activity of SAPK-2 in islets isolated from 12 day-old MF and HC rats was determined using a kit from Upstate Biotechnology according to instructions from the manufacturer.

2.9. Western blot for PDX-1

Equal amounts of protein from nuclear extracts (prepared as described under EMSA) were separated on a 10% SDS-PAGE gel, transferred to nitrocellulose membrane and probed with an anti-PDX-1 antibody. Protein bands were visualized by chemiluminesence and quantitated using a densitometric scanner.

2.10. Protein assay

Protein assays were carried out using the assay kit from Bio Rad according to manufacturer's instructions.

2.11. Statistical analysis

The results are means ± SE of four independent experiments. The significance of the difference between MF and HC groups was analyzed by Students t-test. P value < 0.05 was considered significant.

3. Results

Earlier studies on this rat model showed that there was an immediate onset of hyperinsulinemia (within 24 h) in response to the HC formula given to four day-old rat pups [6]. The immediate onset and persistence of hyperinsulinemia in response to the high carbohydrate milk formula in the HC rat suggests that insulin biosynthesis may be enhanced to sustain this condition. Insulin biosynthesis was measured by the incorporation of L-[4,5-^{3}H]-leucine into insulin in freshly isolated islets from 12 day-old MF and HC rats and is markedly increased (~4-fold) in islets from 12 day-old HC rats compared to age-matched MF islets (Fig. 1A).

To evaluate if increased preproinsulin gene transcription supported the increased insulin biosynthetic capacity in islets isolated from 12 day-old HC rats, mRNA levels of preproinsulin was measured in extracts of HC islets. Northern blot analyses for quantitation of mRNA levels in islets could not be applied in this model because the availability of islets was limiting. In addition, transcription of several of the commonly used housekeeping genes is upregulated in HC islets and could not be used as internal control (unpublished observation). Hence a semiquantitative RT-PCR assay which is very sensitive and requires a smaller number of islets per experiment was adopted for relative quantitation of mRNA levels in this study. Preproinsulin mRNA levels are increased approximately 4.5 fold in HC islets compared to MF islets from12 day-old rats (Fig. 1B).

Preproinsulin gene transcription is regulated by interactions between several transcription factors and their binding to specific cis-acting elements in the insulin promoter. PDX-1 is a β cell specific transcription factor, which in the younger animal has dual functions in pancreatic organogenesis and the efficient transcription of the preproinsulin gene [23–26]. PDX-1 mRNA levels are significantly increased (5.2 fold) in islets from 12 day-old HC rats compared to islets from age-matched MF rats (Fig. 2A). Western blot analysis for PDX-1 protein was carried out to determine if increased PDX-1 mRNA level was associated with increased PDX-1 protein content. Fig. 2B shows that PDX-1 protein levels are substantially increased (~4-fold) in nuclear extracts of HC islets compared to MF islets. To determine whether the increases in PDX-1 protein expression was correlated with increases in the binding activity of PDX-1 for its cis-regulatory element of the insulin gene promoter, EMSA was performed using nuclear extracts of islets from 12 day-old MF and HC rats. Increased DNA binding activity of PDX-1 is evident in the case of HC nuclear extract (Fig. 2C; lane 3 compared to lane 5 for MF nuclear extract). The use of a 100-fold excess of unlabeled oligonucleotide completely abolishes PDX-1 binding to DNA (Fig. 2C; lanes 2 and 4) thereby establishing specificity of the assay.

USF binds to the promoter region of PDX-1 and regulates PDX-1 gene expression [27]. The mRNA level of USF-1 was significantly increased (2-fold) in islets from 12 day-old HC rats compared to age-matched MF rats (Fig. 3).

