0026-895X/95/010065-09$03.00/0

MOLECULAR PHARMACOLOGY, 47:65-73, 1995.

Differential Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin on the "Adipose-Type" and "Brain-Type" Glucose Transporters in Mice

PHILLIP C. C. LIU and FUMIO MATSUMURA

Department of Environmental Toxicology and the Center for Environmental Health Sciences, University of California, Davis, Davis, California 95616

Received May 13, 1994; Accepted October 13, 1994

SUMMARY

One prominent symptom of acute toxicity from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a loss of adipose tissue and body weight, a phenomenon known as the wasting syndrome. In the current study, we examined the effect of TCDD on glucose transport in mice. A single intraperitoneal dose of TCDD (116 μg/kg) resulted in a time-dependent decrease in transport activity in adipose tissue and brains of C57BL/6 mice. Reduction of transport occurred within 24 hr in both tissues. In adipose tissue a slight recovery was observed by 30 days, but in the brains of treated animals glucose transport was significantly decreased even at the latest time. A comparison of dose-response relationships for several tissues between C57BL/6 (TCDD-responsive) and DBA/2J (TCDD-nonresponsive) mice showed parallel curves, with the C57BL/6 animals showing a 10–20-fold greater sensitivity. The estimated ED_{50} values for reduction of transport in adipose tissue were 50 μg/kg and 800 μg/kg for the C57BL/6 and DBA/2J strains, respectively. Treatment of isolated adipose tissue in culture with TCDD and two biphenyl congeners produced a decrease in transport activity that matched the rank order of aryl hydrocarbon receptor affinity for the compounds. Immunoblotting for the adipose-type (type 4) glucose transporter (GLUT) showed a 40% decrease in the membrane fraction of adipose tissue from C57BL/6 mice treated with 116 μg/kg TCDD for 40 hr. A similar decrease in the brain-type GLUT1 was observed in the plasma membrane fraction of brain tissues isolated from the same animals. Analysis of RNA for the corresponding GLUT4 and GLUT1 genes showed a dramatic decrease in GLUT4 mRNA as early as 24 hr after treatment. In contrast, the level of GLUT1 mRNA increased slightly in the brains of treated mice. We conclude that regulation by TCDD of glucose transport activity in mice is an aryl hydrocarbon receptor-dependent process and that the adipose-type GLUT4 appears to be regulated at the mRNA level, whereas the brain-type GLUT1 is affected mainly at the protein level.

TCDD belongs to a large family of toxic, lipophilic, environmentally stable chemicals, the chlorinated polyaromatic hydrocarbons, which includes polychlorinated BPs, dioxins, furans, and naphthalenes. TCDD is the most potent member of this family and has been widely used as a model chemical to study the mechanisms of action of this class of compounds with biological targets. Exposure to TCDD results in widely varying biological responses that depend on the species, gender, age, and genetic background of the individual. These responses include induction of metabolic enzymes, body weight loss, immunosuppression, skin lesions, and changes in cellular growth and differentiation (reviewed in Ref. 1).

One prominent characteristic of the toxic action of TCDD is a reduction of adipose tissue mass, which has been observed in guinea pigs, mice, rabbits, and monkeys (2, 3). The loss of adipose tissue is accompanied by altered serum lipid profiles, such as hyperlipidemia and hypertriglyceridemia (4, 5). Studies of humans exposed to mixtures of polychlorinated aromatic hydrocarbons including dioxins have indicated associations between lipid irregularities, such as elevated serum cholesterol and triglyceride levels, and exposure to these compounds (6).

The precise mechanism for the loss of adipose tissue remains unknown; however, TCDD appears to interfere with systems involved in the regulation of nutritional homeostasis. Early studies demonstrated that TCDD-induced adipose wasting was not caused by malabsorption of intestinal nutrients or increased energy expenditure (7). Reduced feed intake has been postulated to be responsible for TCDD-induced mortality (8), but hypophagia was not observed in mice even at TCDD doses that caused a decrease in adipose tissue and overall loss in body weight (9). A number of studies have indicated an overall shift in the metabolic capability of TCDD-poisoned animals. Metabolism of [^{14}C]glucose by TCDD-treated rats was lower than for controls (10); however, mitochondrial respiration was not

This work was supported by Research Grants ES05233, ES03575, and ES05707 from the National Institute of Environmental Health Sciences. P.C.C.L. is supported by National Institute of Environmental Health Sciences Predoctoral Training Grant ES07059 from the National Institutes of Health.

ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; GLUT, facilitative glucose transporter; Ah, aryl hydrocarbon; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; BP, biphenyl; LPL, lipoprotein lipase; FABP, fatty acid-binding protein; C57, C57BL/6; DBA, DBA/2J.

changed. Additionally, enzymes involved in the gluconeogenic pathway have been shown to be inhibited by TCDD (11). Levels of hormones, such as insulin and thyroid hormone, that regulate metabolic activity are also affected by TCDD (12), but it is not clear whether these changes are primary effects or responses by the animal to reestablish metabolic homeostasis. Because of the complex changes that occur in TCDD-poisoned animals, we have attempted to characterize early changes in adipose tissue function.

One critical point in metabolic regulation is the transport of glucose into cells. This action determines a number of physiological processes, including insulin secretion, glucose metabolism, and glycogen and fatty acid synthesis. Facilitative glucose transport into cells is mediated by a family of proteins known as the GLUTs. Six distinct genes coding for these protein isoforms have been cloned and found to be expressed in a tissue-specific manner (reviewed in Ref. 13). Recently we observed that TCDD decreases glucose uptake in a dose- and time-dependent manner in the adipose tissue and pancreas of male guinea pigs treated *in vivo* (14, 15).

The current investigation has been undertaken to investigate the biochemical mechanisms by which the GLUT is altered by TCDD poisoning in mice. The objectives of this study are to compare the *in vivo* effect of TCDD on glucose uptake in two inbred strains of mice, i.e., C57, a "TCDD-sensitive" strain, and DBA, a "TCDD-nonresponsive" strain, and to correlate the effect of TCDD on function with changes in GLUT protein and mRNA levels.

Experimental Procedures

Materials. TCDD (>99.9% purity) was a gift from the Dow Chemical Co. (Midland, MI). The BP congeners were obtained from Analabs (North Haven, CT). 3-*O*-Methyl-D-[^{3}H]glucose (7 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). Common buffer salts and biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). 4,7-Phenanthroline was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Care and treatment of animals. Male C57 and DBA mice (6–8 weeks old) were purchased from Simonsen Laboratories (Gilroy, CA). They were housed in pairs in stainless steel cages, with free access to food and water, and were maintained under constant temperature and humidity with a 12-hr light cycle. Animals were acclimated for 1 week before use in experiments; thereafter, they were housed individually. After treatment, food was provided in special containers covered with a wire mesh to minimize spillage. In pair-feeding studies, the amount of food eaten by treated animals was measured 16, 24, 40, and 48 hr after treatment and every 24 hr thereafter. Mice were given a single intraperitoneal injection of TCDD dissolved in corn oil/acetone vehicle (7.5 ml/kg).

Measurement of glucose transport. Glucose transport was assayed as described previously (14), with the following modifications. We used 0.5 μCi of 3-*O*-methyl-D-[^{3}H]glucose for each sample, and nonspecific transport was determined by preincubating samples with 20 μM cytochalasin B at 37° for 30 min.

