2000

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Digital Object Identifier (DOI)
10.1093/toxsci/56.2.431

TOXICOLOGICAL SCIENCES **56,** 431–436 (2000)

Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) Is Associated with Hyperinsulinemia and Insulin Resistance

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Received January 12, 2000; accepted April 11, 2000

High exposures of Vietnam veterans to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, a dioxin contained in the herbicide mixture Agent Orange, have previously been demonstrated to be associated with an increased prevalence of diabetes and hyperinsulinemia in non-diabetic subjects. Sixty-nine persons were identified who were in good health and had normal glucose levels during glucose tolerance testing. These subjects lived within 25 miles of the Vertac/Hercules Superfund site located in Jacksonville, Arkansas. The blood sera lipid concentrations of TCDD for the 69 subjects ranged between 2 and 94 ppt. When subjects with blood sera lipid TCDD levels in the top 10% (TCDD > 15 ppt, $n = 7$) were compared to subjects with lower levels (2–15 ppt, $n = 62$), there were no group differences in age, obesity, gender distribution, total lipids, or glucose levels. However, plasma insulin concentrations, at fasting and 30, 60, and 120 min following a 75 g glucose load, were significantly higher in the group with high blood TCDD levels. These finding could not be explained by other known risk factors for hyperinsulinemia. The finding of the TCDD-hyperinsulinemia relationship is consistent with studies of Vietnam veterans and suggests that high blood TCDD levels may cause insulin resistance.

Key Words: **2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Agent Orange; hyperinsulinemia; diabetes; insulin resistance; glucose tolerance; tumor necrosis factor-α.**

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), often referred to as dioxin, is formed as a byproduct of many industrial processes. Probably all persons living in industrialized countries have been exposed to TCDD via the food supply, especially meats, eggs, and dairy products. TCDD has a high solubility in lipid and a half-life estimated to be between 7 and 9 years (Geyer *et al*., 1993; Michalek *et al*., 1996) in humans.

Several studies have reported an association between TCDD exposure and various components of impaired carbohydrate metabolism (Henriksen *et al*., 1997; Pazderova-Vejlupkova *et al*., 1981; Sweeney *et al*., 1992). In one study, 55 workers with heavy industrial exposure to TCDD were followed for 10 years (Pazderova-Vejlupkova *et al*., 1981). Many workers developed chloracne, porphyria cutanea tarda, and hepatic steatosis, and approximately 40% yielded abnormal results when administered glucose tolerance tests. A study of TCDD-exposed U.S. industrial workers found an increased mean TCDD level in diabetic workers compared with non-diabetic workers (Sweeney *et al*., 1992).

To date, the most robust epidemiological study reporting an association between TCDD levels and diabetes is a prospective study of Air Force veterans who were part of Operation Ranch Hand, the unit responsible for aerial spraying of Agent Orange in Vietnam (Henriksen *et al*., 1997). Veterans with high blood TCDD levels demonstrated a greater prevalence of diabetes and a shorter time to onset of diabetes, when compared to veterans with low blood TCDD levels. Non-diabetic veterans with high blood TCDD levels were more likely to be hyperinsulinemic, suggesting that the hyperinsulinemia was the result of insulin resistance. Insulin resistance and hyperinsulinemia can be due to many different conditions, and place individuals at higher risks for the development of type 2 diabetes (Mitchell *et al*., 1992).

The Vertac-Hercules Superfund site is located in Jacksonville, Arkansas. The plant manufactured pesticides from 1948 until its closing in 1986. 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T), the TCDD-containing component of Agent Orange, was manufactured until 1979. Inadequate waste disposal methods and production controls resulted in widespread and persistent TCDD contamination of local streams, parks, and yards (Cranmer *et al*., 1994). In this report, we studied subjects from the neighborhood adjacent to the Vertac site with the intention of determining whether TCDD-exposed subjects manifested evidence of hyperinsulinemia.