Heat shock, arsenite, high glucose, etc. induce stress-related responses [14]. In order to evaluate if the high carbohydrate dietary intervention in neonatal rats elicits stress-induced responses in islets of HC rats, the activity and mRNA levels of the stress activated protein kinase-2 (SAPK-2) were measured in islets from 12 day-old HC rats. The mRNA levels of SAPK-2 are significantly increased in HC islets from 12 day-old HC rats compared to age-matched MF islets (Fig. 4A). The activity of SAPK-2 is also significantly increased in islets from 12 day-old HC rats

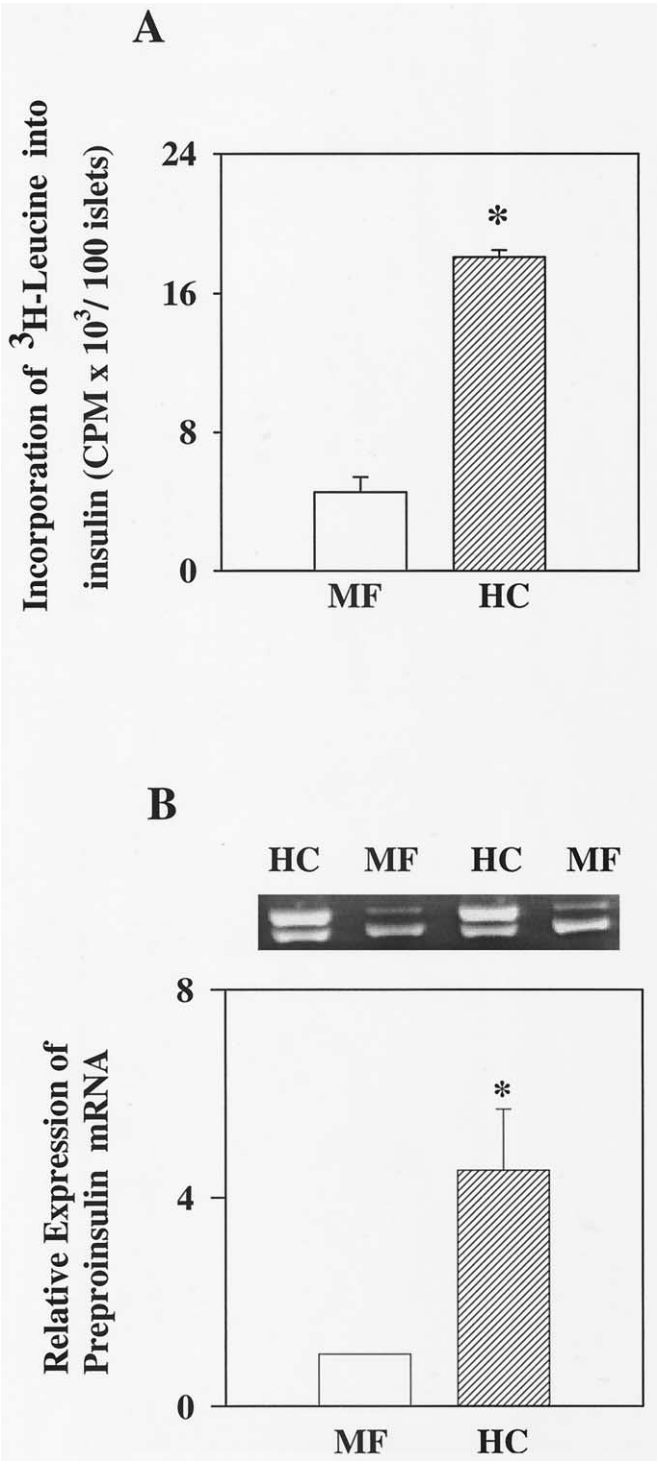

Fig. 1. Insulin biosynthesis (A) and expression of mRNA of the preproinsulin gene (B) in islets isolated from 12 day-old MF and HC rats. A: Equal number of islets [100] from MF and HC rats were incubated for 1 hr with L-[4,5 ^{3}H]-leucine and the incorporation of L-[4,5 ^{3}H]-leucine into immunoprecipitable insulin was monitored. B: mRNA level was quantitated by a semiquantitative RT-PCR assay. Upper Panel: Representative ethidium bromide-stained agarose gel separation for PCR products. The upper band corresponds to preproinsulin cDNA and the lower band corresponds to the competitor DNA for preproinsulin. Lower Panel: The means of relative densitometric values from quantification of preproinsulin mRNA levels. The values in A and B are means ± SE of four independent experiments. * $P < 0.001$.