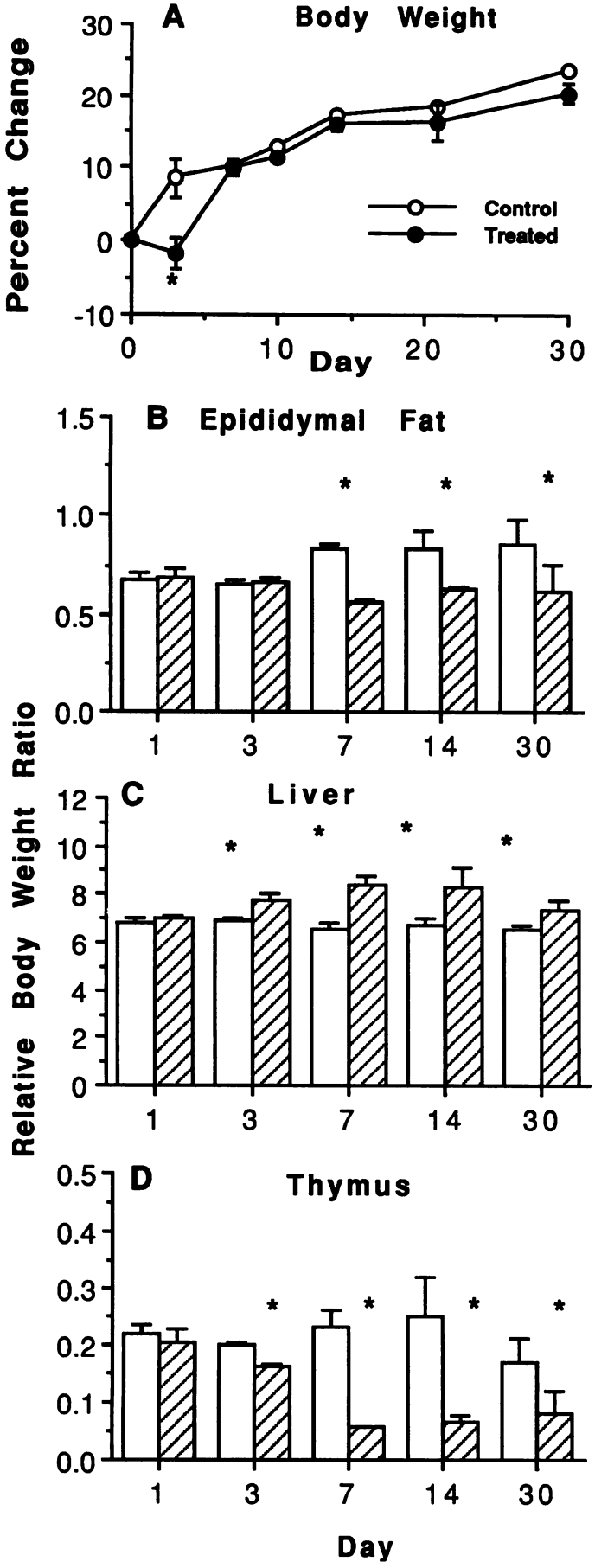
Western blot analysis. Membranes from adipose tissue and brain were isolated as described (15), with minor modifications. Briefly, dissected tissues from C57 mice treated with 116 μg/kg TCDD or with vehicle alone were homogenized in 4 volumes of buffer containing 20 mM HEPES, pH 7.4, 1 mM EDTA, 255 mM sucrose, and a mixture of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, and 2 μg/ml aprotinin). The homogenate was centrifuged at 1000 × *g* to pellet nuclei and whole cells. For total membrane isolation, the supernatant was then centrifuged at 200,000 × *g* for 90 min at 4°, and the pellet was resuspended in 20 mM HEPES/1 mM EDTA. For plasma membrane isolation, the homogenate was first centrifuged at 15,000 × *g* for 20 min at 4°. The pellet was then layered over a cushion of 1.12 M sucrose in HEPES/EDTA buffer and centrifuged at 100,000 × *g* for 1 hr at 4°. The band containing the plasma membranes was removed, pelleted by centrifugation, resuspended in HEPES/EDTA buffer containing 0.25% Triton X-100 and aprotinin, and incubated overnight at 4°, with rotation. Solubilized proteins were separated by centrifugation in a microfuge at 15,000 × *g* for 30 min at 4°. Protein concentrations were determined by the method of Bradford (16), with bovine serum albumin standards.

Protein samples were separated by electrophoresis through a denaturing 10% SDS-polyacrylamide gel (17). After electrophoresis, samples were electroblotted onto a nitrocellulose membrane (Bio-Rad, Richmond, CA) in buffer containing 25 mM Tris·HCl, 192 mM glycine, and 20% methanol, pH 7.4 (18). Before immunoreactions, the membranes were blocked with 5% (w/v) nonfat powdered milk in Tris-buffered saline/Tween (20 mM Tris, 152 mM NaCl, 0.05% Tween-20). Membranes were then incubated with appropriate dilutions of the primary antibody in blocking solution. A rabbit polyclonal anti-GLUT1 antiserum (East Acres Biological, Southbridge, MA) or a rabbit polyclonal anti-GLUT4 antiserum (a gift of Dr. M. Daniel Lane, Johns Hopkins University) was used to probe for the respective forms of the GLUT protein. A horseradish peroxidase-linked goat anti-rabbit antibody was used as the secondary antibody, and the enhanced chemiluminescence system (Amersham) chemiluminescent substrate was used to detect the primary antibody-antigen complexes. After autoradiography, blots were scanned with a densitometer.

Northern blot analysis. Total RNA from tissues was isolated by the single-step acid guanidinium thiocyanate/phenol method of Chomczynski and Sacchi (19). The RNA was fractionated according to size by electrophoresis through a denaturing agarose gel containing 0.66 M formaldehyde. The RNA samples contained ethidium bromide to allow visualization and photography after electrophoresis. The RNA samples were then transferred to a nylon membrane (Schleicher & Schuell, Keene, NH) by capillary blotting; the RNA was cross-linked to the membrane using a UV cross-linker (Fisher model FB-UVXL-1000) and was baked dry at 80°. Membranes were prehybridized for at least 90 min at 65° in a solution containing 1 M NaCl, 100 mM sodium phosphate, pH 6.5, 10 mM EDTA, 1% SDS, 200 μg/ml yeast tRNA, and 200 μg/ml denatured salmon sperm DNA. The DNA probes were labeled using a random priming kit (Pharmacia, Piscataway, NJ) and [^{32}P]-dCTP (3000 Ci/mmol; Amersham). Approximately 1 × 10^6 cpm of probe/ml of hybridizing solution were added and hybridized, with rotation, for 16 hr at 65° in a hybridization oven. Membranes were washed twice at room temperature with 1× standard saline citrate (0.15 M NaCl, 15 mM sodium citrate, pH 7.0)/0.1% SDS and twice under high stringency at 65° with 1× standard saline citrate/0.1% SDS. The blots were autoradiographed with an intensifying screen and quantitated using a radioscanning apparatus (AMBIS, La Jolla, CA).

