MNK Publication

International Journal of Latest Research in Science and Technology **ISSN (Online):2278-5299**
Volume 3, Issue 3: Page No. 194-200, May-June 2014
https://www.mnkpublication.com/journal/ijlrst/index.php

INSULIN GENE EXPRESSION AND ITS DETECTION IN THE RAT KIDNEY

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Abstract- ***Insulin is not only produced by highly specific β pancreatic cells, it is currently known that there are extrapancreatic tissues expressing insulin mRNA such as the brain, thymus and some cells in the olfactory mucosa. Pdx-1 and Nkx6.1 are key transcription factors expressed during pancreatic development, differentiation and maintenance of beta cell function as well as in the synthesis of insulin. These transcription factors are also found in the brain. In this study we analyze the genic expression of insulin Pdx-1 and Nkx6.1 in the rat kidney. We investigated its genic expression by qRT-PCR assay and were confirmed by sequencing the qRT-PCR products. Renal cDNA, was assayed in parallel with rat pancreatic cDNA. Insulin, Pdx-1 and Nkx6.1 were analyzed in the renal tissue by immunohistochemistry. Pdx-1 and Nkx6.1 were also assayed by immunoblot. The resemblance percentages for kidney insulin, Pdx-1 and Nkx6.1 were, 100, 97 and 98% respectively compared to pancreatic insulin and transcription factors. The relative expression level for insulin was lower in the kidney than in the pancreatic tissue. Insulin and its transcription factors were immunodetected in the rat kidney tissue. These results suggest the expression and detection of insulin, Pdx-1 and Nkx6.1. Both transcription factors are involved in the synthesis of insulin. Our findings may be related with insulin function at renal level. However, further research is necessary in order to conclusively prove it. At the renal level, insulin promotes renal sodium reabsorption, participates in the decrease of potassium excretion and renal vascular resistance.***

Keywords **-** Insulin, Pdx-1, Nkx6.1, kidney tubules

I. INTRODUCTION

In the nineteenth century, Claude Bernard was the first to propose that hormones may be produced by non-endocrine organs capable of "internal secretion" as a basic mechanism to regulate homeostasis [1]. Several examples of internal secretion have been described such as osteocalcin, leptin and erythropoietin produced by osteoblasts, adipose tissue and the kidney, respectively [2, 3]. As well, not only pancreatic cells express insulin, so do the brains of rats and rabbits in pre and post-natal pyramidal neurons (in regions such as the olfactory bulb and the hippocampus) in an autocrine manner [4, 5]. Likewise, its expression in epithelial cells from the thymus of mice and other species has been demonstrated [6, 7]. And it has also been found in the rat olfactory mucosa cells [8].

The two transcription factors Pdx-1 and Nkx6.1 implicated in the development of pancreatic cells are highly differentiated. Pdx-1 maintains the phenotype of adult beta cells and is necessary for their operation and survival [9]. It is required for the expression, processing and secretion of insulin as well as glucose homeostatic regulation [10]. On the other hand, Nkx6.1 is essential for the terminal differentiation of β-cells as well as being necessary and sufficient for specifying the production of insulin and maintaining the identity of beta cells [11, 12]. Both are expressed in the brain, the first is expressed during its developmental stage, while Nkx6.1 is expressed during the maturation and migration of hindbrain motor neurons [13].

In kidneys, the genic expression of insulin has not been reported but its key function as an antinatriuretic and its participation in blood pressure have been well documented [14]. It has been shown that insulin is involved in the key function of sodium reabsorption through the activity of Na transport proteins. It has also been proposed that insulin action is carried out at several renal tubular segments. Micropuncture and perfusion studies showed a rise in sodium reabsorption due to an increase of Na^+-K^+-ATPase sensitivity directly caused by insulin in the proximal convoluted tubule of the rat [15]. Gesek and Schoolwerth [16] proved that insulin directly increases the Na^+-H^+ exchanger type 3 activity in proximal tubules of rats, which plays a major role in this segment's apical sodium reentry. The main phosphorous reabsorption in the kidney is carried out in the proximal tubule by the apical sodium-phosphate cotransporter type II, as well, an increased phosphate uptake mediated by insulin has been shown [17, 18]. Studies by Ito et al. [19] have indicated that insulin directly increases sodium chloride reabsorption in the medullary thick ascending limb of rabbit kidneys. A key factor in the maintenance of sodium homeostasis is constituted by the amiloride-sensitive epithelial sodium channel (ENaC) located in the apical membrane of distal convoluted and connecting tubules. Also, insulin concentration in the kidney is higher (5 to 10 times) than in plasma [20]. The objective of this study was to analyze the genic expression of insulin, Pdx-1, Nkx6.1 and its detection in the rat kidney.

