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Effect of dichlorvos on hepatic and pancreatic glucokinase activity and gene expression, and on insulin mRNA levels

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Abstract

Several studies have shown that organophosphate pesticides affect carbohydrate metabolism and produce hyperglycemia. It has been reported that exposure to the organophosphate pesticide dichlorvos affects glucose homeostasis and decreases liver glycogen content. Glucokinase (EC 2.7.1.1) is a tissue-specific enzyme expressed in liver and in pancreatic beta cells that plays a crucial role in glycogen synthesis and glucose homeostasis. In the present study we analyzed the effect of one or three days of dichlorvos administration [20 mg/kg body weight] on the activity and mRNA levels of hepatic and pancreatic glucokinase as well as on insulin mRNA abundance in the rat. We found that the pesticide affects pancreatic and hepatic glucokinase activity and expression differently. In the liver the pesticide decreased the enzyme activity; on the contrary glucokinase mRNA levels were increased. In contrast, pancreatic glucokinase activity as well as mRNA levels were not affected by the treatment. Insulin mRNA levels were not modified by dichlorvos administration. Our results suggest that the decreased activity of hepatic glucokinase may account for the adverse effects of dichlorvos on glucose metabolism.

Keywords: Organophosphate; Pesticides; Dichlorvos; Glucokinase; Insulin

Introduction

Residential and agricultural pesticide use is widespread in the world. Their extensive and indiscriminate use, in addition with their ability to interact with biological systems other than their primary targets constitute a health hazard to both humans and animals. The toxic effects of pesticides include alterations in metabolism, reproduction, development, carcinogenesis and neurotoxicity (Peter and Cherian, 2000; Hodgson and Levi, 1996; Kupfer, 1975).

It has been extensively documented that organochlorine pesticides produce alterations of glucose homeostasis. The hyperglycemic effect of dichloro-diphenil-trichloro-ethane (DDT) was first demonstrated by Stohlman and Lillie, 1948; since that time, numerous investigations have confirmed the fact that DDT and other organochlorine pesticides influence sugar metabolism. The hyperglycemia produced by these compounds is related to an increase in the activity of gluconeogenic enzymes in the liver and in the kidney (Kacew and Singhal, 1973a,b; Bathia et al., 1973; Fox and Virgo, 1986), and to a decrease in glycolytic enzymes (Bathia et al., 1973). Other studies have found that DDT increases glycogen phosphorylase and decreases glycogen synthase (Hikenbottom and Yau, 1974).

Organophosphate compounds are at present responsible for more poisonings than any other single class of pesticides (Sultatos, 1994); furthermore, their use has continued to rise with a 10-fold increase in the past 3 decades (Rosenstock et al., 1991). Several observations indicate that, similar to the detrimental effects of organochlorine pesticides on carbohydrate metabolism, organophosphate pesticides affect glucose homeostasis: hyperglycemia is severely increased in organophosphate poisoning (Seifert, 2001; Hayes et al., 1978; Meller et al., 1981; Namba et al., 1971; Teichert-Kuliszenwska et al.,

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1981). In rat brain, Sarin and Gill (1999) have found that dichlorvos (2,2-dichlorvinyldimethyl phosphate) administration decreased the activities of hexokinase I, phosphofructokinase and lactate dehydrogenase. They also observed that dichlorvos exposure caused an increase in the activity of glycogen phosphorylase accompanied with a significant depletion in brain glycogen content. Decreased hepatic glycogen content and increased blood glucose levels were also observed in several species. In *Macrobrachium lamarrei*, a freshwater prawn, as well as in fresh water fish (*Clarias batrachus*, *Saccobranchus fossilis*, and *Mystus vittatus*) dichlorvos exposure decreased liver glycogen content and affected glucose homeostasis (Omkar and Shukla, 1985; Verma et al., 1983). In rat liver Teichert-Kuliszenwska and Szymcyc (1979) found that a single dose of 50% LD50 increased the activity of glycogen phosphorylase, while UDP-glucose pyrophosphorylase activity was decreased.