Amplification of GLUT genes by PCR. Fragments of the GLUT1 and GLUT4 genes were amplified from brain and adipose cDNA, respectively, by PCR. A degenerate forward primer [CA(GA)TT-(CT)GGITA(TC)AACA(TC)IGGIGT] was used in combination with a specific primer for GLUT1 (TCICCIACGTACATGGIAC) or GLUT4 (AGACATGCTCATGGCTGGACC) in the reactions. The conditions for the PCR were denaturation at 94° for 5 min, annealing at 55° for 5 min, and extension at 72° for 1 min; the cycle was repeated 35 times using 1 min for each step, followed by a final elongation period of 10 min at 72°. The identities of the resulting DNAs were confirmed by sequencing of a portion of the DNA fragment, using the method of dideoxynucleotide incorporation (20) and the Sequenase kit, version 2.0 (United States Biochemicals, Cleveland, OH).

Statistical analysis. Data are expressed as means ± standard errors. Pairwise comparisons were made using two-tailed Student's *t* test. Two-tailed analysis of variance was used to compare multiple groups of data. Differences were judged to be significant at $p \leq 0.05$.



Results

The effect of a single dose of 116 μg/kg TCDD on body weight gain in C57 mice is shown in Fig. 1A. At this dose, the overall body weight gain did not differ significantly between the control and treated groups, and only two animals became moribund by the end of the experiment. However, there were significant differences in the weights of individual tissues. Epididymal fat pad weight, in terms of both epididymal fat pad mass and the ratio of epididymal fat pad to total body weight (Fig. 1B), decreased in the TCDD-treated groups beginning on day 7 and remained lower throughout the time course of the experiment. The liver weights (Fig. 1C) in treated groups increased significantly, compared with the control group, over the same period. Thymus weight (Fig. 1D) was markedly lower in the treated group. These data indicate that at this dose, although body weight did not differ between TCDD-treated and control groups because of counterbalanced increases and decreases in different tissues, a significant decrease in adipose tissue occurred. At higher doses, losses in total body weight have been reported in this mouse strain (9, 21).

The effect of a single injection of TCDD on glucose transport over a period of 30 days was assayed in the adipose tissue of the C57 mice. As shown in Fig. 2a, a significant decrease in glucose transport by adipose tissue was apparent 24 hr after the injection and transport remained suppressed for at least 21 days. Nonmoribund survivors showed some recovery in glucose uptake on day 30. The decrease in glucose transport activity cannot be attributed to decreased feeding by TCDD-treated mice, because hypophagia was not observed in these animals (data not shown).

Next we compared the relative sensitivities of male C57 and DBA mice by measuring glucose transport in adipose tissue 24 hr after a single dose of TCDD. In both strains, we found dose-dependent decreases in the rate of glucose transport (Fig. 2b). In C57 mice we observed a significant decrease in adipose tissue at a dose of 12 μg/kg, whereas for DBA mice a significant difference was seen at 116 μg/kg. The estimated ED_{50} values for adipose tissue for the two strains were 50 μg/kg and 800 μg/kg for C57 and DBA mice, respectively, giving a 16-fold difference in sensitivity.

In a previous report (12) we showed that glucose uptake in tissues other than adipose tissue was affected *in vivo* in guinea pigs; therefore, we also measured the *in vivo* effect of TCDD in several other tissues of C57 mice over this time course (Fig. 3). An early decrease in the liver transport activity was observed, followed by recovery to control values. This pattern of effects differed from our previous observations in guinea pigs, in which a transient increase in glucose uptake was found in the liver (14). The significance of these species differences with respect to toxicity is not yet known. One surprising finding is that in the brain glucose transport remained depressed throughout the term of the study.

Fig. 1. Changes in body weight gain and tissue weights of control and TCDD-treated C57 mice. C57 mice received a single injection of 116 μg/kg TCDD in corn oil/acetone or vehicle alone. Mice were weighed every 4–5 days, and the amount of chow eaten was measured every other day. The values represent the mean ± standard error (seven animals), except in the 30-day-treated group (five animals; two moribund animals were removed from the group). Open bars represent control group; hatched bars represent TCDD-treated. *, significant difference from the control group ($p < 0.05$).

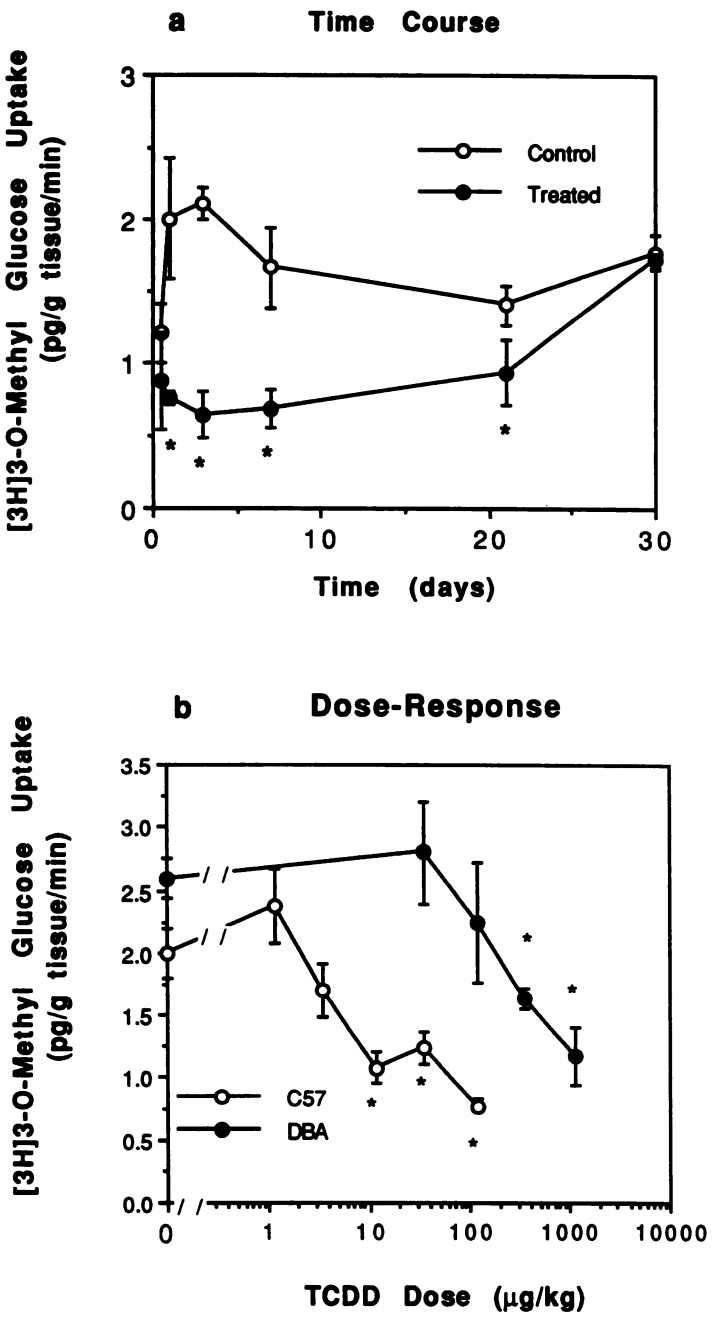


Fig. 2. Effect of TCDD on adipose tissue glucose transport. a, Time course of the effect of TCDD on glucose transport in C57 mouse adipose tissue. C57 mice were treated with a dose of 116 μg/kg TCDD and killed at the specified times. Glucose transport was measured as described in Experimental Procedures, using 0.5 μCi of 3-*O*-methyl-D-[^{3}H]glucose and 13.3 mM D-glucose in each sample. Nonspecific transport was measured by preincubating tissue samples with 20 μM cytochalasin B for 30 min. Each *point* represents the average of five animals, except on day 30, when only three treated animals were used. Tissues from two moribund animals in the 30-day group were not sampled. b, Dose-response curve. C57 and DBA mice were dosed with the TCDD concentrations shown, in corn oil/acetone, whereas controls received vehicle alone. The animals were killed 24 hr after treatment and their adipose tissues were dissected. Each *point* represents the mean ± standard error of three animals of each mouse strain. *, Significant differences from the control group ($p < 0.05$).