II. METHODS

All reagents used were analytical grade. They were obtained from Sigma Chemical Co. (St. Louis, MO., USA),

Publication History
Manuscript Received : 26 June 2014
Manuscript Accepted : 28 June 2014
Revision Received : 29 June 2014
Manuscript Published : 30 June 2014

Promega (Madison, WI), Qiagen (Germany), or from local suppliers (Merck and J.T. Baker, México).

The experiments in this study were performed following the guidelines stated in "Principles of Laboratory Animal Care" (NIH publication #86-23, revised 1996) and Mexican regulations "Norma Oficial Mexicana NOM-062-ZOO-1999, Especificaciones técnicas para la producción, cuidado y uso de animales de laboratorio" (published in December, 1999). The studies were approved by the ethical committee of the Biological Sciences Division Council of our University.

A. Experimental Animals

Male Wistar rats (200-220 g of b. wt.) were obtained from our animal facility, fed with Purina standard chow, and maintained at 20-22°C with 12-h light /dark cycles.

B. Tissue collection

For sacrifice, animals were administered a dose of sodium pentobarbital (50 mg/kg b.w., i.p.). The kidneys and pancreas were dissected through an incision in the abdominal cavity.

C. RNA isolation

RNA was isolated from a fragment of kidney or pancreatic tissue (50 mg) from each animal and prepared using the column method according to the manufacturer‘s protocol PROMEGA (Madison, WI). Briefly, the tissue was homogenized in 1000 µL of lysis buffer. 350 µL of dilution buffer was added to 175 µL of lysate. The sample was heated at 70 °C for 3 minutes and then centrifuged for 10 minutes. 250 µL 95% ethanol was added to cleared lysate and mixed. The lysate was transferred to the column and centrifuged for 1 minute. 600 µL of wash solution was added and centrifuged for another minute. 50 µL of DNase was applied to the column and incubated for 15 minutes at room temperature. After this time, 200 µL of DNase stop solution was added and centrifuged for 1 minute and washed two times. To eluate the RNA, 100 µL of nuclease free water was added to membrane column.

D. Real-time RT-PCR (qPCR)

Total RNA was quantified spectrophotometrically at 260/280 nm. The integrity of RNA samples were evaluated on a 1% agarose gel. For qPCR analysis, RNA isolated from five individual animals for each genotype was used for independent qPCR analysis. The cDNA of each sample was synthesized from 2 µg of total RNA in a total volume of 25 µL using oligo-$(dT)_{20}$ primers and the PROMEGA kit (Madison, WI) at 37 °C for 60 min according to the manufacturer’s protocol. The cDNA samples were diluted at 35 µg/µL as a final concentration and 2 µL was used for each qPCR reaction. cDNA was amplified in real time PCR using the Rotor Gene Q System (Qiagen, Germany). Pancreatic cDNA was diluted 1:10000.

qPCR analysis was performed using SYBR Green PCR master mix (Qiagen, Germany) according to the manufacturer’s protocol. The PCR mixture contained 0.25 µL (0.25 µM) of each primer, 10 µL of master mix and 7.5 µL of nuclease-free water. Ribosomal 18s primer was used as an internal control for the qPCR analysis.

Primer sequences and the size of each amplified PCR product are shown in Table 1. All oligonucleotide primers were synthesized and used to prime the amplification of the cDNA template, based on the previous published sequences (NCBI) of insulin (C07149), Pdx1 (NM_022852), Nkx6.1 (NM_031737) and ribosomal 18 s. The following standard PCR conditions were used: one cycle at 95 °C for 5 min; 40 cycles at 95 °C for 10 s; 60 °C for 20 s; and 74-95 °C for 60 s for a melting curve. A cycle threshold (CT) was assigned at the beginning of the logarithmic phase of PCR amplification, and duplicate CT values were analyzed using Microsoft Excel. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression of the gene in each sample [21]. PCR products were confirmed by direct sequencing. A BLAST search against the rat genome using the electronic PCR program from the NCBI Genome Database of the primer pairs confirmed that no pseudogene. PCR products were then detected by 1.7% agarose gel electrophoresis and was visualized by ethidium bromide staining.

