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Insulin Expression in Rats Exposed to Cadmium[1]

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Objectives To investigate the effects of cadmium exposure on insulin expression in rats. **Methods** Eighteen adult SD rats were administered cadmium subcutaneously (0.5, 1.0, and 2.0 mg/kg • bw). The effects on endocrine of pancreas were assessed. The levels of cadmium and zinc in pancreas, blood and urine glucose, serum insulin and urine NAG (N-acyetyl-β-glucosaminidase) were determined. The gene expressions of metallothionein (MT) and insulin were also measured, and the oral glucose tolerance tests (OGTT) were carried out. **Results** The contents of cadmium in pancreas in cadmium-treated rats were higher than that in the control group, which was associated with slight increase of zinc in pancreas. Cadmium-exposed rats (1.0 and 2.0 mg/kg • bw) demonstrated a marked glucose intolerance. But the levels of serum insulin did not change significantly after cadmium administration, and the UNAG had no change in Cd-treated group. The gene expression of insulin decreased in 1.0 and 2.0 mg/kg • bw cadmium-exposed groups, compared with the control group. The expression of MT-I was higher in the groups exposed to 1.0 and 2.0 mg/kg • bw cadmium while the expression of MT-II was higher in the group exposed to 2.0 mg/kg • bw cadmium. **Conclusions** Cadmium may be accumulated in the pancreas, resulting in the change of the expression of insulin, MT-I and MT-II genes. Cadmium can influence the biosynthesis of insulin, but does not induce the release of insulin. The dysfunction of pancreas occurs earlier than that of kidney after administration of cadmium.

Key words: Cadmium; Endocrine; Insulin; Metallothionein; Oral glucose tolerance test

INTRODUCTION

Cadmium (Cd) is an element belonging to group IIB in the periodic table of elements. It is a toxic transition metal with a wide variety of adverse effects on both humans and rodents. Cd is nonbiodegradable and has a very long biological half-life (10-30 years) in humans[1-2]. Cd accumulates preferentially in the liver, kidney, and reproductive system in animals, the major target organs of Cd toxicity[3]. Acute Cd poisoning produces primarily hepatic and testicular injury, whereas chronic exposure results in renal damage and osteotoxicity[4-8]. Cadmium in appreciable quantities is also found in the pancreas. In spite of its accumulation in the pancreas with a decrease in pancreatic function is a characteristic of Itai-Itai disease, there are few investigations into the effects of this metal on the pancreas[9].

Owing to its location the pancreas is virtually impossible to palpate. Therefore, life-threatening lesions often are not detectable until they begin to encroach on other structures, such as intestines or vertebral column. Additionally, the pancreas has a large endocrine and exocrine reserve, so signs and symptoms of the disease may not become apparent until they are very advanced[10]. Thus great importance should be attached to the effect of pancreas.

Insulin plays a key role in glucose homeostasis by virtue of its actions[11]. Long-term exposure to cadmium can lead to its accumulation in the pancreas and hyperglycemia[12]. In rabbits, accumulation of cadmium in the pancreas is associated with a decrease in the percentage of beta cells relative to alpha cells. This change in cell-type ratio may reflect the selective destruction of beta cells. Such a decrease in the number of functional beta cells can indicate a decrease in insulin secretory activity[9].

The objective of this study was to assess the effects of cadmium on pancreatic function in rats, including the levels of insulin, blood glucose, glucose tolerance, and related gene expression in pancreas.

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MATERIALS AND METHODS

Animals and Treatment

Thirty Sprague-Dawley rats weighing 180-220 g were purchased from the Animal Center of Fudan University. All these animals were kept in the SPF animal facilities, and acclimated to laboratory conditions for 1 week prior to experiment. The animals were allowed free access to standard rodent chow and tap water in 12 h light/dark cycle at 22℃ ± 2℃. One week later, the rats were divided into diabetes group (6 animals) injected intraperitoneally (*i.p.*) with streptozotocin (STZ) at a dose of 20 mg/kg body weight for seven days and non-diabetes group (18 animals) injected subcutaneously with cadmium chloride ($CdCl_2$) for ten days. The non-diabetes group were divided into 3 subgroup injected with $CdCl_2$ at doses of 0.5, 1.0, and 2.0 mg/kg • bw, daily. At the same time, the control group (6 animals) received saline solution.