Dose-response curves for liver, spleen, and thymus (Fig. 4) in the two strains were parallel, but the DBA curves were shifted to the right, compared with those of the C57 strain, indicating a relatively lower sensitivity to TCDD. The differences in ED_{50} values among these tissues between the two strains varied from 8- to 25-fold. These differences in sensitivity fall within the range reported by other investigators (9, 22) for a number of toxic endpoints in the same strains of mice. This magnitude of interstrain difference in the ED_{50} values for inhibition of glucose uptake by TCDD is consistent with an Ah receptor-mediated effect, because the difference in ligand binding affinity of the Ah receptor from these strains is approximately 10-fold (23).

To further test this hypothesis, we used an adipose tissue culture model to investigate the relative potencies of BP congeners and a putative Ah receptor antagonist in glucose transport. After a 3-hr incubation in culture with each specified agent, adipose tissue was assayed for glucose transport. In culture, TCDD showed an inhibitory effect at a dose as low as 1 nM (Table 1). When the tissue was incubated with 4,7-phenanthroline (a putative receptor antagonist) (24) by itself, there was no difference in transport, compared with the control group; however, a 30-min preincubation with 4,7-phenanthroline followed by a 2.5-hr incubation with a high dose (10 nM) of TCDD blocked, although not completely, the suppressive effect of TCDD on glucose transport. A structure-activity comparison of two BP congeners (coplanar 3,3′,4,4′-BP and noncoplanar 2,2′,5,5′-BP) and TCDD showed that the rank order of potency for glucose uptake suppression followed the rank order of potency for ligand binding of these congeners by the Ah receptor (25). The 3,3′,4,4′-BP suppressed glucose transport at a 100-fold higher dose than required for TCDD. Based on these data, we conclude that the effect of TCDD on glucose transport in adipose tissue and other tissues is dependent upon binding to the Ah receptor.

Because GLUTs are subject to several levels of *in vivo* regulation by hormones and other growth factors, we attempted to determine whether the effect of TCDD on transport activity was caused by a change in GLUT numbers. We examined the level of specific immunoreactive GLUT proteins in membrane fractions from two tissues, adipose and brain. We chose these tissues for examination because both tissues showed a consistent decrease in transport activity after *in vivo* exposure and the GLUT genes and their corresponding gene products have been well characterized in these two tissues. Plasma membranes isolated from the brains of C57 mice treated with 116 μg/kg TCDD for 40 hr showed a smaller amount of 45-kDa protein immunoreactive with an anti-GLUT1 antibody (Fig. 5A). Although we found variations in the amounts of transporter between different preparations of membranes, we consistently observed that TCDD treatment caused a decrease in the amount of immunoreactive protein, compared with the corresponding control, within a given preparation. Densitometric scans of the blots showed a 35% decrease in the intensity of the band corresponding to GLUT1.

Total membranes isolated from the adipose tissue of the same mice also showed a marked decrease in GLUT4, the insulin-regulatable form of the protein (Fig. 5B). Densitometric scanning revealed that the band from the TCDD-treated sample was 40% less intense than that from the corresponding control sample. GLUT4 exists in two cellular compartments

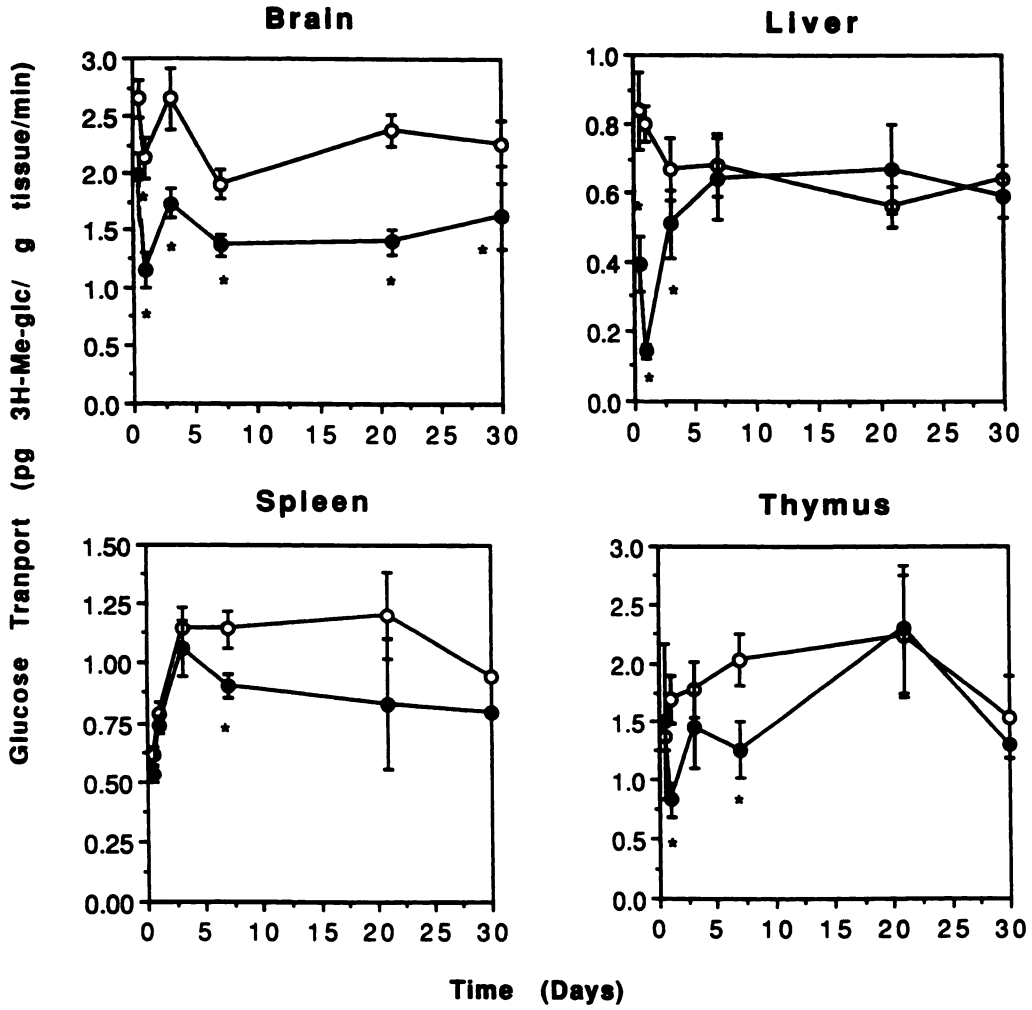


Fig. 3. Time course of the effect of TCDD on glucose transport in C57 mouse tissues. Animals were treated and glucose transport assays were conducted as decribed for Fig. 2. *Me-glc*, 3-*O*-methyl-D-glucose. ○, control; ●, TCDD-treated.

within adipocytes, the plasma membrane and low density microsomes (13). Because we did not separate these two membrane fractions, our experiment does not allow determination of which pool of transporters is affected by TCDD. However, we previously found that the number of transporters is decreased in the plasma membranes of guinea pig adipocytes, as measured by cytochalasin B binding assays (15).