Glucokinase (EC 2.7.1.1), also known as hexokinase IV, is a tissue-specific enzyme present in hepatocytes, in pancreatic beta cells and in certain neuroendocrine cells of the brain and gut (Weinhouse, 1976; Bedoya et al., 1986; Jetton et al., 1994). Glucokinase plays a crucial role in glucose homeostasis. In the liver its activity is critical for glucose uptake and glycogen synthesis (Granner and Pilkis, 1990; Ferre et al., 1996; Seoane et al., 1996; O'Doherty et al., 1996), in beta cells glucokinase plays a key-role regulating insulin secretion in response to glucose (Meglasson and Matschinsky, 1986; Matschinsky, 1996). The comparison of the pancreatic and hepatic glucokinase gene transcripts reveals a tissue-specific control of their expression as well as the existence of two distinct promoters in a single glucokinase gene (Magnuson and Shelton, 1989; Magnuson, 1990). The existence of alternative promoters suggests that separate factors regulate glucokinase transcription in the two tissues.

In this work we investigated the effect of dichlorvos [20 mg/kg body weight (i.e. 25% LD50)] on hepatic and pancreatic glucokinase activity and mRNA levels. Since pancreatic glucokinase activity is a determinant of glycolysis and therefore of insulin mRNA synthesis (German, 1993), we also analyzed the effect of the pesticide on the insulin message.

Materials and methods

Animals

Male Wistar rats, weighing approximately 200–300 g, were housed in an environmentally controlled room and a 12-h light–dark cycle. All animals had unrestricted access to a standard diet (Teklab, Global Diet 2018 A, Harlan, Indianapolis, IN) and water. Housing conditions consisted of two rats kept in a plastic cage ($42 \times 27 \times 16$ cm) with a steel lid. Half of the rats were treated with a corn oil solution containing 20 mg/kg body weight (i.e. 25% LD50) of dichlorvos (Proveedora Agroindustrial de Sinaloa, Sinaloa, Mexico) and half of them received corn oil as vehicle. After 1 or 3 days of treatment rats were anesthetized with 63 mg/kg of pentobarbital (Anestesal, Pfizer, Mexico City, Mex). Approximately 600 mg of liver was excised to analyze hepatic glucokinase activity and mRNA levels. Pancreatic islets were isolated by collagenase digestion as previously reported (Romero-Navarro et al., 1999). Rats were staged and treated in accordance with the animal use protocol approved by the national and institutional guidelines for the animal welfare: Institutional Animal Care and Use Committee of the Instituto de Investigaciones Biomedicas based on the outdated Mexican Official Norm NOM-062-2OO-1999 and Institute for Laboratory Animals Research (NOM/GCUAL). The Animal Care Committee of the Biomedical Research Institute approved the protocols used in these experiments.

Glucokinase assay

400–500 islets were harvested and centrifuged at 1200 rpm. Tissue pellets were lysed in 500 μl of Reporter Lysis Buffer (Promega, Madison, WI, USA), vortexed and cell membranes disrupted by three freeze-thaw cycles. 500 μl of GK buffer consisting of 50 mM Tris (pH 7.6), 4 mM EDTA, 150 mM KCl, 4 mM Mg_2SO_4, and 2.5 mM dithiothreitol were added. To analyze hepatic glucokinase 500 mg of liver were resuspended in 1 mL of GK buffer and homogenized with a Polytron®, Kinematica AG, Littau, Switzerland. The lysates from liver and islets were then centrifuged at 4° for 1 h at 53 000 $\times g$, in a Beckman ultracentrifuge model Optima (Palo Alto; CA, USA). Supernatants were recovered, and enzymatic activity was assayed as previously described by Walker and Parry (1966), using NADP (Sigma, MO) as coenzyme. Glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides (Sigma, St. Louis, MO, USA), was used as coupling enzyme. Correction for hexokinase activity was applied by subtracting the activity measured at 0.5 mM glucose from the activity measured at 100 mM glucose. Protein concentrations were determined by Bradford assay (1976).

Messenger RNA analysis

Glucokinase, insulin and actin mRNA were quantified using RT-PCR. Total RNA was isolated with the single-step method based on guanidine isothiocyanate/phenol/chloroform extraction using TRIzol reagent (Gibco BRL, NY). RNA concentration was determined by absorbance at 260 nm and its integrity was verified by electrophoresis on 1.1% denaturing agarose gels in the presence of 2.2 M formaldehyde. Total RNA was reverse transcribed to synthesize single strand cDNA. 10 μl of the reverse transcription reaction were subjected to PCR in order to simultaneously amplify glucokinase, insulin, and actin gene as an internal control. The sequences of the specific primers for glucokinase amplification fragment were 5′-[GCT TCA CCT TCT CCT TCC C]-3′ in the sense primer and 5′-[CCC ATA TAC TTC CCA CCG A]-3′ in the antisense . The primers used for actin gene amplification were 5′-[GGG TCA GAA GGA TTC CTA TG]-3′ in the sense and 5′-[GGT CTC AAA CAT GAT CTG GG]-3′ in the antisense. The primers used for insulin gene amplification region were 5′-[ATT GTT CCA ACA TGG CCC TGT]-3′ in the sense and 5′-[TTG CAG TAG TTC TCC AGT TGG]-3′ in the antisense. The 50 μl PCR