Body weights of rats were recorded everyday to determine the effect of Cd and STZ on the general health of rats. Ten days after STZ and Cd administration, the rats were placed in metabolic cages to collect urine for 24 h. Tissue and blood were collected after 24 h of final injection and overnight fasting. Urine and blood samples were stored at -20℃ for analysis. Samples of pancreas were removed and stored at -70℃.

Morphology

A complete necropsy with inspection of pancreas for gross morphology changes was performed on all animals. Tail region of pancreas comprised of higher islets from each animal was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined under light microscope.

Oral Glucose Tolerance Test (OGTT)

The rats underwent an oral glucose tolerance test after an overnight fast as described elsewhere[13]. Two grams of glucose per kilogram of body weight was administered orally with an 18-gauge gavage needle. Blood was drawn from a tail vein at 0, 30, 60, 120, and 180 min for measurement of blood glucose. The area under the blood concentration versus time curve (AUC) was calculated from the following formula: AUC=15(G0+2G30+3G60+4G120+2G180)[14]. Blood glucose was measured with a Glutrend II (ACCU-CHEK, Roche, Germany).

Insulin Determination

Serum insulin was determined with a commercially available enzyme linked immuno sorbent assay (ELISA) kit (Mercodia, Sweden). All procedures were carried out in accordance with the descriptions in the manual. The intra- and inter-assay variation coefficients of the internal quality control pools were all within 5%.

Tissue Digestion

Rat pancreas samples were digested by heating with concentrated nitric acid. The digests were analyzed for cadmium and zinc (Zn) using an atomic absorption spectrometer (PE Co., USA)[15].

RNA Preparation and RT-PCR

The RNA extraction procedure described in the manual (Molecular Research Center, Inc.) was carefully followed. Briefly, 50-100 mg of tissue was homogenized using a glass-Teflon with 1 mL TRI regent. The homogenate was stored at room temperature to permit the complete dissociation of nucleoprotein complexes and supplemented with 0.2 mL chloroform per 1 mL TRI regent. The samples were covered tightly and shaken vigorously for 15 s. The mixture was kept at room temperature for 5 min, followed by centrifugation at 12000×*g* (4℃) for 15 min. After centrifugation, the aqueous phase was carefully transferred into a fresh tube and mixed with an equal volume of isopropanol. The samples were stored at room temperature for 5-10 min and centrifuged at 12000×g (4℃) for 10 min. RNA precipitate formed a gel-like or white pellet on the side and bottom of the tube. The supernatant was discarded and the RNA pellet was washed with 75% ethanol and centrifuged at 7500 g (4℃) for 5 min. After removal of the ethanol, the RNA pellet was washed, air-dried for 3-5 min, and then dissolved in 80 μL DEPC-treated water at 55℃ for 10 min. The purity of RNA was determined from the ratio of the absorbance at 260 and 280 nm by spectrophotometry (Beckman DU 650). The RNA yield was calculated based on the absorbance at 260 nm (1 U of A260= 40 μg RNA).

Semi-quantitative RT-PCR

One μg of total RNA was used to synthesize cDNA, 0.5 μg of Oligo $(dT)_{18}$ primer was added. The samples were incubated at 70℃ for 5 min and subsequently chilled on ice. The denatured RNA was mixed with reaction mixture containing 4 μL of 5×reaction buffer, 10 mmol/L dNTP mixture, and 20 U of RNase inhibitor. The samples were incubated at 37℃ for 5 min, 1 μL of 200 U M-MuLV reverse transcriptase was added to give a final volume of 20 μL. The reaction was performed at 42℃ for 60 min