To determine whether the decreases in protein were coincident with decreases in the levels of mRNA, we probed samples of total RNA isolated from brain or adipose tissue with DNA sequences complementary to the GLUT1 (Fig. 6A) and GLUT4 (Fig. 6B) genes, respectively. GLUT1 is found in many tissues, including endothelial cells lining the blood-brain barrier, but is present at only very low levels in adipose tissue (13), whereas GLUT4 is located exclusively in insulin-responsive tissues such as adipose tissue and not in brain (13). This tissue specificity is demonstrated in Fig. 5C. The GLUT1 probe hybridized strongly to a mRNA in the brain sample. We did not detect any GLUT1 in adipose tissue, possibly because it is expressed at very low levels; in rats, it has been estimated that <10% of total transporter protein is GLUT1 (26). The GLUT4 probe detected mRNA only in the adipose tissue sample. A time course experiment after a single dose of TCDD (116 μg/kg) in C57 mice adipose tissue showed that the decrease in GLUT4 mRNA occurred within 24 hr and persisted for at least 7 days. The maximal difference in GLUT4 mRNA levels between control and treated samples occurred between 24 and 72 hr, in accordance with transport activity. In brain there was not a correlation between mRNA levels and either transport activity or membrane GLUT1 levels. GLUT1 mRNA showed no difference between control and treated samples at 24 hr, when the data were normalized to β-actin levels, and then increased slightly in TCDD-treated animals at 72 hr and 1 week, even though transport activity was decreased at those time points.

TCDD was found to significantly decrease the mass of epididymal adipose tissue; therefore, we were interested in determining whether the expression of fat-specific genes, besides GLUT4, was affected at early time points. The data in Fig. 6D indicate that TCDD caused a decrease in the levels of LPL mRNA in a manner similar to that seen for GLUT4. The message for FABP was also decreased, but this became apparent at a much later time (7 days). This reduction of cellular mRNA appeared to be specific for genes involved in differentiated adipocyte function. There was not an overall suppression of mRNA levels or destabilization of message, as in the case of fasted animals (27). In our study, non-fat-specific messages, such as β-actin, were unaffected by TCDD. Additionally, TCDD induced the expression of the *CYP1A1* gene (data not shown).

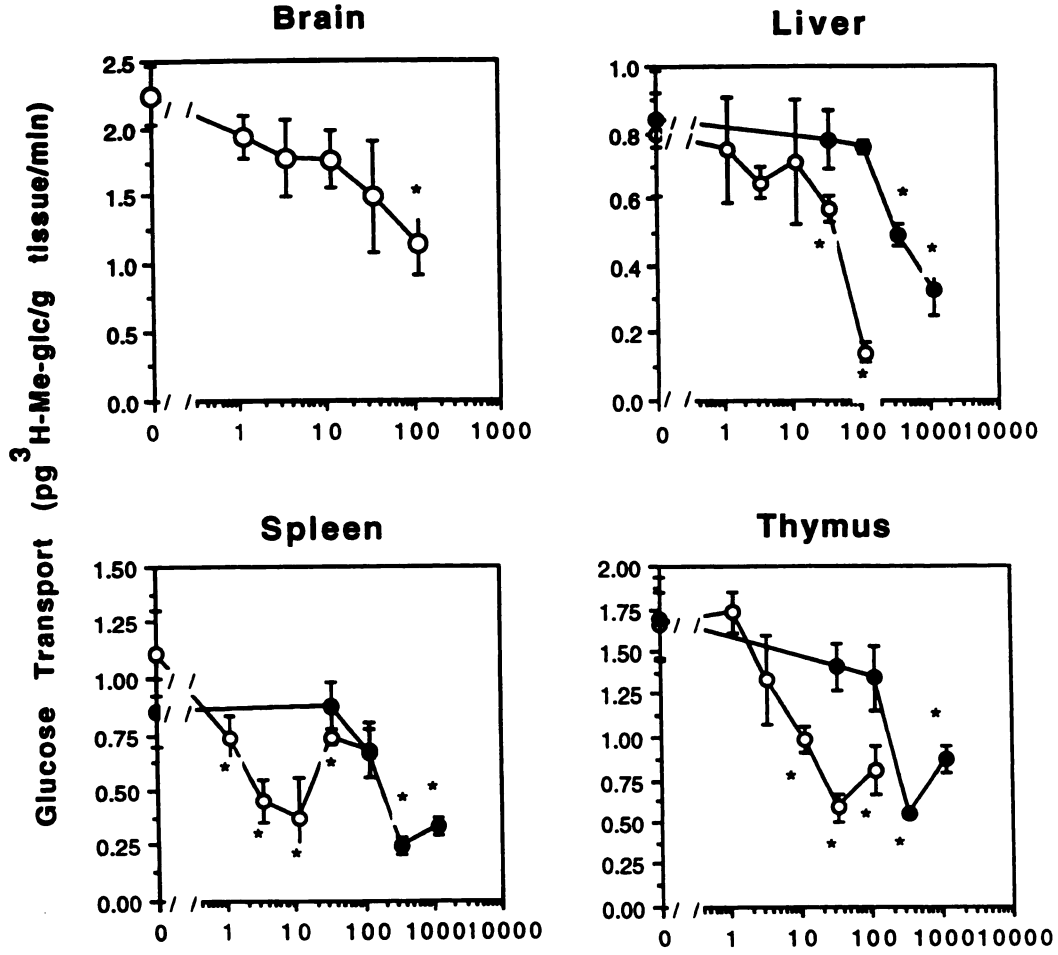


Fig. 4. Comparison of the effect of TCDD on glucose transport in various tissues from C57 and DBA mice. The effects of TCDD dose on glucose uptake by brain (C57 only), liver, spleen, and thymus are shown. The experiments were conducted as described in Fig. 2. ○, C57 strain; ●, DBA strain. Each data point represents the mean ± standard error of three individual mice. *Me-glc*, 3-*O*-methyl-D-glucose.

TABLE 1

Effects of TCDD and BP congeners on glucose transport in C57 mouse adipose tissue

Adipose tissue from untreated mice was dissected and treated in culture (*in situ*) with TCDD or other dioxin congeners, at the specified doses, for 3 hr. After this incubation, tissues were rinsed, and glucose transport assays were performed as described for Fig. 2. The effect of the receptor antagonist 4,7-phenanthroline was tested by adding it 30 min before TCDD addition and then continuing the incubation for an additional 3 hr. The data represent a total of six replicates/sample, from two separate experiments.

Sample	Glucose transport	
	pg of 3-O-methyl-D-[^{3}H]glucose/ g of tissue/min	*% of control*
Control	3.57 ± 0.18	100 ± 5
TCDD (0.1 nM)	3.56 ± 0.20	99 ± 5
TCDD (1 nM)	2.44 ± 0.40[a]	68 ± 11
TCDD (10 nM)	2.11 ± 0.35[a]	59 ± 9
TCDD (10 nM) + 4,7-phenanthroline (1 μM)	3.05 ± 0.54	85 ± 15
4,7-phenanthroline (1 μM)	3.16 ± 0.74	89 ± 20
3,3′,4,4′-BP (1 μM)	2.16 ± 0.36[a]	61 ± 10
3,3′,4,4′-BP (10 nM)	3.48 ± 0.95	97 ± 26
2,2′,5,5′-BP (1 μM)	3.92 ± 0.59	109 ± 16
2,2′,5,5′-BP (10 nM)	4.31 ± 0.14[a]	121 ± 4

[a] Statistically different from control group, using Student's *t* test ($p < 0.05$).