reaction included: 1 μl of previously synthesized insulin cDNA, or 2 μl of previously synthesized glucokinase or actin cDNA, 20 mM Tris–HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, and 2.5 U of Taq DNA polymerase. Negative controls without RNA and with non-retrotranscribed RNA were included in all the experiments. After the initial denaturation step at 95 °C for 5 min, PCR reaction was performed for 12, 25, and 20 cycles in the cases of insulin, pancreatic glucokinase, hepatic glucokinase and actin respectively. The cycle amplification profiles were 20 for the insulin gene: 94 °C, 1 min; 57 °C, 2 min; and 72 °C, 1 min; for the pancreatic glucokinase gene: 94 °C, 1 min; 62 °C, 1 min; and 72 °C, 1 min; and for the actin gene: 94 °C, 0.5 min; 55 °C, 0.5 min, and 72 °C, 0.5 min. A final extension cycle was performed at 72 °C for 7 min in all cases. The number of performed cycles was within the exponential phase of the amplification process. All PCR products were always studied and analyzed together throughout the experiments. 20 μl of PCR products were separated on 1% agarose gel and stained with ethidium bromide. The image was captured under a UV transilluminator with a Type 665 negative film (Polaroid Co., Cambridge, MA). The intensity of the glucokinase, insulin and actin bands was quantified by densitometry using a Scanjet 5300 (Hewlewtt Packard, Sunnyvale, CA) apparatus and the Scion Image software (Frederick, MD). Insulin and glucokinase expression level was normalized to that of actin.

Statistics

Data are presented as mean±S.E. Multiple comparisons were evaluated using one-way ANOVA. The significance level chosen was $P<0.05$.

Results

Hepatic glucokinase activity and mRNA levels

We investigated the effect of dichlorvos administration (25% LD50) on hepatic glucokinase. As shown in Fig. 1, administration of dichlorvos decreased glucokinase activity.

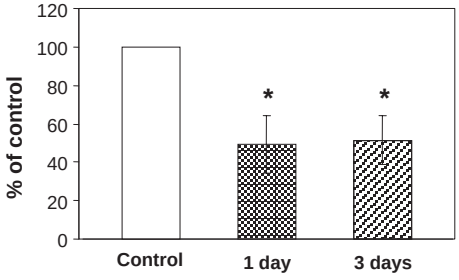

Fig. 1. Effect of dichlorvos on hepatic glucokinase activity. Rats were treated with either dichlorvos 20 mg/kg body weight (25% LD50) or vehicle as described in Materials and methods. Glucokinase activity was determined in liver homogenates. Data are expressed as mean percentages±SE of glucokinase activity in control rats (activity of glucokinase in control rats=5.5+0.85 mU/mg protein); experiments were performed by duplicate ($n=3$). Multiple comparisons were evaluated by one-way ANOVA analysis of variance. (*); $P\leq0.05$.

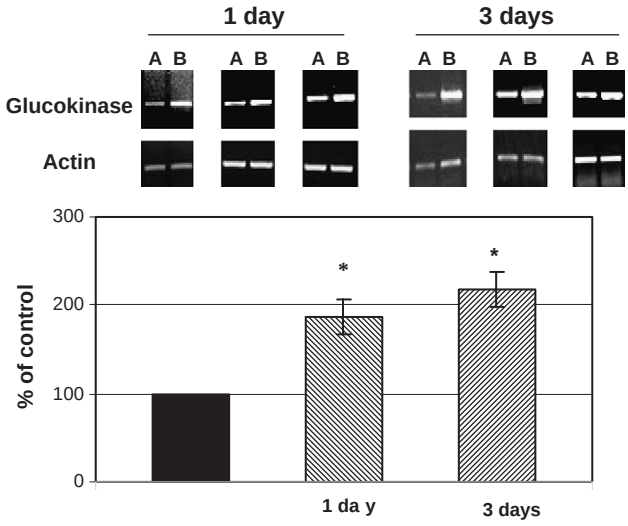

Fig. 2. Effect of dichlorvos on hepatic glucokinase mRNA levels. Glucokinase and actin mRNA were quantified using RT-PCR as described in Materials and methods. Each sample was standardized to actin. Lane A: vehicle; Lane B: dichlorvos. Data are expressed as relative to that measured in vehicle-treated rats. Each value represents the mean±SE of three different experiments. (*); $P\leq0.05$.