and the samples were then incubated at 70℃ for 10 min to inactivate the enzyme. cDNA from each sample was used in PCR in a total volume of 25 μL containing 12.5 μL of 2×PCR Master Mix (an optimized ready-to-use PCR mixture of recombinant TaqDNA polymerase, PCR buffer, $MgCl_2$, and dNTPs), 0.5 μmol/L of forward and reverse primers. The primers used are shown in Table 1. PCR amplification was performed in a Perkin–Elmer 9600 PCR thermocycler on the following thermocycling conditions: at 94℃ for 5 min (initial denaturation), at 94℃ for 30 s, at 55℃-65℃ for 30 s, at 70℃ for 50 s (25-35 cycles), and at 72℃ for 10 min (final extension). After PCR, 5 μL products was subjected to electrophoresis on 2% agarose gels containing ethidium bromide. The image was visualized and photographed under UV transillumination (Techtumlab AB). Relative band intensities of each sample were calculated after being normalized with the band intensity of β-actin.

TABLE 1

DNA Sequence of Forward and Reverse Primers and PCR Conditions

Gene Identity	Primers	Sizes (bp)	Annealing Temperature	No. of Cycles
MT-1	Forward: ACT GCCTTC TTG TCG CTT A Reverse: TGG AGG TGT ACG GCA AGA CT	312	56	35
MT-2	Forward: CCA ACT GCC GCC TCC AAT CG Reverse: GAA AAA AGT GTG GAG AAC CG	297	56	35
INS	Forward: CCG TCG TGA AGT GGA GGA Reverse: CAG TTG GTA GAG GGA GCA GAT	154	59	30
β-actin	Forward: CCT CTA TGC CAA CAC AGT GC Reverse: GTA CTC CTG CTT GCT GAT CC	210		

Statistical Analysis

Statistical analysis was carried out using software SPSS11.0. One-way ANOVA test was used to test for the statistical significance of the means of all groups. $P<0.05$ was considered statistically significant.

RESULTS

Concentration of Cadmium and Zinc in Tissues of Rats Treated with Cadmium

After Cd administration, Cd was significantly increased in the liver (LCd), kidney (KCd), and pancreas (PCd). But the lowest Cd was found in pancreas (Fig. 1).

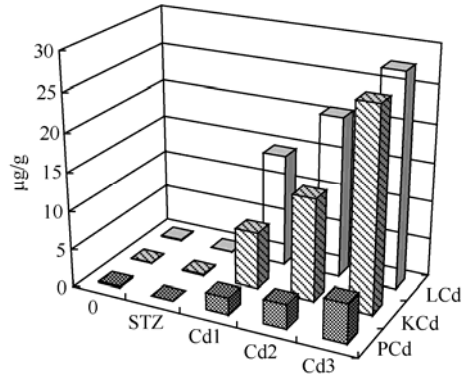

FIG. 1. Concentration of cadmium in different tissues of rats treated with $CdCl_2$.

The contents of Zn in tissues increased after Cd exposure, especially in the liver (LZn) and kidney (KZn). It was increased in the pancreas (PZn), but there was no significant difference (Fig. 2). It should also be noted that the basal content of Zn in the pancreas was much higher than that in the liver and kidney. Significant increases in serum Zn (BZn) levels in 1.0 and 2.0 mg /kg • bw Cd-exposed groups, but such increases could only be detected in urine (UZn) of 1.0 mg /kg • bw and STZ groups (Fig. 3).

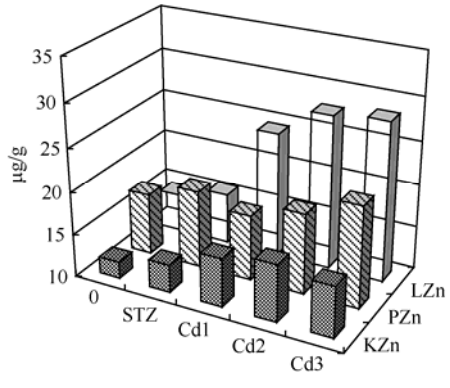

FIG. 2. Contents of zinc in different tissues of rats treated with $CdCL_2$.