E. Sequentiation of PCR products

The Automated DNA sequencing of each PCR product was carried out on capillary-based electrophoresis sequencers. The unit utilizes an ABI Prism 310 (1-capillar) and an ABI Prism 3100 (16-capillary) Genetic Analyzers from Applied Biosystems, with Big Dye Terminator Cycle Sequencing chemistry.

GENE	PRODUCT LENGTH	FORWARD PRIMER	REVERSE PRIMER
Insulin	**232** bp	CCAGTTGG	CACCTTT
		*T*AGAGGGA	GTGGTCC
		GCAG	TCACCT
Pdx-1	247 bp	GGGACCGC	GGCTTAA
		TCAAGTTT	CCTAAAC
		GTAA	GCCACA
Nkx6.1	209 bp	GGGCTTGT	ACTTGGC
		TGTAATCG	AGGACCA
		TCGT	GAGAGA
18s RNA r	140 bp	GTAACCCG TTGAACCC CATT	CCATCCA ATCGGTA GTAGCG

Applied Biosystems, with Big Dye Terminator Cycle Sequencing chemistry.

Table 1 Primer sequences and product size of genes that were further tested with real-time PCR (qPCR).

F. Western Blot Analysis

A fragment (40 mg) of right kidney or pancreas was homogenized in 10 mL of an ice-cold isolation solution (250 mM sucrose, 10 mM triethanolamine, 1 mg/mL leupeptin, and 1 mg/mL phenylmethylsulfonyl fluoride, pH 7.6) at 15,000 rpm with 3 strokes for 15 sec with a tissue homogenizer (IKA Works, Inc. Wilmington, NC). After homogenization, total protein concentrations were measured by Bradford protein assay (Bio-Rad Laboratories Inc., Hercules, CA). Samples were stabilized by adding 1 vol of 2X Laemmli sample buffer to 1 vol of sample and heating at 70 °C for 10 min. 100 micrograms of protein from each sample were loaded into individual lanes and electrophoresed on 12% polyacrylamide-SDS minigels using a Mini-PROTEAN Tetra Cell electrophoresis apparatus (Bio-Rad Laboratories Inc.). The proteins were then transferred electrophoretically to PVDF membrane (Immobilon –P, Millipore, Billerica, MA, USA). After blocking with 5% skim milk in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and 0.2% Tween-20, pH7.5) for 1 hour, the membranes were probed overnight at 4°C with primary antibodies (1:1000) (Zymed Labs, Inc, San Francisco) and then incubated with secondary horseradish peroxidase conjugated antibody (1:12000) (Zymed Labs, Inc, San Francisco). Sites of antigen-antibody reaction were viewed using enhanced chemiluminescence substrate (Immobilon Western, Millipore, Billerica, MA, USA) and analyzed in EDAS 290 (Kodak, Rochester, NY, USA).

G. Insulin, Pdx-1 and Nkx6.1 immunodetection

Fragments of kidney and pancreatic tissue were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. After deparaffination, 5 μm thick tissue sections were treated with boiling 0.01 M citrate buffer (pH 6.0) for 20 min for antigen retrieval. Then, these tissue sections were incubated in 0.2% IgG-free albumin in phosphate buffered saline (PBS) for 30 min. Sections were then labeled with mAbs anti-insulin (Zymed Labs, Inc, San Francisco, CA) (1:100), mAbs anti-Pdx-1 (Millipore Corp. Temecula, CA) (1:500) and mAbs anti Nkx6.1 (Santa Cruz Biotechnology Inc. CA) (1:250), respectively, for 2 hours at room temperature. Secondary antibodies conjugated with peroxidase were used for detection. HRP-rabbit anti-guinea pig IgG (1:100), HRP-goat anti-rabbit IgG (1:100), HRP-rabbit anti-goat IgG (1:100) (Zymed Labs, Inc, San Francisco) respectively for 2 hours at room temperature. 3,3'-diaminobenzidine (DAB) 0.06% in PBS was used as the staining solution. These sections were stained with hematoxylin. Then, they were mounted in Entellan (Merck, Darmstadt, Germany) and analyzed with a 100x objective on a DM-1000 Leica microscope.