After 1 day of treatment with dichlorvos the hepatic glucokinase activity decreased by 49.30±14.88%; similar decrease were observed at 3 days of treatment (51.48±12.84 %). We also determined the effect of dichlorvos administration (25% LD50) on hepatic glucokinase mRNA levels. In contrast to the decreasing effect of dichlorvos on glucokinase activity, the treatment with the pesticide for 1 day produced a relative increase of 86.55±37.2% on glucokinase RNA levels. This stimulatory effect was also observed at 3 days of treatment (Fig. 2). No changes on total protein or total mRNA concentration were observed with dichlorvos administration (data not shown).

Pancreatic glucokinase activity and mRNA levels

We also determined glucokinase activity and mRNA levels in pancreatic islets isolated from either dichlorvos or vehicle-treated rats. As depicted in Table 1, after 1 and 3 days of

Table 1
Effect of dichlorvos administration on pancreatic glucokinase activity and mRNA levels and on insulin mRNA

Glucokinase				Insulin	
Activity percent of control		mRNA percent of control		mRNA percent of control	
1 day	3 days	1 day	3 days	1 day	3 days
106.8±7.9%	82.8±12.0%	98.4±16.3%	90.7±5.8%	115.5±21.7%	98.3±2.0%
$n=3$	$n=4$	$n=4$	$n=3$	$n=3$	$n=3$
$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$

Data are expressed as mean percentages±SE.
Control glucokinase activity; 54.5±9 pmol/h/islet. Each value represents the mean±SE of the number of experiments indicated in the table performed by duplicate.
Multiple comparisons were evaluated by ANOVA one way analysis of variance.

treatment with the pesticide, no significant ($P>0.05$) changes in glucokinase activity or glucokinase mRNA levels were observed. Dichlorvos administration did not affect islet total protein or mRNA content (data not shown).

Insulin expression

The effect of dichlorvos on insulin mRNA was also determined. As shown in Table 1, the treatment with 25% LD50 of the pesticide did not affect insulin mRNA levels.

Discussion

In the present study we found that the organophosphate pesticide, dichlorvos affects pancreatic and hepatic glucokinase activity and expression differently: in the pancreatic islets, neither glucokinase activity nor mRNA levels were affected by the treatment with dichlorvos. In contrast, in the liver the pesticide decreased the enzyme activity and augmented mRNA levels. The different response of hepatic and pancreatic glucokinase isoenzyme expression and activity to diverse factors has been previously documented; indeed, we (Fernandez-Mejia and Davidson, 1992; Fernandez-Mejia et al., 2001) and others (Bedoya et al., 1986; Liang et al., 1992) have previously shown that glucokinase isoenzymes are regulated differently in the pancreatic beta cell and in the liver, supporting the concept that the alternate promoters and dissimilar first exons confer differential isoenzyme regulation to these tissues. However, the present report is the first study to show that differential regulation of glucokinase is produced simultaneously in vivo.

We found that in contrast to the increasing effect of dichlorvos on hepatic glucokinase mRNA levels, enzyme activity was decreased. Several studies have found that modifications of glucokinase mRNA levels are not necessarily reflected on glucokinase activity: the positive effect of glucocorticoids on hepatic glucokinase mRNA levels (Minderop et al., 1987) is countered by the enhancement of glucokinase protein degradation (Sibrowski and Seitz, 1980). The protein levels and activity of pancreatic glucokinase are not affected by thyroid hormone despite the negative effect of this hormone on glucokinase mRNA levels (Fernandez-Mejia and Davidson, 1992; García-Flores et al., 2001). In the pancreatic cell line RIN 1046-38, biotin induction on pancreatic glucokinase mRNA levels is not paralleled by increases on glucokinase activity (Borboni et al., 1996). These observations suggest that additional mechanism(s) should regulate glucokinase protein synthesis, protein turnover or translocation (Magnuson and Matschinsky, 2004). Whether the increase observed in glucokinase mRNA levels in response to dichlorvos is the result of a physiologic compensatory effect to overcome decreased glucokinase activity, or weather it is a consequence of a direct effect of the pesticide on the different molecular mechanisms involved in glucokinase regulation deserves further research.