Discussion

In this study we examined the effects of TCDD in C57 mice, with respect to changes in glucose transport activity and GLUT protein and mRNA levels. GLUTs in adipose tissue were affected more severely than were those in brain but, surprisingly, transport function in the brain remained suppressed even at the latest time points. The levels of GLUT1 protein decreased in concordance with transport activity in the brain. Within the brain, GLUT1 is found almost exclusively in endothelial cells that comprise the blood-brain barrier (28). Although glucose flux across the blood-brain barrier is not considered to be rate-limiting for glucose utilization in the brain, the loss of transporters has been associated with several disease states, including type I diabetes (29). It is surprising that levels of GLUT1 mRNA did not change in the same direction as the membrane protein and activity levels in the brain. However, this type of discordance between protein and mRNA levels has been noted in diabetic rats (30). It is not yet known whether the neuronal form of the GLUT (GLUT3) is affected by TCDD.

In contrast to previous studies (8), we did not observe hypophagia in any treatment group. Therefore, in our study decreased feeding was not the cause of the loss of GLUT4 mRNA

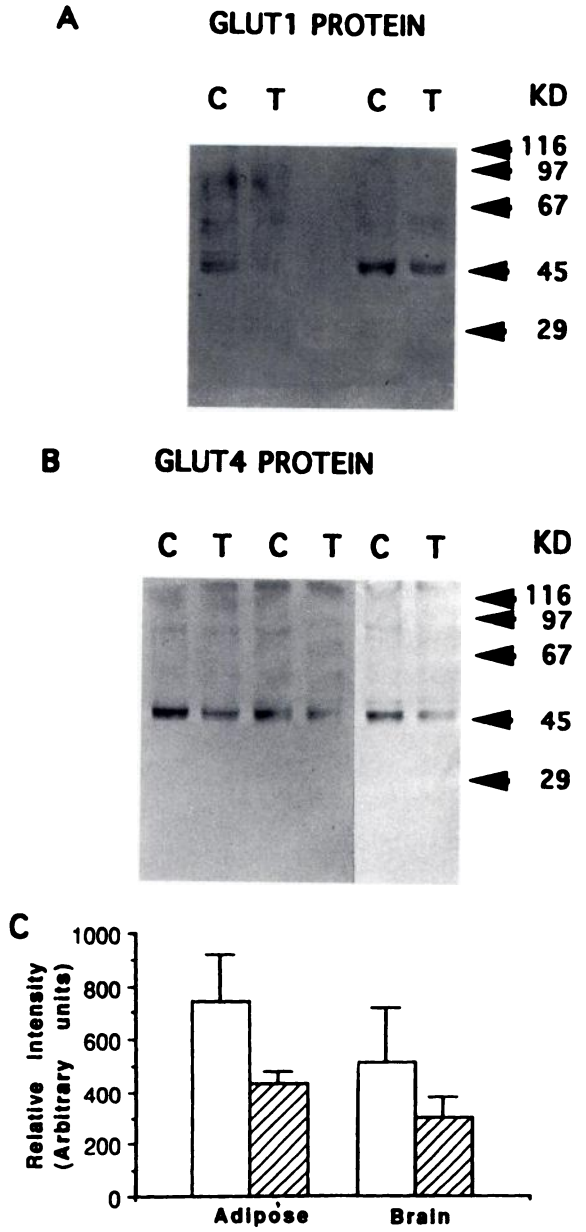


Fig. 5. Comparison of the effect of TCDD on GLUT1 protein levels in brain and GLUT4 protein levels in adipose tissue of C57 mice. A, Plasma membranes were isolated from the brains of control (*C*) or TCDD-treated (116 μg/kg, 40 hr) (*T*) mice. Protein samples (15 μg/lane) were electrophoresed, blotted onto nitrocelluose membranes, and subjected to immunoblotting using a polyclonal anti-GLUT1 antiserum primary antibody, as described in Experimental Procedures. Each pair of control and treated samples represents a separate membrane preparation experiment. *Numbers to the right*, molecular weight standard proteins. B, Immunoblots of the total membrane fraction of adipose tissue isolated from mice treated as described for A are shown. A polyclonal anti-GLUT4 antibody was used to detect the adipose-type GLUT. Each lane represents pooled adipose tissue from three mice. C, Densitometric analysis of the immunoblots is shown. The mean values and ranges of three separate experiments for adipose tissue and two experiments for brain are shown.

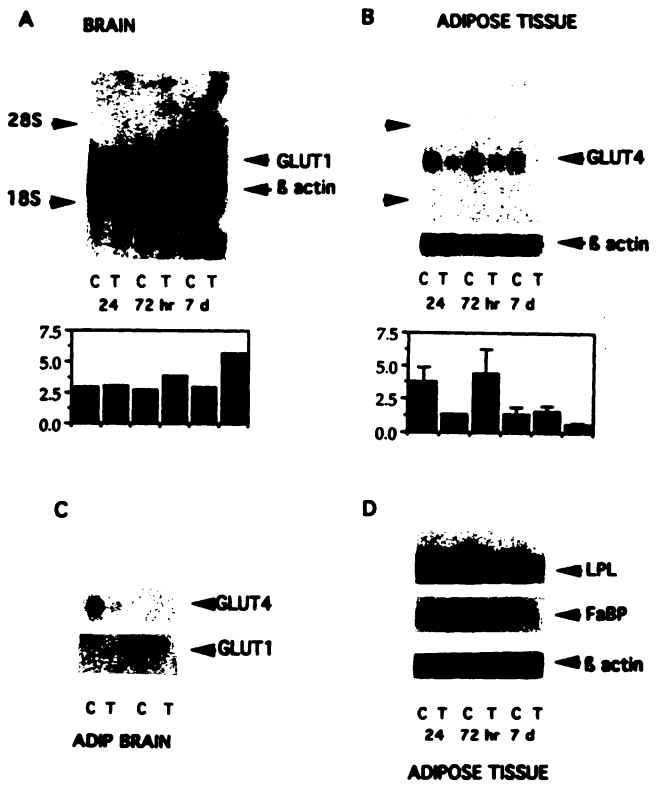


Fig. 6. Northern blot analysis of GLUT mRNA in control and TCDD-treated C57 mice. A, Time course of effect of TCDD treatment on GLUT1 mRNA levels in the brain. Mice were treated with 116 μg/kg TCDD in acetone/corn oil (*T*) or with vehicle alone (*C*) and were killed at the specified times. Twelve micrograms of total RNA were electrophoresed and blotted onto nylon membranes as described in Experimental Procedures. Blots were hybridized to ^{32}P-labeled cDNA probes for GLUT1 and β-actin, which was used to normalize loading inequalities. The amount of radioactivity was quantitated by radioimaging scanning (AMBIS) and is depicted in the histographs. The units are (GLUT1 cpm/β-actin cpm) × 10. B, Time course of effect of TCDD treatment on GLUT4 mRNA levels in adipose tissue isolated from the same mice as described in A. A ^{32}P-labeled cDNA probe for murine GLUT4 or β-actin was used. The quantitation of blots by radioimaging in two independent experiments is shown in the histograph. The units are (GLUT4 cpm/β-actin cpm) × 10. C, Demonstration of the specificity of the GLUT1 and GLUT4 cDNA probes. The blot was hybridized sequentially with the GLUT1, GLUT4, and β-actin probes. D, Blots of adipose tissue RNA from the time course experiment (Fig. 6B), probed sequentially with cDNAs for LPL, FABP, and β-actin.

