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Long lasting cadmium intake is associated with reduction of insulin receptors in rat adipocytes

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Abstract

The effects of chronic cadmium exposure on adipose tissue have not been extensively reported. In adult Wistar male rats we investigated *in vivo* effect of 6 weeks lasting cadmium intake in drinking tap water ($CdCl_2$ 9,7 mg/l). Insulin receptors in isolated adipocytes from epididymal fat and glucose transporter protein GLUT4 content in fat tissue plasma membranes were determined. Control and Cd treated rats had similar water intake with subsequent heavy augmentation of Cd content in liver of experimental animals. In comparison with controls, Cd intake did not influence body mass increment and fat cell size, but significantly increased serum glycemia and moderately elevated insulinemia. Cadmium intake significantly reduced ($\sim 50\%$) both, total insulin receptors number and density of the receptors in fat cells. No differences in the content of GLUT4 in crude plasma membranes of adipose tissue were observed. Diminished insulin receptors in adipocytes could account for diabetogenic effect of long lasting cadmium intake.

Introduction

Cadmium is a xenobiotic with still unknown physiologic function. The consequences of cadmium compounds to human endocrine, reproductive and immune functions remain unclear. Increased exposure for humans represents occupational risks of several specialized industries (nickel-cadmium batteries, metallurgical alloys, pigments and stabilizers of plastic), intake of contaminated food and cigarette smoking. The source of contaminated food (root vegetables, rice, bread and pastries) is the soil as the result of industrial emissions or fertilization (Korzun & Heck 1990). The dietary origin of cadmium is also from seafood (oysters, shrimps, crabs). Smoking is an important source of cadmium exposure, one cigarette contains approximately 0–6.67 μg cadmium (Smith *et al.* 1997) and its content in the fat tissue of smokers is four times higher than that of nonsmokers (Mussalo-Rauhamaa 1986). In the body cadmium is bound to metallothioneins (MT) and the complex is distributed to various tissues and organs, it accumulates predominantly in kidney, liver, reproductive tissues, pancreas and lungs. As a consequence of binding to MT, cadmium is very slowly eliminated from the body, that explains an extremely long biological half-life exceeding 10 years (Kelley 1999) and makes cadmium a cumulative toxin. A severe intoxication clinically manifests as osteomalacia, aminoaciduria and glycosuria (Verougstraete *et al.* 2002).

In 1993 IARC classified cadmium as a group I carcinogen (IARC 1993). Long-term cadmium exposure is associated with an increased risk of cancer such as lung (Verougstraete *et al.* 2002), pancreas (Schwartz 2000), kidney, liver, stomach, testes, prostate, adrenals and hematopoietic system (Waalkes *et al.* 1992; Waalkes 2002). In the literature, there are rare information on Cd effects on isulin receptors and insulin action in adipose tissue. Addition of Cd (1 mM) to intact rat adipocytes did not affect the insulin receptor kinase activity, but stimulated glucose transport without changing the amount of glu-

cose transporter in crude plasma membranes (Ezaki 1989). The stimulatory effect of Cd on glucose transport was also confirmed in cell culture model and again, no effects on GLUT4 protein was observed (Harrison 1991). It seems that aforementioned findings on Cd induced glucose transport could explain previously described *in vitro* insulin mimetic effect of cadmium on glucose lipogenesis (Yamamoto *et al.* 1984) and glucose oxidation (Yamamoto *et al.* 1986) in rat adipocytes.