(Fig. 4B). The role of phosphatidyl inositol 3 kinase (PI3 kinase) on preproinsulin transcription has been reported [15]. The mRNA level of PI3-kinase is significantly increased in HC islets from 12 day-old rats (3-fold over MF value; Fig. 4C).

4. Discussion

In the HC rat model the nutritional intervention in the form of a switch from high fat-derived calories in rat milk to a high carbohydrate-derived milk formula during the suckling period causes a wide spectrum of adaptive changes in the islets of HC neonates. These adaptive responses result in the immediate onset and persistence of hyperinsulinemia in these rats [6,16]. Since the suckling period is a critical window in the ontogeny of the pancreas in the rat [5], it is vulnerable to a nutritional challenge during this period. The overlap of this vulnerable period, when the β cells are rapidly dividing, with the high carbohydrate nutritional intervention in our rat model causes irrevocable changes in HC islets. We recently reported alterations in the cellular architecture and in the insulin secretory pattern of the islets from 12 day-old HC rats compared to islets from age-matched MF rats [8–10]. In this study we report substantial alterations at the molecular level in HC islets from 12 day-old HC rats compared to islets from age-matched MF rats. The present results indicate that these early molecular adaptations may form the basis for the altered cellular structure and insulin secretory capacity of HC islets [8–10].

The immediate replenishment of insulin stores occurs via an increase in insulin biosynthesis by an increase in the translation rate of the preproinsulin mRNA. The chronic hyperinsulinemic state of the HC rat suggests alterations in insulin biosynthetic capacity, as the insulin secreted must be compensated for at the translational level. Insulin biosynthesis as measured by the incorporation of ^{3}H-leucine was significantly increased in islets from 12 day-old HC rats compared to islets from age-matched MF rats. Glucose has been shown to regulate proinsulin biosynthesis in isolated rat pancreatic islets [28]. Basal hyperinsulinemia observed in the Zucker fatty and the Zucker diabetic rats has been attributed to an increase in the rate of insulin biosynthesis [29]. This report supports the increased insulin biosynthesis and the development of basal hyperinsulinemia observed in the HC rats.

Although short-term compensation for the insulin secreted occurs through translation of the pre-existing mRNA, it is clear that the chronic hyperinsulinemic state of the HC rat warrants adaptations at the molecular level via alterations in the transcription of the preproinsulin gene and related transcription factor genes. Preproinsulin mRNA level was markedly increased in HC islets. That preproinsulin gene expression is modulated according to demands is illustrated by an increase in preproinsulin mRNA level in corticosteroid-induced insulin resistance and after pancrea-