Several investigations have reported that dichlorvos exposure decreases glycogen content (Sarin and Gill, 1999; Omkar and Shukla, 1985; Verma et al., 1983) and affects polysaccharide metabolism. The pesticide decreases UDP-glucose pyrophosphorylase activity (Teichert-Kuliszenwska and Szymcyc, 1979), an enzyme that participates in glycogen synthesis. On the contrary, dichlorvos exposure increases the activity of glycogen phosphorylase (Sarin and Gill, 1999; Teichert-Kuliszenwska and Szymcyc, 1979), a key enzyme in glycogen breakdown. Hepatic glucokinase activity plays an important role in glycogen synthesis (Granner and Pilkis, 1990; Ferre et al., 1996): increased glucokinase activity has a potent enhancing effect on glycogen synthesis (Seoane et al., 1996; O'Doherty et al., 1996); in contrast, impaired hepatic glycogen synthesis is observed in glucokinase deficiency (Postic et al., 1999). Our finding that dichlorvos decreases hepatic glucokinase activity is in accordance with the detrimental effect of the pesticide on glycogen content and on glycogenesis.

Studies on the effect of dichlorvos administration during the oral glucose loading test in rabbits revealed that after 1 h of dichlorvos administration, treated animals presented hyperinsulinemia (Teichert-Kuliszenwska et al., 1981). In the present study we found that insulin mRNA levels were not affected by dichlorvos administration. The cholinergic effects of dichlorvos (Ehrich et al., 1997; Kobayashi et al., 1986) can account on these effects: acetylcholine increases glucose-induced insulin secretion by rapid mechanisms that involve a rise in free cytosolic Ca2+ concentration and a marked protein kinase C-mediated increase in the efficiency of Ca2+ on exocytosis (Gilon and Henquin, 2001). However, Ca2+ and activation of protein kinase C do not affect insulin mRNA levels (Welsh et al., 1988).

Several observations indicate that hyperglycemia is severely increased in organophosphate poisoning (Teichert-Kuliszenwska et al., 1981; Seifert, 2001; Hayes et al., 1978; Meller et al., 1981; Namba et al., 1971). Studies by Teichert-Kuliszenwska et al. (1981) revealed that dichlorvos administration developed hyperglycemia not compensated by insulin despite its increased secretion. The authors propose that the pesticide may affect glucagon release inhibiting glycogen synthesis and activating glycogenolysis (and gluconeogenesis), two actions that could result in transient hyperglycemia. It has been reported that acetylcholine is a potent secretagogue of both insulin and glucagon (Gilon and Henquin, 2001; Duttaroy et al., 2004); therefore, as proposed by Teichert-Kuliszenwska et al., the cholinergic effects of dichlorvos may perhaps involve changes in the insulin/glucagon ratio. Alternatively, dichlorvos-induced hyperglycemia, which is not compensated by hyperinsulinemia, may be linked to detrimental effects of the pesticide on insulin sensitivity. These two hypotheses will require to be tested.

Diabetes is rapidly emerging as a major health problem in the world (Zimmet et al., 2001; Onkamo et al., 1999). The prevalence of diabetes is rising due to the Westernized life style which includes excessive energy intake and physical inactivity (Zimmet et al., 2001). However, several drugs (Ferner, 1992) and environmental factors also account for the development of diabetes (Ferner, 1992; Yoon et al., 1987; Department of Veterans Affairs, 2001). The increasing use of organophos-

phate pesticides with their adverse effects on glucose metabolism, may well be an additional factor in the speedy expansion of diabetes in the world.

Conclusion

In this study we report that dichlorvos affects pancreatic and hepatic glucokinase activity and expression differently. In the liver the pesticide decreased the enzyme activity; on the contrary glucokinase mRNA levels were increased. In contrast, pancreatic glucokinase activity as well as mRNA levels were not affected by the treatment. The decreased activity of hepatic glucokinase may account for the adverse effects of dichlorvos on glucose metabolism.

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