in adipose tissue. Moreover, pair-fed control animals showed expression of GLUT4 mRNA nearly equal to that of controls fed *ad libitum*, whereas treated animals had virtually undetectable levels of the message (data not shown). The timing of the loss of GLUT4 mRNA agrees well with the functional assay data; however, our data show a difference in the quantitative decreases in protein and mRNA levels of GLUT4. A potential explanation may be the significantly longer half-life (>40 hr) of the GLUT4 protein (31), compared with the half-life (6–12 hr) of the mRNA (32). Even complete blockage of transcription may not result in a corresponding decrease in protein content until much later. This discrepancy is consistent with prior observations that diabetogenic agents and fasting also exert greater effects on mRNA levels than on membrane protein levels in rat adipose tissue (30).

Mobilization of fat from adipose tissue, leading to decreased adipose tissue weight, has been previously observed in mice by several other investigators (9, 21). The decreased levels of GLUT4 and LPL mRNA are the first examples of decreased gene expression in adipose tissue after TCDD toxicity. The relatively quick response of these two genes further documents that adipose tissue is a primary target of the toxic effects of

TCDD. Transport of glucose into fat cells is critical for fatty acid synthesis, because glycerol-3-phosphate is derived mainly from glucose metabolism in adipose tissue. Therefore, we postulate a model to explain changes in adipose tissue function at early time points, as follows. The earliest biochemical effects we have observed in adipose tissue after TCDD exposure are decreases in LPL (33) and GLUT4 activities. The suppression of these activities may impair the ability of adipocytes to synthesize and store triglycerides from available fatty acids in the serum. As the level of fatty acids decreases in the adipocytes, genes for other fat-specific proteins, such as FABP, may also be down-regulated in response. These actions, combined with the overall shift in metabolism from carbohydrates to fats (34, 35), may lead to significant loss of tissue mass in fat pads.

It was recently reported that hepatic phosphoenol pyruvate carboxykinase mRNA is decreased after 4 days in rats treated with a dose of 125 μg/kg TCDD (36). Taken together with our results, these data suggest that there is a second class of genes related to energy metabolism, whose expression is decreased by TCDD poisoning and which stand in opposition to the xenobiotic-metabolizing "Ah battery" of genes whose expression is induced by TCDD directly through interactions of the Ah receptor with dioxin-responsive enhancer elements (37). How these energy-metabolizing genes are regulated by TCDD remains to be determined.

The mechanism by which TCDD reduces GLUT4 mRNA levels in adipose tissue appears, like many other documented effects of TCDD, to depend on the Ah receptor. Our data showing the 8–25-fold difference in sensitivity between the C57 and DBA strains, together with the structure-activity data, are consisent with a mechanism of action that is dependent upon TCDD binding to the Ah receptor. The C57 and DBA mice are different at multiple loci other than the *Ah* locus (22), but a recent study (21) of the effects of TCDD on organ weights and lipid parameters in congenic C57 mice differing only at the *Ah* locus found good agreement between the results from the congenic mice and results from earlier C57 and DBA interstrain comparisons (9).

Although our data suggest that TCDD affects expression of the GLUT4 gene, we cannot conclude that this effect is directly at the level of DNA. TCDD may indirectly affect GLUT by changing the level or activity of an endogenous regulator of the GLUT gene, such as insulin. This possibility is not likely in the case of isolated cultures of adipose tissue, which retain sensitivity to TCDD (Table 1 and Ref. 14). Moreover, in previous studies of rats hypoinsulinemia was not seen until 7 days after treatment (12), whereas the decrease in GLUT4 was quite apparent by 24 hr in this study. Finally, chronic hyperglycemia, which would be expected as a result of hypoinsulinemia, has not been reported in mice; rather, a decrease (9) or no change (21) in serum glucose levels has been observed. Other hormones and modulators of glucose transport, such as growth factors and cytokines, may warrant further investigation.

Another potential explanation is that TCDD may also affect the regulation of these proteins, particularly GLUT1, by post-transcriptional modification. The GLUTs have been shown to be phosphorylated by both cAMP-dependent and Ca^{2+}-dependent protein kinases *in vitro*, but direct phosphorylation has not been correlated with changes in transporting activity (38). Transporting activity in isolated adipoctyes has been shown to increase in the presence of okadaic acid, a phosphatase inhibitor (39), suggesting that phosphorylation may indirectly affect transporter regulation through other regulatory proteins. For example, the signal transduction pathway of insulin to the GLUT involves phosphorylation, but specific kinases and their substrates in this chain of events have yet to be fully elucidated. It has been reported that phoshorylation of specific proteins in adipose tissue is altered by TCDD (40), but whether these phosphoproteins are involved in glucose transport or fat metabolism remains to be determined.

Aside from diabetogenic agents such as streptozotocin, few exogenous chemicals have been reported to decrease the level of GLUT proteins or gene expression. Thus, the ability of TCDD to impair the function and regulation of this system may provide a biochemical probe for elucidating the mechanism of TCDD-induced perturbations in metabolic homeostasis. Further investigations into this mechanism may also yield clues for understanding the development of diabetic-like symptoms after TCDD exposure.

Acknowledgments

We thank Dr. Howard Green (Harvard Medical School) for sharing plasmids containing the β-actin and FABP cDNAs and Dr. M. Daniel Lane (Johns Hopkins University) for providing the anti-GLUT4 antibody. We appreciate the expert technical assistance of Ms. Debra Dunlap. We thank Dr. Essam Enan and Dr. Michael S. Denison for helpful discussions and Dr. Marjorie Phillips for critical reading of the manuscript.