In pancreatic islets of obese hyperglycemic mice low cadmium concentration evoked basal (Nilsson *et al.* 1986) and glucose stimulated (Nilsson *et al.* 1987) insulin response. In contrast, high Cd concentration significantly inhibited the secretory response to glucose (Nilsson *et al.* 1986). *In vivo* rat intake of cadmium resulted in lower glycemia accompanied with higher serum insulin value (Nakamura *et al.* 1983). Further discrepancies in cadmium effects on glucose homeostasis and insulin levels are results of hyperglycemia and inhibition of insulin release from rat pancreas in rats exposed to cadmium (Ghafghazi *et al.* 1977; Merali & Singhal 1980). Incompatibility of literary data on cadmium effect is based on both, experimental approach (*in vivo* vs. *in vitro* studies) and the various metal concentrations used. Low doses of Cd used in experiments mimic low or moderate levels of environmental contamination. High cadmium accumulation in adipose tissue of smokers and proposed diabetogenic effect of cadmium initiated our study to investigate the long lasting *in vivo* effect of moderate cadmium concentration intake on glucose homeostasis and insulin receptor status in fat cells of rats. The functional insulin receptors in target tissues substantially participate in insulin stimulated glucose disappearance and metabolism as well as in insulin degradation processes.

Materials and methods

Chemicals

All the chemicals, until indicated otherwise, were of Sigma grade (Sigma, St. Louis, USA).

Animals

Male (SPF) Wistar rats obtained from Charles River (Anlab, Prague, Czech republic, 150.8 ± 3.6 g initial body mass) were drinking ad libitum $CdCl_2$ (9.7 mg/l) in tap water for 6 weeks. The control rats received tap water only. Cadmium content in tap water was 0.211 μg/l (data from The National Institute of Epidemiology and Occupational Hygiene, Bratislava, Slovak Republik). The water consumption was monitored daily. The animals had free access to standard laboratory diet chow (ST1, Velaz, Prague, Czech Republic). Three rats per cage were kept in animal room with controlled temperature (21–23 °C), relative humidity (45%) and light-dark cycle (12–12 h). Control and experimental group consists of 6 rats each. The animals were killed by decapitation between 09:00 and 09:30 after overnight food deprivation. Serum was prepared by centrifugation of the blood collected, liver tissue was removed, rinsed in cold saline, blotted dry and the samples were stored frozen until analysis. Principles of laboratory animal care and all procedures were approved by the Animal Care Committee of the IEE SAS Bratislava (Slovak Republic). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Isolation of adipocytes

Adipocytes were isolated from epididymal fat tissue by collagenase digestion (collagenase Type II, 2 mg/g of adipose tissue) according to Rodbell (1964).

Insulin binding

The binding of mono-(Tyr-A-14) 125I insulin (Zorad *et al.* 1985) to adipocytes was estimated by the method of Kasuga *et al.* (1977). Competition binding curves transformed according to Scatchard (1949) were analyzed using the Ligand computer program (Munson & Rodbard 1980). Insulin binding capacity (number of receptors) was calculated from each curve for individual animal. Statistical mean of 6 values for each group is presented in Table 1.

Western blot analysis of GLUT4 protein

Crude plasma membranes were isolated from frozen epididymal adipose tissue (after excision immediately immersed in liquid nitrogen, then stored at -80 °C) as described in details in Golda *et al.* (2001). Solubilized membranes (~ 30 μg protein/sample) were subjected to SDS gel electrophoresis and electrophoretic transfer to nitro-cellulose membrane Hybond C (Amersham). GLUT4 bands were immunodetected by incubation with rabbit anti-Glut4 (East-Acres, USA) antibody,

Table 1. Characteristics of animals.

	Control	$CdCl_2$	$P <$
Weigh gain (+g)	237 ± 7.8	255 ± 9.6	NS
Average water intake (ml/day/rat)	20.3 ± 0.6	20.5 ± 0.2	
Cd content in liver (μg/g of wet tissue)	< 0.02	6.19 ± 0.62	0.001
Glycemia (mmol/l)	5.2 ± 0.1	6.64 ± 0.3	0.05
Insulinemia (ng/ml)	0.47 ± 0.05	0.68 ± 0.1	NS
Fat cell size (d = μm)	84.2 ± 2.9	88.9 ± 1.4	NS
IR density (number/1 μm^2)	9.51 ± 1.7	3.96 ± 0.6	0.05

X±SE, $n = 6$ rats per each group, IR – insulin receptors, $P <$ – statistical significance, NS – not significant.

followed by binding of peroxidase-coupled monoclonal anti-rabbit imunnoglobulins (Sigma). Labeled bands were revealed by ECL procedure (Amersham) and the images were analysed by Ultralum KS 4000 camera (Ultra-Lum. Inc., CA).