Correlation analysis also indicated that there was an association between the contents of Zn and Cd in the liver ($r=0.640$, $P<0.01$), kidney ($r=0.533$, $P<0.01$) and pancreas ($r=0.286$, $P<0.01$) after Cd administration.

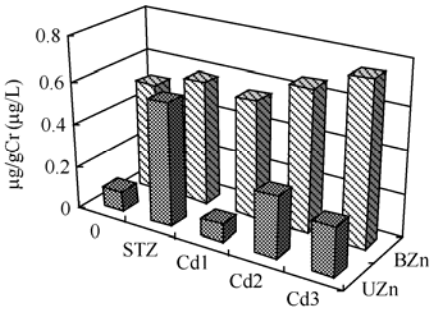

FIG. 3. Contents of zinc in serum and urine of rats treated with $CdCl_2$.

Pancreas Morphology

There was a marked involution of pancreatic islets in STZ group and Cd-treated groups, and the area markedly decreased after administration. The endothelioid cells were swollen and increased in number in blood vessels of pancreas in STZ group and Cd-treated groups.

Blood Glucose, Serum Insulin, Urine Glucose, and UNAG

After Cd administration, blood glucose was significantly increased in male rats of STZ group and 1.0 and 2.0 mg Cd /kg • bw groups on the seventh day (Table 2), but all in normal extent. On the tenth day, the blood glucose increased in male rats of 2.0 mg Cd /kg • bw group and in female rats of STZ group.

TABLE 2

Cadmium-induced Variation of Blood Glucose Levels ($\bar{x} \pm s$)

Group	*n*	Male		*n*	Female	
		7 d	10 d		7 d	10 d
0	4	2.65±0.25	3.18±0.46	3	3.57±0.21	3.77±0.38
STZ	3	3.40±0.26**	4.20±0.46**	3	12.95±11.10*	5.90±2.40*
0.5 mg Cd/kg • bw	3	3.13±0.42	3.27±0.51	3	3.57±0.15	3.00±0.20
1.0 mg Cd/kg • bw	3	4.07±0.35**	3.77±0.12	3	3.37±0.38	2.77±0.21
2.0 mg Cd/kg • bw	3	3.53±0.29**	3.97±0.15*	3	3.43±0.23	3.33±0.12

Note. * $P<0.05$ *vs*. control group. ** $P<0.01$.

No significant changes in serum insulin were observed in all groups. Positive urine glucose appeared in 1.0 and 2.0 mg Cd /kg • bw and STZ groups (Table 3).

TABLE 3

Cadmium-exposed Variation of Serum Insulin and the Positive Ratio of Urine Glucose

Group	*n*	Serum Insulin (ng/mL) ($\bar{x} \pm s$)	Positive Ratio of Urine Glucose (%)
0	7	1.486±0.466	0 (0/7)
STZ	6	1.275±0.288	100 (6/6)**
0.5 mg Cd/kg • bw	6	1.703±0.295	0 (0/6)
1.0 mg Cd/kg • bw	6	1.103±0.481	33.3 (2/6)
2.0 mg Cd/kg • bw	6	1.274±0.351	16.7 (1/6)

Note. ** $P<0.01$ *vs*. control group.

At the end of the study, UNAG was determined to check the function of kidney. No significant change was found in cadmium groups, but significant elevation occurred in STZ group (Fig. 4). No correlation was found between urine glucose and UNAG.

Linear regression analysis of blood glucose, serum insulin, cadmium, and zinc was carried out. Positive correlation was found between urine cadmium and blood glucose ($r=0.875$, $P<0.05$), and negative correlation was found between blood glucose and serum insulin ($r=0.400$, $P<0.006$) in male rats. But no correlation was found in female groups.