References

1. Poland, A., and J. C. Knutson. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: examinations of their mechanisms of toxicity. *Annu. Rev. Pharmacol. Toxicol.* **22**:517–554 (1982).
2. Gupta, B. H., J. G. Vos, J. A. Moore, J. G. Zinkl, and B. C. Bullock. Pathologic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in laboratory animals. *Environ. Health Perspect.* **5**:125–150 (1973).
3. McConnell, E. E., J. A. Moore, J. K. Haseman, and M. W. Harris. The comparative toxicity of chlorinated dibenzo-*p*-dioxins in mice and guinea pigs. *Toxicol. Appl. Pharmacol.* **44**:335–356 (1978).
4. Gasiewicz, T. A., and R. A. Neal. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin tissue distribution, excretion, and effect on clinical chemical parameters in guinea pigs. *Toxicol. Appl. Pharmacol.* **51**:329–339 (1979).
5. Brewster, D. W., and F. Matsumura. TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) reduces lipoprotein lipase activity in the adipose tissue of the guinea pig. *Biochem. Biophys. Res. Commun.* **122**:810–817 (1984).
6. Walker, A. E., and J. V. Martin. Lipid profiles in dioxin-exposed workers. *Lancet* **1**:446–447 (1979).
7. Neal, R. A., P. W. Beatty, and T. A. Gasiewicz. Studies on the mechanisms of toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *Ann. N. Y. Acad. Sci.* **320**:204–213 (1979).
8. Kelling, C. K., B. J. Christian, S. L. Inhorn, and R. E. Peterson. Hypophagia-induced weight loss in mice, rats, and guinea pigs treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Fundam. Appl. Toxicol.* **5**:700–712 (1985).
9. Chapman, D. E., and C. M. Schiller. Dose-related effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in C57BL/6J and DBA/2J mice. *Toxicol. Appl. Pharmacol.* **78**:47–157 (1985).
10. Weber, L. W. D., H. Greim, and K. Rozman. Metabolism and distribution of [^{14}C]glucose in rats treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *J. Toxicol. Environ. Health* **22**:195–206 (1987).
11. Weber, L. W. D., M. Lebofsky, H. Greim, and K. Rozman. Key enzymes of gluconeogenesis are dose-dependently reduced in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats. *Arch. Toxicol.* **65**:119–123 (1991).
12. Goraki, J. R., and K. Rozman. Dose-response and time course of hypothyroxinemia and hypoinsulinemia and characterization of insulin hypersensitivity in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats. *Toxicology* **44**:297–307 (1987).
13. Burant, C. F., W. I. Sivitz, H. Fukumoto, T. Kayano, S. Nagamatsu, S. Seino, J. E. Pessin, and G. I. Bell. Mammalian glucose transporters: structure and molecular regulation. *Recent Prog. Horm. Res.* **47**:349–388 (1991).
14. Enan, E., P. C. C. Liu, and F. Matsumura. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin causes reduction of glucose transporting activities in the plasma membrane of adipose tissue and pancreas from the guinea pig. *J. Biol. Chem.* **267**:19785–19791 (1992).
15. Enan, E., P. C. C. Liu, and F. Matsumura. TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) causes reduction of glucose uptake through glucose transporters on the plasma membrane of the guinea pig adipocyte. *J. Environ. Sci. Health B Pestic. Food Contam. Agric. Wastes* **27**:495–510 (1992).
16. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).

17. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–682 (1970).
18. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
19. Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159 (1987).
20. Sanger, F., S. Nicklen, and A. R. Coulson. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467 (1977).
21. Birnbaum, L. S., M. M. McDonald, P. C. Blair, A. M. Clark, and M. W. Harris. Differential toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in C57BL/6J mice congenic at the *Ah* locus. *Fundam. Appl. Toxicol.* **15**:186–200 (1990).
22. Poland, A., and E. Glover. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: segregation of toxicity with the *Ah* locus. *Mol. Pharmacol.* **17**:86–94 (1980).
23. Okey, A. B., L. M. Vella, and P. A. Harper. Detection and characterization of a "low affinity" form of cytosolic Ah receptor in livers of mice "nonresponsive" to induction of cytochrome P1–450 by 3-methylcholanthrene. *Mol. Pharmacol.* **35**:823–830 (1989).
24. Mahon, M. J , and T. A. Gasiewicz. Chelatable metal ions are not required for aryl hydrocarbon receptor transformation to a DNA binding form: phenanthrolines are possible competitive antagonists of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Arch. Biochem. Biophys.* **297**:1–8 (1992).
25. Safe, S., S. Bandiera, T. Sawyer, B. Zmudzka, G. Mason, M. Romkes, M. A. Denomme, J. Sparling, A. B. Okey, and T. Fujita. Effects of structure on binding to the 2,3,7,8-TCDD receptor protein and AHH induction: halogenated biphenyls. *Environ. Health Perspect.* **61**:21–33 (1985).
26. Zorzano, A., W. Wilkinson, N. Kotliar, G. Thoidis, B. E. Wadzinkski, A. E. Ruoho, and P. F. Pilch. Insulin-regulated glucose uptake in rat adipocytes is mediated by two transporter isoforms present in at least two vesicle populations. *J. Biol. Chem.* **264**:12358–12363 (1989).
27. Kahn, B. B., S. W. Cushman, and J. S. Flier. Regulation of glucose transporter-specific mRNA levels in rat adipose cells with fasting and refeeding. *J. Clin. Invest.* **83**:199–204 (1988).
28. Pardridge, W. M., R. J. Boado, and C. R. Farrell. Brain-type glucose transporter (GLUT-1) is selectively localized to the blood-brain barrier. *J. Biol. Chem.* **265**:18035–18040 (1990).
29. Choi, T. B., R. J. Boado, and W. M. Pardridge. Blood-brain barrier glucose transporter mRNA is increased in experimental diabetes mellitus. *Biochem. Biophys. Res. Commun.* **164**:375–380 (1989).
30. Camps, M., A. Castello, P. Munoz, M. Monfar, X. Testar, M. Palacin, and A. Zorzano. Effect of diabetes and fasting on GLUT-4 (muscle/fat) glucose-transporter expression in insulin-sensitive tissues. *Biochem. J.* **282**:765–772 (1992).
31. Sargeant, R. J., and M. R. Paquet. Effect of insulin on the rates of synthesis and degradation of GLUT1 and GLUT4 glucose transporters in 3T3-L1 adipocytes. *Biochem. J.* **290**:913–919 (1993).
32. Kaestner, K. H., J. R. Flores-Riveros, J. C. McLenithan, M. Janicot, and M. D. Lane. Transcriptional repression of the mouse insulin-responsive glucose transporter (GLUT4) gene by cAMP. *Proc. Natl. Acad. Sci. USA* **88**:1933–1937 (1991).
33. Brewster, D. W., and F. Matsumura. Reduction of adipose tissue lipoprotein lipase activity as a result of *in vivo* administration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to the guinea pig. *Biochem. Pharmacol.* **37**:2247–2253 (1988).
34. Muzi, G., J. R. Gorski, and K. Rozman. Mode of metabolism is altered in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats. *Toxicol. Lett.* **47**:77–86 (1989).
35. Lentnek, M., O. W. Griffith, and A. B. Rifkind. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin increases reliance on fats as a fuel source independently of diet: evidence that diminished carbohydrate supply contributes to dioxin lethality. *Biochem. Biophys. Res. Commun.* **174**:1267–1271 (1991).
36. Stahl, B. U., D. G. Beer, L. W. D. Weber, and K. Rozman. Reduction of hepatic phosphoenolpyruvate carboxykinase (PEPCK) activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is due to decreased mRNA levels. *Toxicology* **79**:81–95 (1993).
37. Sutter, T. R., and W. F. Greenlee. Classification of members of the *Ah* gene battery. *Chemosphere* **25**:223–226 (1992).
38. Schurmann, A., G. Mieskes, and H. G. Joost. Phosphorylation of the adipose/muscle-type glucose transporter and its relationship to glucose transport activity. *Biochem. J.* **285**:223–228 (1992).
39. Lawrence, J. C., J. F. Hiken, and D. E. James. Stimulation of glucose transport and glucose transporter phosphorylation by okadaic acid in rat adipoctyes. *J. Biol. Chem.* **265**:19768–19776 (1990).
40. Enan, E., and and F. Matsumura. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin induced alterations in protein phosphorylation in guinea pig adipose tissue. *J. Biochem. Toxicol.* **8**:89–99 (1993).

Send reprint requests to: Fumio Matsumura, Department of Environmental Toxicology, University of California, Davis, Davis, CA 95616.