METHODS

Study subjects. A previous exposure study had examined blood sera lipid levels of TCDD in 177 persons. Some of these subjects lived near the Vertac/Hercules Superfund site, while others lived approximately 25 miles away, in Mabelvale, Arkansas (Cranmer *et al*., 1994). The range of blood sera

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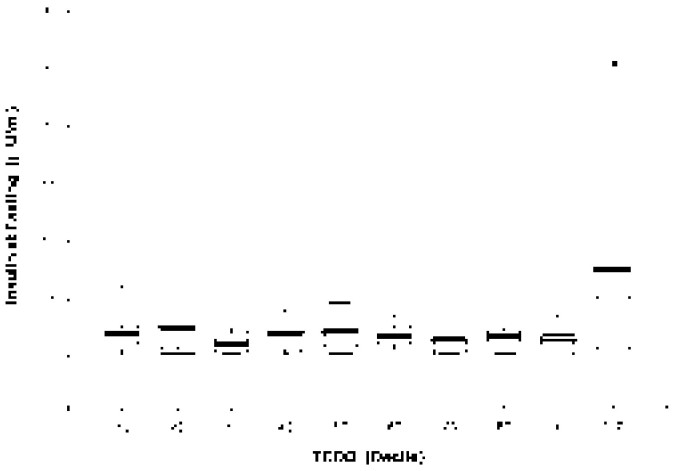

FIG. 1. Fasting insulin values in each TCDD decile. The 69 subjects were divided into deciles according to their TCDD levels. Each group contained 7 subjects, except the 10th percentile group, which contained 6 subjects. The fasting insulin values for the subjects in each decile are shown. The plots are composed of bars and boxes. The top and bottom bars represent the 95th and 5th percentiles, respectively. The top of the box is the 75th percentile and the bottom of the box is the 25th percentile. Within the boxes, the wide bar is the mean, and the thin bar is the median. *$p < 0.05$ using Kruskal-Wallis one way ANOVA on ranks.

lipid TCDD levels varied considerably among these subjects (range: 2 to 95 ppt). These 177 persons served as the pool from which the subjects reported on herein were selected. Although only subjects living close to the Superfund site had very high blood sera TCDD levels, many near-site subjects had normal levels.

Participants in the exposure study (Cranmer *et al*., 1994) were re-sampled and measured for TCDD levels in 1991, 1994, and 1995. Blood sera lipid TCDD levels remained remarkably constant during this four year span (Cranmer *et al*., 2000), suggesting that TCDD body burdens remained reasonably constant for the study group.

Study design and subject recruitment. The intention of the present study was to determine, in subjects with a normal glucose response to a glucose tolerance test, whether persons with high levels of blood sera lipid TCDD were at an increased risk of hyperinsulinemia. Persons reporting either a history of diabetes or previous treatment with oral hypoglycemic drugs or insulin were excluded. Routine laboratory tests were performed to exclude subjects with subclinical hepatic, renal, thyroid, or other chronic diseases. A total of 69 healthy subjects with normal glucose metabolism by glucose tolerance testing and known TCDD levels met the study entry selection criteria, volunteered, and were evaluated for hyperinsulinemia by glucose tolerance testing.

Oral glucose tolerance tests (OGTTs). All subjects fasted overnight prior to the OGTT. The OGTTs were initiated prior to 7:30 A.M., and utilized a 75 g glucose challenge. Measurement of plasma glucose and insulin were made at 0 time (pre-challenge) and 30, 60, and 120 min after challenge.

Clinical laboratory methods. Serum glucose was measured by the glucose oxidase method (Beckman Synchron), and serum insulin was measured by radioimmunoassay (Count-A-Count®) and expressed as μIU/ml.

Analytical laboratory methods. Blood lipid TCDD was measured in collaboration with the Centers for Disease Control and Prevention (CDC, Atlanta). The CDC laboratory used high-resolution gas chromatography with high-resolution mass spectrometric analysis (Patterson *et al*., 1987). The CDC laboratory also conducted TCDD measurements on the blood of veterans participating in the Air Force Health Study (Henriksen *et al*., 1997).

Statistical analysis. Estimates of the total amount of insulin released during the OGTT were calculated for the entire 2 h period by the Area Under the Curve (AUC) Method, and expressed as μIU/ml-hr. Statistical and data analyses were performed with programs included in Sigmastat (Jandel Scientific, San Rafael, CA).

RESULTS

As described in the Materials and Methods section, 69 subjects met the study criteria which included: no history of diabetes or glucose intolerance; no drug use known to influence glucose or insulin levels; the demonstration of a normal fasting glucose; and normal glucose levels after a 75 g glucose challenge. Normal glucose tolerance included a fasting glucose of < 110, and a 2 h glucose of < 140. Glucose and insulin levels were obtained for samples taken at 0 (fasting) and 30, 60, and 120 min after challenge with glucose. Risk factors for hyperinsulinemia (including age, gender, body mass index [BMI], and total lipids) were determined.