There was no correlation between the contents of Zn in rats and other effect indexes although cadmium

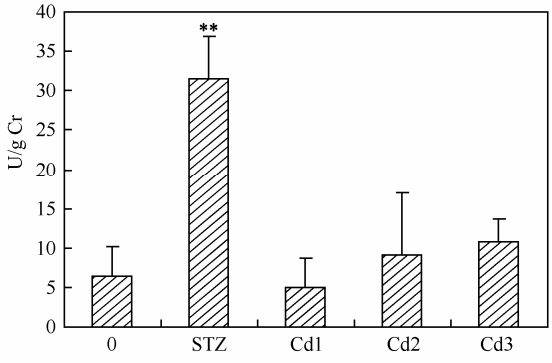

FIG. 4. Influence of cadmium and STZ on UNAG. **, $P<0.01$ *vs*. control group.

influenced the levels of zinc.

OGTT

Blood glucose concentrations were determined before and after 30, 60, 120, and 180 min of glucose load (2.0 g/kg • bw). In both groups, blood glucose levels after oral glucose loading reached a peak at 0.5 h and then gradually decreased. Rats pretreated with cadmium 1.0 and 2.0 mg/kg • bw displayed hyperglycemia and reduced glucose tolerance as indicated by significant elevation of blood glucose values over those attained from control rats at 30, 60, 120, and 180 min after glucose load. Similar changes occurred after glucose load in STZ group. The results are shown in Table 4.

TABLE 4

Values of AUC for OGTT After STZ and Cd Treatment (mmol/L •min)

Dose Group	*n*	GM [a]	95%CI [b]
0 mg Cd/kg • bw	7	837.53	804.56-872.44
0.5 mg Cd/kg • bw	6	909.91	849.65-970.15
1.0 mg Cd/kg • bw	6	997.70**	855.42-1152.18
2.0 mg Cd/kg • bw	6	954.99*	883.21-1027.19
STZ	6	1614.36**	1282.81-1985.99

Note. [a]Values shown as geometric means (GM); [b]95% confidence interval (95%CI). * $P<0.05$ *vs.* control group. ** $P<0.01$.

RT-PCR

The effects of cadmium on MT-1 and MT-2 mRNA levels in pancreas were investigated by RT-PCR. After cadmium administration the levels of MT-1 and MT-2 mRNA increased. The MT-1 mRNA expression in pancreas was induced in all Cd-exposure groups. The MT-2 mRNA expression was induced just in 2.0 mg/kg • bw group (Fig. 5).

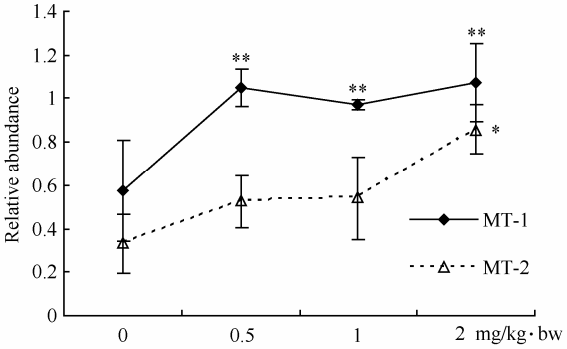

FIG. 5. Effects of cadmium on MT-1 and MT-2 mRNA levels in rat pancreas. Cadmium administration up-regulated of MT-1 in all Cd-exposed groups and MT-2 in 2.0 mg/kg • bw group. * $P<0.05$ *vs.* control group. ** $P<0.01$.

Cd administration resulted in a significant reduction of INS mRNA in 1.0 and 2.0 mg/kg • bw groups (Fig. 6). Positive correlation was found between serum insulin and INS mRNA expression. The correlation coefficient was 0.483 ($P<0.05$).