Other determination

For cadmium analysis the liver tissues were weighed, placed in platinum crucibles and dry-ashed in muffle furnace at 460–500 °C for 24 h. The ash was solubilized with 3 M HCl. Appropriately diluted samples were analyzed by atomic absorption spectrometry (AAS) using model SpectrAA 220 FS instrument (Varian Australia Pty Ltd.). Analytical accuracy was monitored by assaying recovery samples, as well as reference samples of NBS standard reference material 1577 Bovine Liver. Serum glucose concentrations were measured by glucoanalyser SUPER GL (Dr Müller, Germany), serum insulin levels by radioimmunoassay Rat insulin RIA kit (Linco Res. Inc., USA). Fat cell size (diameter) in each isolated batch of individual rat was measured by light microscope after staining with crystal violet (Belzung *et al.* 1993).

Data are presented as means ± SEM, the statistical significance of differences between groups was determined by computer unpaired *t*-test.

Results

Table 1 presents animal characterization. 6 weeks lasting intake of cadmium did not affected body mass increment of experimental animals, at the end of experiment the weight gain was comparable to that of control rats. Equal volume of water intake in both groups is in favor of tissue cadmium content. Nearly 300 times elevated Cd content in liver of experimental group is based on metal intake per se, and not on different amount of water intake. Fat cell size of experimental animals did not differ to that of control rats. Statistically significant higher fasting glycemia in cadmium treated rats was accompanied by moderate elevation of insulinemia.

Due to similar fat cells size, the results of insulin receptors studies are expressed per standard cell number (1×10^6 adipocytes). Insulin specific binding competition curves (Figure 1A) were constructed after substraction of nonspecific binding (C = 3.32 ± 1.0, Cd = 3.5 ± 0.5, NS, e.g. % of total binding) indicate diminished insulin specific binding in adipocytes of Cd treated rats. Scatchard curves (Figure 1B) processed by transforming binding data from Figure 1A determine insulin binding capacity at the intercept with axis X. The figure displayes the average curves constructed as the mean of 6 curves from individual rats in each group. Less insulin receptors (insulin binding capacity $R_0/10^6$ cells: C = 335 ± 47 vs. Cd = 168 ± 27 fmol, $P < 0.05$) were detected in adipocytes of cadmium treated rats. The difference remains similar while expressing the density of insulin receptors (number of receptors per square μm), data presented in Table 1.

Immunoblots of fat cells crude plasma membranes (Figure 2A) revealed the presence of GLUT4 protein in the same quantity in both, control and Cd groups. The examination was proved by densitometric measurement of 46 kDa protein bands (Figure 2B).

Discussion

Long lasting elevated cadmium exposure elicits diabetogenic effect (Ghafghazi *et al.* 1977; Merali & Singhal 1980). Harmful cadmium effect on elevated glycemia is the consequence of liver disturbances characterized by enhanced gluconeogenesis (Merali & Singhal 1980), activation of key gluconeogenic enzymes (Chapatwala *et al.* 1982) and reduction of glycolytic enzymes activities (Kielan *et al.* 1989). Glycosuria is produced by kidney damage. In our experimental setting, after six weeks intake of cadmium, we found in overnight fasting rats $\sim 27\%$ increase in serum glucose concentration with moderately elevated insulin level. This combination of the values indicates the presence of disturbances in glucose homeostasis produced either by elevated glucose