III. STATISTICAL ANALYSIS

T test was used to compare the kidney $2^{-\Delta\Delta Ct}$ values against the $2^{-\Delta\Delta Ct}$ pancreas values (SPSS, Chicago, Ill. USA). A difference was considered significant when $p<0.05$.

IV. RESULTS

A. Insulin, Pdx-1 and Nkx6.1 gene expression

The RNA samples were used for RT-PCR(qPCR) amplification to look for insulin, Pdx-1 and Nkx6.1 genes, in the kidneys and pancreas. RT-PCR(qPCR) products of kidney RNA, for insulin, showed the same band size that was found in pancreatic RNA (232 bp). Transcription factors, Pdx-1 and Nkx6.1, revealed the same fragment length, of the corresponding pancreatic transcription factors, 247 bp for Pdx-1 and 209 bp for Nkx6.1 as was shown in figure 1. RT-PCR products of these genes were sequenced to determine the percentage of homology with pancreatic insulin, Pdx1, and Nkx6.1 genes. A BLAST search against the rat genome using the electronic PCR program from the NCBI Genome Database showed that RT-PCR products of RNA rat kidney presented a homology with respect to the same pancreatic rat genes of 100% for insulin (195/195); 97% for Pdx-1 (119/123) and 98% (178/181) for Nkx6.1 (Table 2). The relative mRNA level for each gene determined as $2^{-\Delta\Delta Ct}$ was shown in fig 2. These results showed that these values were lower in the kidney for insulin (pancreas = 0.045 ± 0.007; kidney = 0.006 ± 0.002) (Fig. 2A) and for the two transcription factors, Pdx-1 (pancreas = 2.181 ± 1.174; kidney = 0.394 ± 0.207) and Nkx6.1 (pancreas = 4.356 ± 1.049; kidney = 1.214 ± 0.234) (Figs. 2B, 2C).

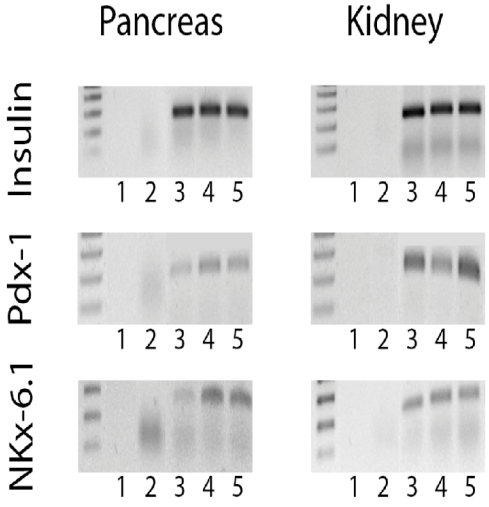

Fig. 1 Insulin, Pdx-1 and Nkx6.1 RT-PCR products. Samples from three animals (lanes 3,4,5) representing insulin, Pdx-1 and Nkx6.1 expression are shown. Lane 1 is the negative control (free nucleases water) and lane 2 is a total RNA control.

% IDENTITY	GENBANK ACCESSION NUMBER	BLAST RESULT
100 195/195	NM_019129.3	Rattus norvegicus Insulin 1 mRNA.
97 119/123	NM_022852.3	Rattus norvegicus Pancreatic and duodenal homeobox 1 (Pdx1), mRNA
98 178/181	NM_031737.1	Rattus norvegicus NKx6.1 homeobox 1 (Nkx6-1), mRNA

Table 2 Sequence results of qPCR products for kidney genes of insulin, Pdx-1 and Nkx6.1

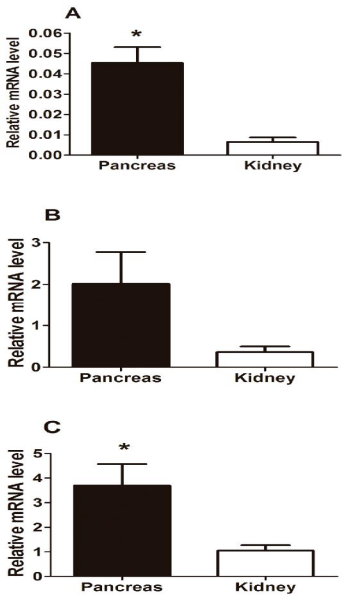

Fig. 2 Insulin, Pdx-1 and Nkx6.1 mRNA levels in pancreas and kidney. A) Insulin, B) Pdx-1, C) Nkx6.1. Each bar represents the mean ± S.E.M. n=5. $p < 0.05$.