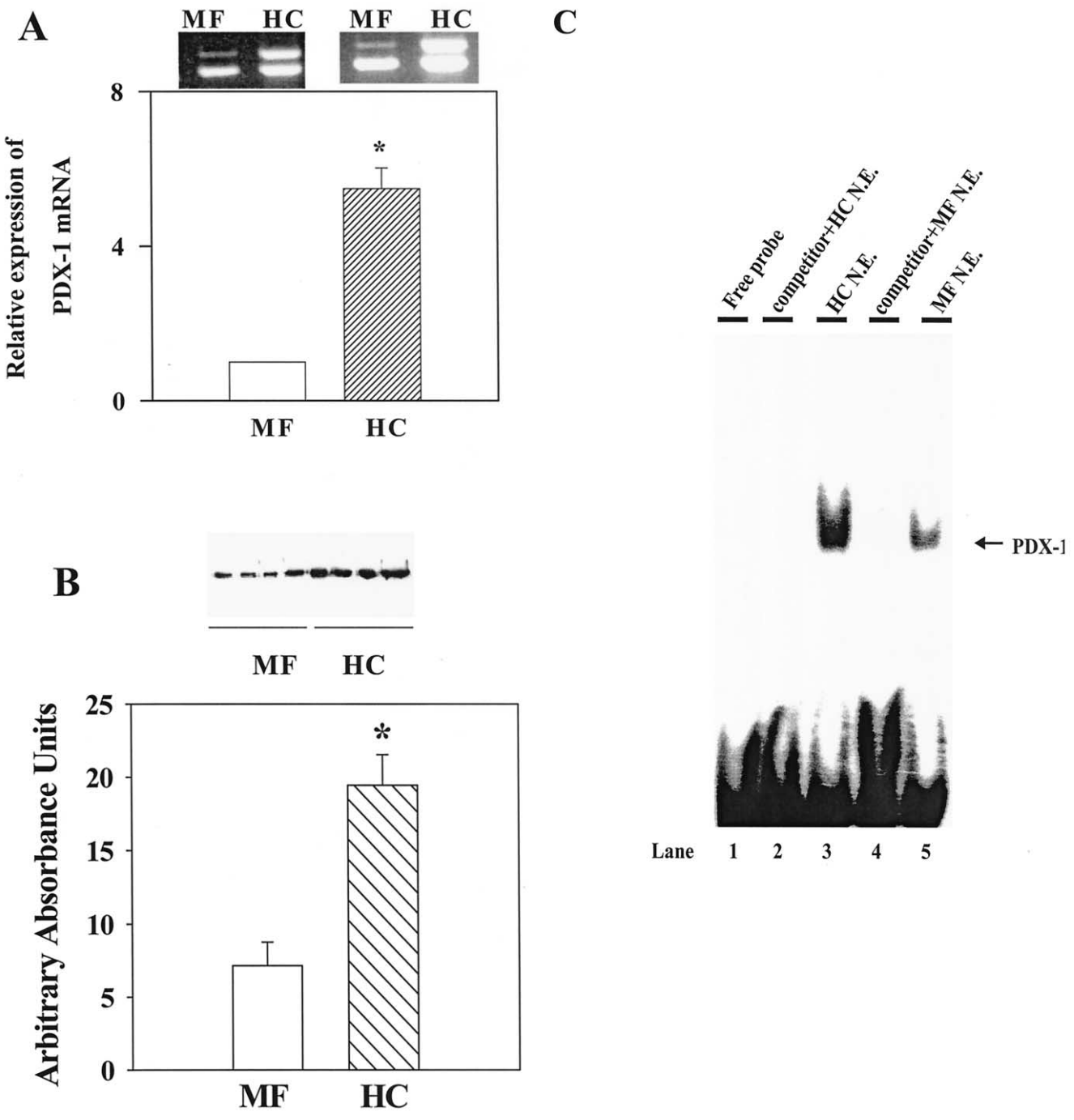

Fig. 2. Expression of mRNA of the PDX-1 gene (A), western blot analysis of PDX-1 protein (B) and EMSA analysis of PDX-1 binding activity in nuclear extracts (C) of islets isolated from 12 day-old MF and HC rats. A: mRNA level was quantitated by a semiquantitative RT-PCR assay. Upper panel: Representative ethidium bromide-stained agarose gel separation for PCR products. The upper band corresponds to PDX-1 cDNA and the lower band corresponds to the competitor DNA for PDX-1. Lower panel: The means of relative densitometric values from quantification of PDX-1 mRNA levels B: Equal amounts of protein from nuclear extracts of MF and HC islets were analyzed for PDX-1 protein content using a specific anti-PDX-1 antibody. Upper panel represents the autoradiogram for identification of PDX-1 protein. Lower panel is the means of the densitometric scan of the bands corresponding to PDX-1 protein. The values in A and B are means ± SE of four independent experiments. * $P < 0.001$. C: EMSA analysis of PDX-1 binding activity was performed using the A3 site of the rat preproinsulin gene promoter as probe. PDX-1 binding activity is highlighted by arrow. The free probe and the competition assay lanes for the MF and HC samples were as indicated.