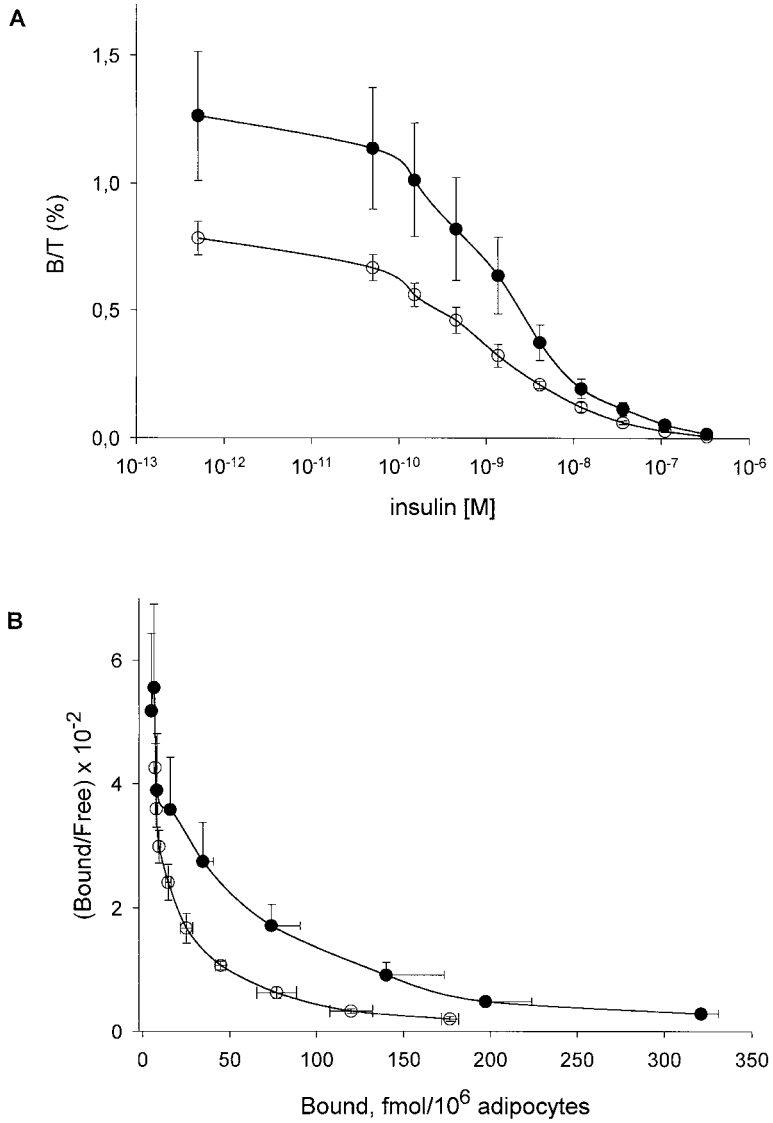

Fig. 1. A – insulin binding displacement curves in isolated adipocytes from control (●) and $CdCl_2$ (◯) treated rats. B – Scatchard plots, transformation of binding curves from Figure 1A. Control group (●), $CdCl_2$ group (◯).

production in liver or decreased glucose metabolism at the periphery. Since insulin receptors in muscles and adipose tissue substantially participate in insulin stimulated glucose lowering effect through stimulated translocation of glucose transporter GLUT4, the investigation of insulin receptors status and GLUT4 protein content in isolated adipocytes was performed. We found less total insulin receptors as well as lower receptor density in adipocytes of cadmium treated animals. The significant reduction in binding sites of Cd group for about $\sim 50\%$ indicate lowered insulin effects, what could substantially contribute to diminished glucose metabolism. Overall cadmium toxicity is characterized by diminished DNA synthesis, DNA strand breaks, inhibition of DNA repair and intracellular membrane damage (Beyersmann & Hechtenberg 1997; Waalkes & Misra 1996; Watanabe & Suzuki 2002). Based on the above data it is likely that the changes at the level of DNA and gene encoding receptor protein could be responsible for synthesis of less insulin receptors due to cadmium presence in fat cells. At now, the study proving this hypothesis is under the investigation. Other mechanisms like increased receptor degradation, not proper insertion of mature recep-