B. Pdx-1 and Nkx6.1 protein expression

Western blot analysis showed Pdx-1 and Nkx6.1 protein expression in kidney tissue at the same molecular weights of these pancreatic transcription factors (Fig. 3). The intensity of kidney bands was lower than the pancreatic bands. This may be due to a minor genic expression of the transcription factors observed in kidney tissue.

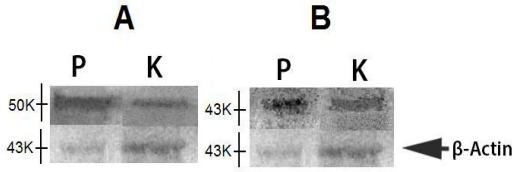

Fig. 3 Representative Western blot analysis. (P) Rat pancreatic and (K) renal tissue for Pdx-1, Nkx6.1 transcription factors and β-actin. A) Pdx-1, B) Nkx6.1.

C. Insulin, Pdx1 and Nkx6.1 immunodetection

Kidney tissue showed a positive immunoreactivity for insulin, Pdx-1 and Nkx6.1 in cortex and medulla zones in proximal, distal and Henle's loop tubular cells, respectively. At renal cortex zone it was observed immunoreactivity for insulin, Pdx-1 and Nkx6.1 only in both, proximal and distal tubules and located at luminal side (Fig. 4). There was no observation of immunolabelling in the glomeruli cells (data not shown). The renal medulla zone also presented immunoreactivity for insulin and for both transcription factors, which was observed on the luminal side of Henle's loop and tubular cells (Fig. 5). Figure 6 shows pancreatic tissue immunoreactivity (Langerhans islets) for insulin, Pdx-1 and Nkx6.1.

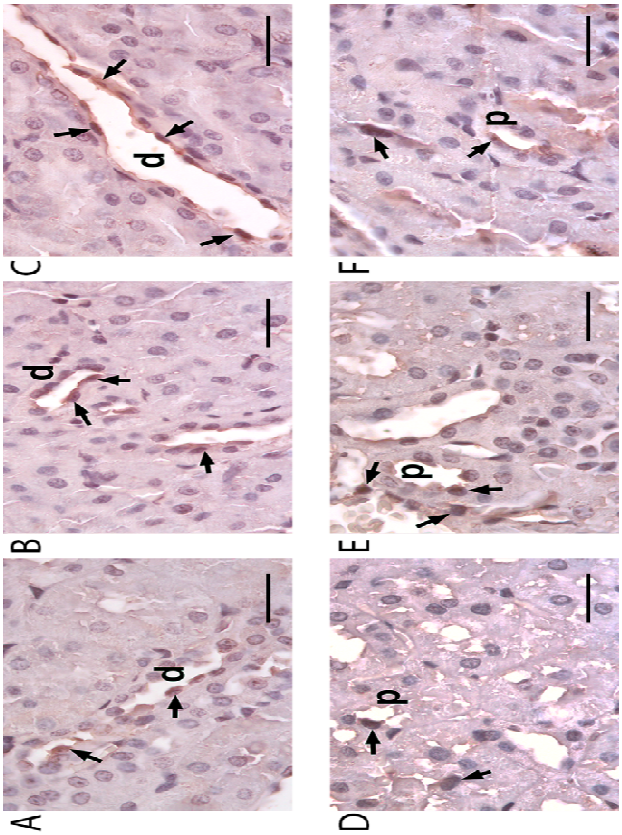

Fig. 4 Immunoperoxidase labeling of rat renal cortex (X 1000 magnification). Staining (arrows) for A) Insulin, B) Pdx-1 and C) Nkx6.1 in distal (d) tubules. D) Insulin, E) Pdx-1 and F) Nkx6.1 in proximal (p) tubules is shown. Labeling was found at the apical side of the tubules. Scale bars 5μm.