tectomy [30,31]. The half-life of preproinsulin mRNA was three-fold higher in islets cultured in 17 mM glucose compared to islets cultured in 3.3 mM glucose [32]. Glucagon-like peptide-1 (GLP-1), a potent stimulator of insulin secretion, also increases the rate of transcription of the preproinsulin gene by mechanisms involving cAMP [12]. Earlier we had reported a significant increase in the plasma level of the active form of GLP-1 and a concomitant in-

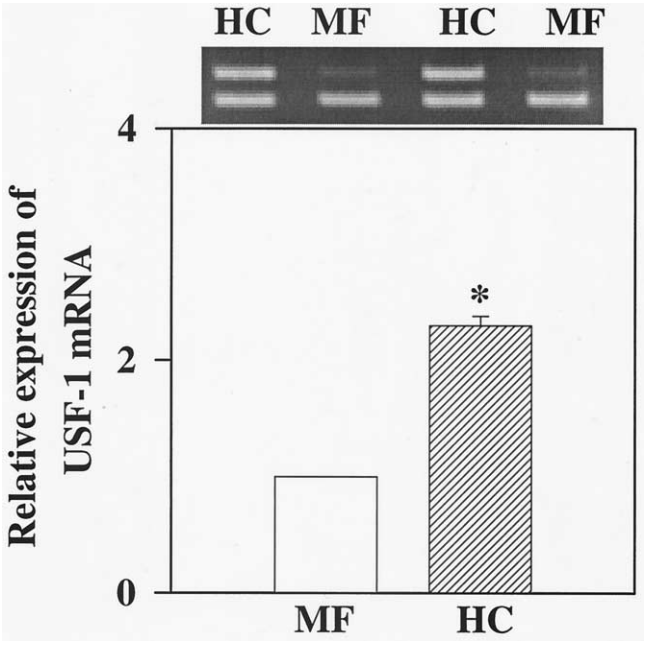

Fig. 3. Expression of mRNA of the USF-1 gene in islets from 12 day-old MF and HC rats. The mRNA level was quantitated by a semiquantitative RT-PCR assay. Upper Panel: Representative ethidium bromide-stained agarose gel separation for PCR products. The upper band corresponds to USF-1 cDNA and the lower band corresponds to the competitor DNA for USF-1. Lower Panel: The means of relative densitometric values from quantification of USF-1 mRNA levels. The values are means ± SE from four independent experiments. * $P < 0.001$.

crease in the mRNA level of its receptor in islets from 12 day-old HC rats indicating that GLP-1-mediated effects on preproinsulin gene transcription may be important in HC islets [10].

PDX-1 is an important transactivator of the preproinsulin gene and functions as the glucose-sensitive factor in preproinsulin gene transcription [25,26]. The levels of PDX-1 mRNA are markedly increased in islets from 12 day-old HC rats compared to islets from 12 day-old MF rats (Fig. 2A). The increased DNA binding of PDX-1 and the increased protein content of PDX-1 together with the increased mRNA levels emphasize the pivotal role of PDX-1 in preproinsulin gene regulation in HC islets. PDX-1 regulates preproinsulin gene promoter transcription via the A-elements of the preproinsulin gene and may function by virtue of its DNA-binding properties, thus bringing other DNA-bound regulatory proteins into juxtaposition with the preinitiation complex [33]. Recently it has been shown that PDX-1 synergizes with the E-box binding protein complex (HLH factors composed of heterodimers of Pan1/2, BETA 2/Neuro D) in preproinsulin gene transcription [34].