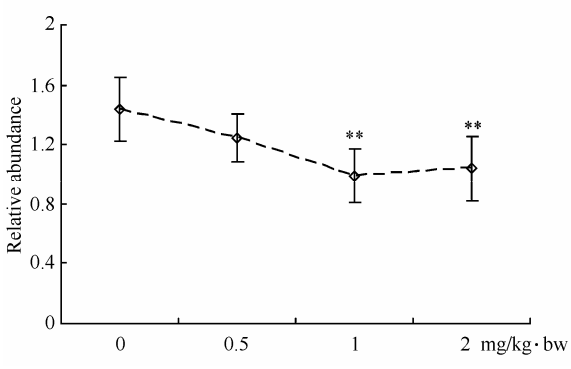

FIG. 6. Effects of cadmium on INS mRNA levels in rat pancreas. After cadmium administration, a significant depression was seen in Cd-exposed group and STZ group. ** $P<0.01$ *vs.* control group.

The expressions of MT-1 and MT-2 also increased in STZ group, but no significant difference was found. Significant suppression of INS mRNA was seen in STZ group.

DICUSSION

In this study, the effects of cadmium exposure on morphology of pancreas, blood glucose, serum insulin, OGTT, zinc concentration, MT mRNA, and INS mRNA expressions in pancreas were investigated.

Streptozotocin (STZ; N-nitroso derivative of glucosamine) is a broad-spectrum antibiotic extracted from *Streptomyces acromogenes*. It is a pancreatic β-cell toxin that induces rapid and irreversible necrosis of β-cells and is widely used in developing experimental animal models of IDDM and NIDDM[16]. STZ induces MT synthesis, which is known to occur in response to glucagons and glucocorticoids that are elevated in diabetes mellitus, also in response to the increase of oxidative stress. Here we used STZ to serve as a positive control to compare the effect of cadmium on pancreas. Blood glucose was significantly increased in STZ group and the change was unstable in female rats after STZ administration. The male rats were more sensitive than females to the change of blood glucose caused by cadmium.

Cadmium administration resulted in accumulation of this metal in the rat pancreas, proving that pancreatic tissue has an extremely high

affinity for cadmium. No gross or light microscopical morphological changes were observed in the study. The volumes of urine and drinking water in STZ group markedly increased at the end of the study. The urine volume also increased in 1.0 mg/kg and 2.0 mg Cd/kg • bw groups, not significantly different from that in control group.

It is well known that long-term exposure to cadmium by various routes may give rise to its accumulation in the kidney and consequent nephrotoxicity[17]. UNAG is the most sensitive biomarker of cadmium. So we determined the levels of UNAG to check the renal function after Cd administration in an attemp to find whether the dysfunction of pancreas is earlier than that of kidney. Nephrotoxicity caused by cadmium was not seen in the Cd groups. But blood and urine glucose increased in some groups pretreated with cadmium, suggesting that the metabolic effect of cadmium is more responsive than nephrotoxicity, which has not been reported in previous studies.

In this study, administration of 1.0 and 2.0 mg/kg • bw cadmium produced glucose intolerance in intact rats of either gender, demonstrating that cadmium induces suppression of pancreatic function. Slight change of serum insulin was also observed, but whether it is associated with decreased pancreatic secretory activity remains unknown. So we assayed the relative abundance of insulin mRNA. Insulin gene transcription is crucial for the maintenance of pancreatic β-cell differentiation and insulin production[18]. Pancreatic β-cells have the unique capacity of producting and regulating secretion of insulin in response to metabolic needs[19]. Although we did not find any significant change in serum levels of insulin, but INS mRNA was decreased in pancreas after Cd exposure, suggesting that biosynthesis of insulin is inhibited by destabilizing insulin mRNA, while insulin release is not inhibited after exposure to cadmium. The metabolic alteration caused by exposure to cadmium may be due, at least in part, to lack of insulin.