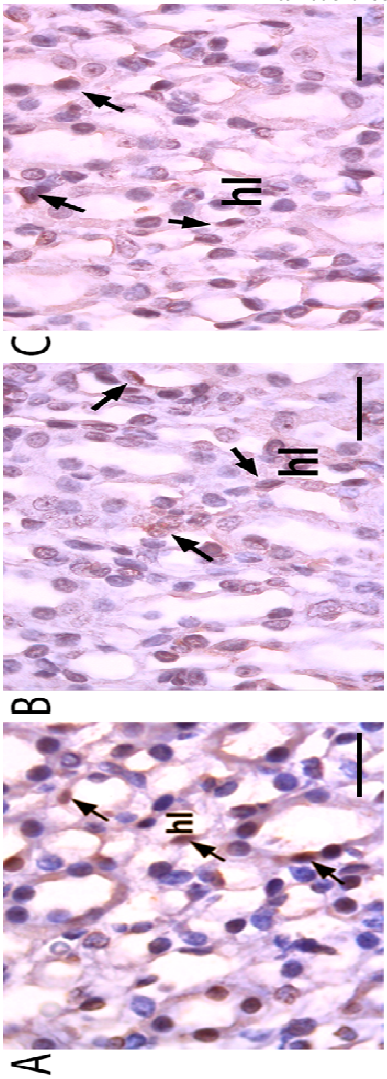

Fig. 5 Immunoperoxidase labeling of rat renal medulla (X 1000 magnification). Staining (arrows) for A) Insulin, B) Pdx-1, C) Nkx6.1 in tubules and Henle's loop (hl) is shown. Labeling was found at the apical side of the tubules. Scale bars 5µm.

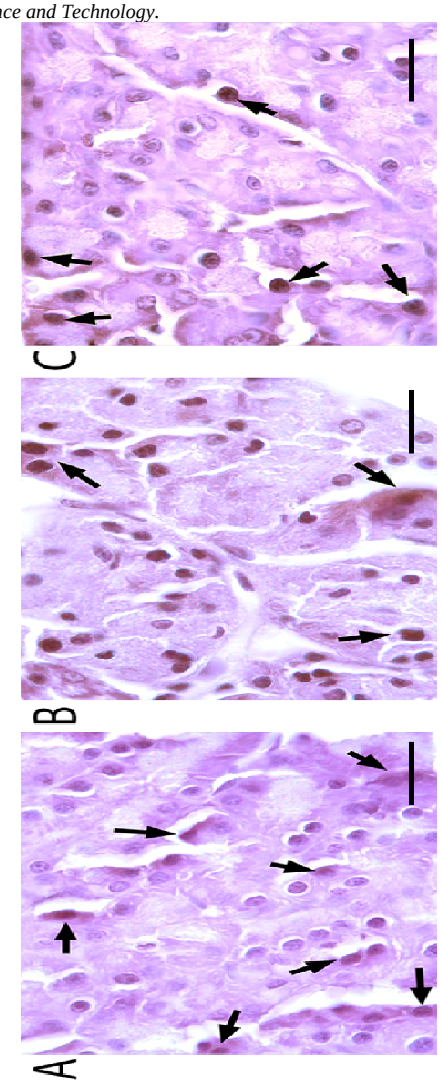

Fig. 6 Immunoperoxidase labeling of rat pancreatic tissue (Langerhans islets) (X 1000 magnification). Staining (arrows) for A) Insulin, B) Pdx-1, C) Nkx6.1 is shown. Scale bars 5µm.

V. DISCUSSION

This study's main finding is the evidence of insulin and transcription factors, Pdx-1 and Nkx6.1 gene expression and protein expression in the rat kidney, and its detection through immunohistochemistry and immunoblot (for Pdx-1 and Nkx6.1). These results have not been previously reported. Some authors have described how both, *in vitro* and *in vivo* insulin, participate in renal sodium reabsorption for maintaining this ion's homeostasis, which plays a crucial role in the compartmental distribution of body fluids [22, 23]. Insulin increases kidney sodium reabsorption through the activation of its receptors which are located on the basolateral side along the entire length of the renal tubules with the greatest density found in the thick ascending limb of Henle's loop and in the distal convoluted tubule [24].