In addition to its effect on preproinsulin gene transcription, PDX-1 plays a very important role in pancreatic organogenesis by committing the pluripotent epithelial cell to an endocrine lineage [35]. The homozygous PDX-1 null mouse and a child homozygous for an inactivating mutation in the PDX-1 gene failed to develop pancreas indicating the importance of PDX-1 for proper organ development [24,36]. Our recent observations on morphological adaptations in islets of 12 day-old rats indicate that there are significant changes in the size (smaller islets) and number (increased) of islets in the HC pancreas [8]. Considering that PDX-1 mRNA levels and its protein content are markedly increased in islets from 12 day-old HC rats, it is reasonable to suggest that the observed morphological adaptations in HC pancreas may be modulated by PDX-1.

The increase in the mRNA levels and activity of SAPK-2 in neonatal HC islets suggests that the high carbohydrate dietary treatment may be akin to a stress-like situation in the HC rats and hence induces stress-related response in these islets. Macfarlane *et. al.* [14,37] have suggested from studies using human islets and MIN 6 cells that preproinsulin gene transcription is augmented by high glucose by activation of PDX-1 gene transcription and its translocation to the nucleus via a pathway involving SAPK-2. Burns et. al. [38] have shown a role for SAPK-2 in the proliferation of MIN 6 cells. The HC dietary intervention causes significant changes in the activity and gene expression of SAPK-2 in HC islets suggesting that it may be related to activation of PDX-1 in the HC rat. The role of PI3 kinase in the regulation of preproinsulin gene has been reported [15] and the increase seen in the gene expression of PI3 kinase in islets from 12 day-old HC islets is in agreement with these published results.

USF is a ubiquitous transcription factor [39]. USF has been shown to specifically bind to the PDX-1 promoter and regulates expression of the PDX-1 gene in β cells [27]. USF-1 mRNA level was significantly increased in islets from 12 day-old HC rats and may function as an upstream regulator of PDX-1 gene expression in HC islets.

In contrast to the hyperglycemia-induced modulation of insulin levels observed in fetuses of diabetic mothers and in diabetic patients, in the HC rat model the onset of hyperinsulinemia is driven by an increase in the availability of high-carbohydrate-derived calories (without the development of hyperglycemia) during the critical window of pancreatic development. It appears that the system senses a need for an increase in the demand for insulin to metabolize the increased availability of carbohydrate-derived calories and responds by adaptations in islet functions. The results from the present study indicate that several molecular events occur during this critical period in pancreatic organogenesis in response to this nutritional intervention and may be important for the increased preproinsulin gene expression, which is essential to sustain the chronic hyperinsulinemic condition in the HC rat. These molecular events regulate both the ontogeny of the pancreas as well as the insulin secretory capacity of the islets in the HC rats. Considering that hyperinsulinemia predisposes the organism to the onset of several pathological conditions in adulthood (obesity,