Insulin release is a calcium-dependent phenomenon. A rapid influx of calcium ions can initiate insulin secretion. The opening of [Ca^{2+}] channels apparently is the final common pathway in the activation of insulin secretion. The factors regulating the activity and expression of [Ca^{2+}] channels may provide a better understanding of insulin secretion in normal and diabetic states[20]. Previous studies have shown that cadmium can block the calcium channel[21]. In view of the similarities in the ionic radii and chemical properties of Cd^{2+} and Ca^{2+}, these two metals may exert an antagonistic effect. Cd^{2+} can substitute for Ca^{2+} in Ca^{2+}-binding protein. The major route of Cd^{2+} influx is voltage-gated [Ca^{2+}] channel. Cadmium combined with-SH of membrane protein inhibits the [Ca^{2+}] influx, decreasing free [Ca^{2+}] in cells. This results in the changes of calcium-dependent functions, but we think that this change should be a chronic effect of cadmium. In addition, the pancreas seems a critical organ in the metabolism of Zn because it accumulates Zn. Many physiological functions need Zn, such as synthesis of insulin. In this study, the content of Zn in pancreas did not increase significantly, but was slightly elevated, probably contributing to the synthesis of insulin. As this is a short-term study and the changes of insulin and Zn are a progressive process, we could not find their significant changes.

MT is a nonenzyme protein of low molecular mass (6-7kDa) that acts as a biological chelator of heavy metals through the formation of metallothiolate bonds with their cysteine residues. Physiological induction of MT appears principally involved in detoxification of heavy metals and metabolism of several essential trace elements. Four major types of MT have been found in mammals. MT-1 and MT-2 are similarly regulated in rodents, and the proteins are thought to be functionally equivalent[22-23]. However, MT-2 differs from MT-1 not only in amino acid composition, which results in differences in preference for metals (MT-1 is rich in Cd whereas MT-2 in Zn). In the present study, the level of MT-1 mRNA was two-fold higher than that of MT-2 in rat pancreas after cadmium treatment. MT may be required for maintenance of normal pancreatic function because the pancreatic MT levels fluctuate in response to a variety of physiological changes[24]. In our study, after cadmium administration, the contents of Cd in pancreas significantly increased but the same change in Zn did not occur, which may explain why MT-1 mRNA is expressed at higher levels in pancreas than MT-2 mRNA. The delicate balance between intracellular contents of Zn and Cd may be a key factor in regulating the expression of MT mRNA in the pancreas[25]. On the contrary, increased MT gene expression also influences the levels of these two metals in organs. Indirectly it may result in the functional changes associated with Cd and Zn. Increased MT mRNA expression in pancreas can protect the pancreas against the toxicity of cadmium. Our study revealed that cadmium could induce atrophy of islets, suggesting the decrease in cell number and the change in cell form. These influences can change the function of islets, but we did not find the overt change of serum insulin. The high expression of MTs may exert some physiological functions such as storage of zinc to participate in the synthesis of insulin and detoxification of cadmium in pancreas.

In adult vertebrates, insulin is exclusively expressed in β-cells of the pancreatic islets of Langerhans and plays a key role in the control of energy metabolism, and more specifically of glucose homeostasis[26]. The insulin gene is mainly expressed in islet β-cells in pancreas. Our results revealed that the expression of INS mRNA was also inhibited after administration of cadmium, illuminating that the level of transcription of INS gene in pancreas is influenced by cadmium. A correlation could be found between the relative abundance and the level of serum insulin. So it may be inferred that decreased INS mRNA expression after Cd-treatment is one reason why the change of insulin level was not exhibited in our study.

In conclusion, cadmium can induce hyperglycemia. Glucose regulates insulin biosynthesis and secretion of adult β-cells by transcriptional and posttranscriptional mechanisms[26-27]. Although its association with the lack of insulin was not evidenced, the Cd-induced down-regulation of INS gene was exhibited in rat pancreas. The results indicate that cadmium may play a role in disrupting the balance between serum insulin and blood glucose. The levels of MT mRNA in pancreas may be up-regulated after cadmium treatment, which may be attributed to the protective role of pancreas. But pancreas is a complicated organ, the function of which results from complex interactions among multiple metabolic organs, including liver, muscles, and adipose tissue. Therefore, it is difficult to define the direct effect of cadmium on the pancreas and further study is needed.

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