It has been proposed that insulin's action is carried out at several renal tubular segments and it stimulates some mechanisms for sodium reabsorption: Na^+-K^+-ATPase, Na^+-H^+ exchanger type 3, sodium-phosphate cotransporter type II and epithelial sodium channels.

Pancreatic β-cells have been considered as uniquely responsible for the transcription of the gene encoding insulin [25] because of the crucial participation of its transcription factors Pdx-1 and Nkx6.1 which play a key role in the early development, differentiation and function of β-pancreatic cells. As well, a regulatory action in insulin genic expression has been suggested [26]. Nkx6.1 transcription factor participates in an essential manner at the end stage of β-cell differentiation [11]. We found insulin gene expression in the rat kidney tissue. The qPCR products sequence study showed 100% of identity with the rat pancreatic insulin. Its presence was identified by immunohistochemistry assays.

To support these findings, we looked for the transcription factors, Pdx-1 and Nkx6.1 that in the pancreas are conducive to β-cell differentiation and insulin expression. Both transcription factors were found to be expressed in the kidney and sequence studies showed 97 and 98% resemblance with the pancreatic rat transcription factors, respectively. The genic expression for kidney insulin and transcription factors Pdx-1 and Nkx6.1 was lower than the pancreatic expression (pancreatic cDNA was diluted 1:10000) and may be related to this important role. In this study, these transcription factors were identified by western blot and immunohistochemistry assays in the renal tissue. The renal immunoreactivity found for insulin and transcription factors, was located at the apical side of proximal and distal tubules and Henle's loops on the side of the membrane where it had been described for kidney sodium reabsorption mechanisms. This finding might be associated with the insulin antinatriuretic key function in this organ.

In accordance with our findings, Söderberg et al. [27] reported insulin-like peptide production in *Drosophila* renal tubules and suggested a role as a renal function autocrine regulator. Insulin expression was reported in other organs, in thymus it was observed by Jolicoeur et al. [6] in perinatal mice which persisted until 12 weeks of age. Intensity of bands from the thymus cDNA samples which were tested for insulin transcripts were reported as dilution of the pancreas sample and was lower than a 1:3000 dilution (3,000 to 10,000 times lower than that in pancreatic β-cells.). It has been proposed that small intrathymus insulin amounts play an important role of insulinspecific self tolerance of T cells and its deficit is related with type 1 diabetes mellitus [28].

As well in the brain, a small amount of insulin transcripts were found and insulin immunoreactivity was noted only as a diffuse staining [4]. Insulin's function in this organ is not fully understood. There are some findings that associate it with glucose metabolism and with the growth, maturation and myelinization of the central nervous system. In addition, it has been proposed that insulin promotes the growth of neurites and neuronal differentiation [29, 30]. Transcription factors Pdx-1 and Nk6.1 also were found in the brain [31, 32]. Both organs have presented higher concentrations of insulin than in plasma [33]. Lacroix et al. [8] demonstrated insulin synthesis at olfactory mucosa cells and provided evidence that insulin modulates the most peripheral step of odor detection at the olfactory mucosa level. In this study, they also found the transcription factor Pdx-1.

All these findings suggest that insulin production in other tissues is related with the organ function. In this study we present evidence of insulin, Pdx-1 and Nkx6.1 expression and their detection in rat kidney tissue. Both transcription factors are involved with ß-cell development, differentiation and insulin synthesis. These results may be related to the crucial function of insulin at the renal level and therefore ensuring its adequate supply. However, further research is necessary in order to conclusively prove the hormone's synthesis in this tissue.

VI. CONCLUSION

It has been reported that under normal conditions insulin can be produced in several extrapancreatic tissues. This study provides evidence to suggest insulin expression and its detection in the rat kidney –specifically by tubular and Henle's loop cells– which could be related with sodium reabsorption that participates in the regulation of ion-water homeostasis. In support of these findings, kidneys also express transcription factors typically related to the development and function of β-cells.

Acknowledgments. Authors wish to thank M.B. Félix Aguirre for his support in sequencing analysis of PCR products.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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