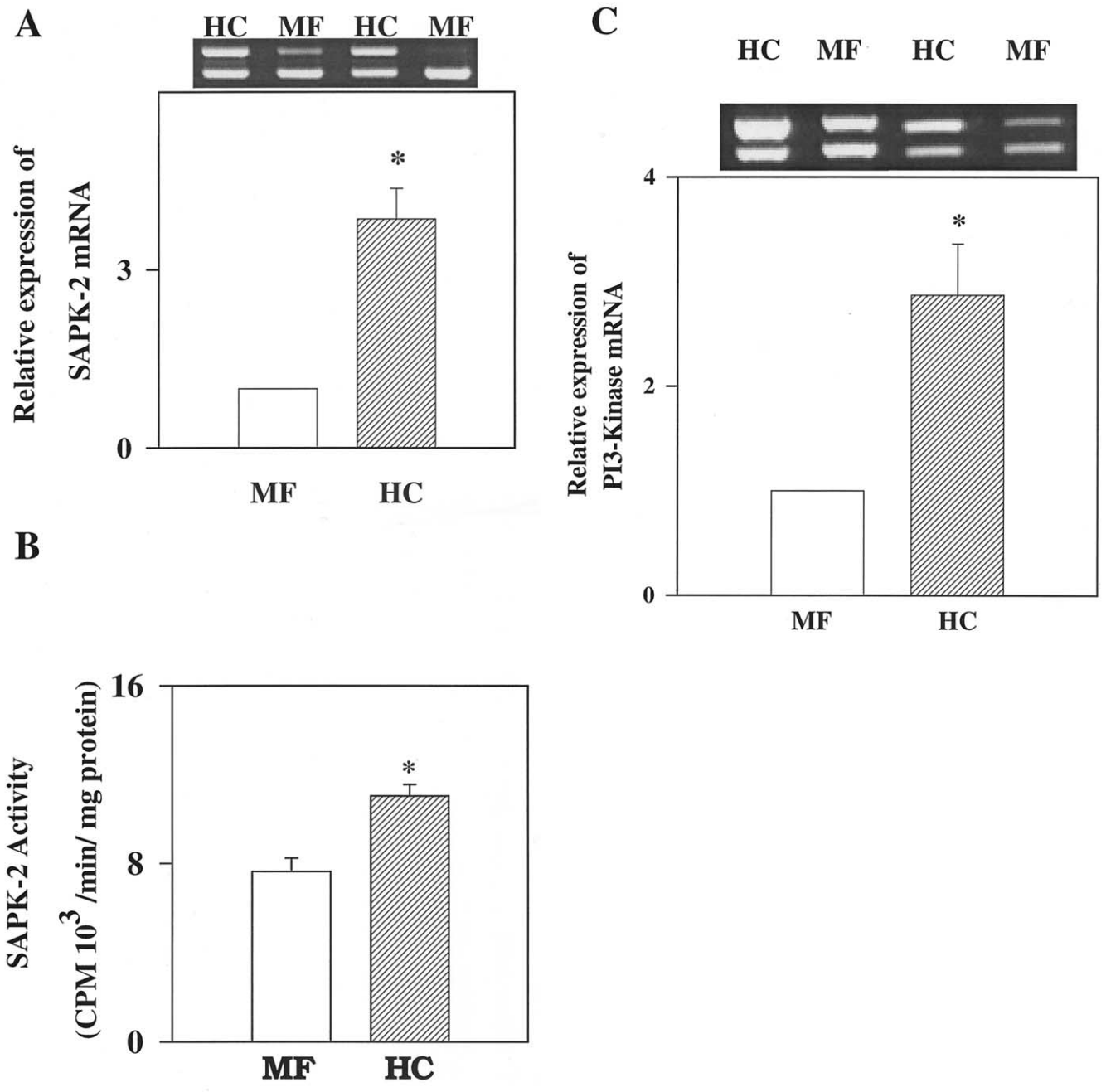

Fig. 4. Expression of mRNA of SAPK-2 gene (A), enzyme activity of SAPK-2 (B) and expression of mRNA level of the PI3 kinase gene (C) in islets isolated from 12 day-old MF and HC rats. A: mRNA level of SAPK-2 gene was quantitated by a semiquantitative RT-PCR assay. Upper panel: Representative ethidium bromide-stained agarose gel separation for PCR products. The upper band corresponds to SAPK-2 cDNA and the lower band corresponds to the competitor DNA for SAPK-2. Lower panel: The means of relative densitometric values from quantification of SAPK-2 mRNA levels. B: SAPK-2 activity was measured in MF and HC islets using a kinase cascade assay kit. C. mRNA level of PI3 kinase gene was quantitated by a semiquantitative RT-PCR assay. Upper Panel: Representative ethidium bromide-stained agarose gel separation for PCR products. The upper band corresponds to PI3-kinase cDNA and the lower band corresponds to the competitor DNA for PI3-kinase. Lower Panel: The means of relative densitometric values from quantification of PI3-kinase mRNA levels. The values in A, B and C are means ± SE from four independent experiments. * $P < 0.001$.

NIDDM, hypertension, etc.), the study of the molecular mechanisms for the onset of hyperinsulinemia is essential to evaluate the early events that lead to adult-onset clinical syndromes. In this context the HC rat model provides a unique opportunity to evaluate these early molecular events under physiological conditions.

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