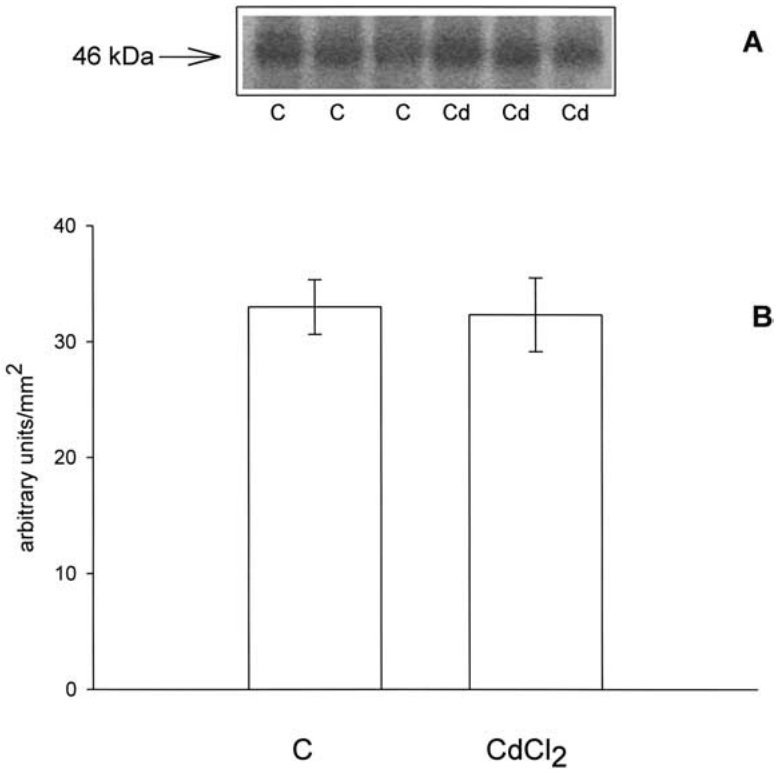

Fig. 2. A – Western blot analysis of GLUT4 protein in crude plasma membranes of adipocytes. Each 46 kDa band corresponds to different rat tested. One representative image of three separate runs is presented. B – Densitometric measurements of GLUT4 protein bands from Figure 2A.

tor into plasma membrane, or increase insulin-receptor complex internalization might also contribute to diminished insulin receptors in isolated fat cells. *In vitro* stimulatory effect of cadmium on glucose transport in isolated adipocytes was observed, but this alteration was not correlated with changes in glucose transporter protein GLUT4 (Ezaki 1989; Harrison *et al.* 1991). Our results obtained by *in vivo* cadmium effect on total basal GLUT4 protein content in adipocyte plasma membranes are consistent with the former results (Ezaki 1989; Harrison *et al.* 1991). Thus it seems unambiguous, that cadmium either *in vitro* or *in vivo* does not modulate GLUT4 protein in plasma membranes of unstimulated adipocytes. This finding does not exclude possible altered insulin stimulated GLUT4 translocation from intra-cellular vesicular storage sites to the plasma membrane in the presence of cadmium. It is expected that due to diminished insulin receptors in adipocytes of our experimental animals, less intracellular signal(s) will evoke translocation of fewer glucose transporter proteins followed by decreased glucose input into the cells. Cd^{2+} interacts at the cell membrane and is taken up by mechanisms that include Ca^{2+} channels and divalent cation transporter (Endo *et al.* 1999). Thus intracellular cadmium content increased on the account of calcium may deteriorate the effect of Ca^{2+} on insulin stimulated GLUT4 translocation and fusion with the plasma membrane (Whitehead *et al.* 2001) and hence contribute to increased glycemia. The precise mechanism of cadmium intervention with GLUT4 in adipocytes calls for further investigation.

The principal finding of the present study – reduction of insulin receptors in rat adipocytes as the result of *in vivo* cadmium effect, could account for observed diabetogenic effect of cadmium. The observations of the present animal study require similar investigation in human adipose tissue with elevated cadmium content as the result either of smoking or occupational exposure.

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