Because all subjects have measurable blood sera TCDD levels, we hypothesized that a dose-response relationship may exist between blood TCDD levels and insulin resistance. Hence, we divided the range of TCDD levels into deciles, and examined fasting insulin levels in each decile (6–7 subjects) of blood TCDD. Figure 1 plots fasting insulin levels versus TCDD by decile on an arithmetic scale. In none of the lowest nine deciles were the mean fasting insulin levels greater than 2.5 μIU/ml. However, subjects with the highest decile of TCDD (corresponding to a TCDD >15 ppt) had significantly higher fasting plasma insulin (mean 7.0 μIU/ml) than any other group ($p < 0.05$; Kruskal-Wallis one way ANOVA on ranks). Therefore, a high TCDD level (i.e., a TCDD level associated with higher fasting insulin levels) was defined as above the

TABLE 1
Clinical and Biochemical Characteristics of Subjects when Divided According to TCDD Levels

	TCDD > 15 ppt $n = 7$	TCDD < 15 ppt $n = 62$
Age (years)	55 ± 11.6	51 ± 13.2
Gender M:F	3:4 (0.75)	25:37 (0.73)
BMI	28 ± 4.3	27 ± 4.4
Total Lipids (mg/dl)	677 ± 79.8	624 ± 127
Glucose (mg/dl)		
Fasting	103 ± 5.7	100 ± 8.6
30 min	149 ± 18.4	146 ± 28.7
60 min	134 ± 39.6	138 ± 34.5
120 min	118 ± 23.7	110 ± 28.5
Insulin (μIU/ml)		
Fasting	7.0 ± 8.4*	2.0 ± 2.5
30 min	412 ± 780*	79 ± 113
60 min	325 ± 317*	100 ± 159
120 min	294 ± 431*	65 ± 166

Note. Subjects were divided according to the highest decile of TCDD (> 15 ppt) and lower TCDD levels (< 15 ppt). All data are mean ± S.D; *$p < 0.05$ vs. TCDD < 15 ppt group, Student's ^{t}-test and Mann Whitney Rank Sum test.

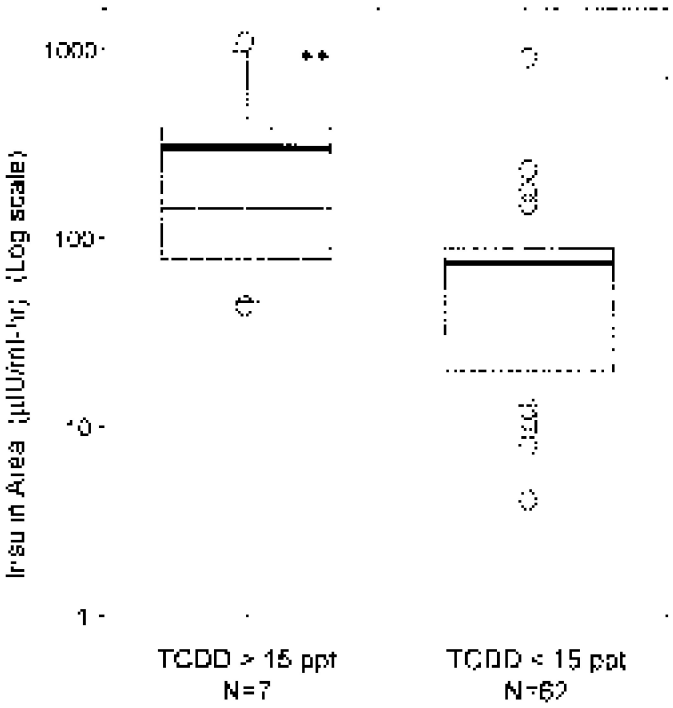

FIG. 2. Total insulin area under the curve in subjects with high TCDD (> 15 ppt) versus all other subjects. See legend to Figure 1 for descriptions of the bars and boxes. The plots are composed of bars and boxes. The top and bottom bars represent the 95th and 5th percentiles, respectively. The top of the box is the 75th percentile and the bottom of the box is the 25th percentile. Within the boxes, the wide bar is the mean, and the thin bar is the median. $^{**}p < 0.05$ using Kruskal-Wallis one way ANOVA on ranks.

90th percentile of the 69 persons participating and corresponding to a level > 15 ppt. Persons with TCDD levels below 15 ppt were classified as normal.

Because fasting hyperinsulinemia could be due to other factors, we examined the characteristics of subjects in the groups with TCDD levels more than or less than 15 ppt (Table 1). There were no statistically significant differences between the two groups for gender distribution, age, BMI, or total lipids. There were no differences in fasting blood glucose, or glucose levels after a 75 g glucose challenge. However, subjects with high TCDD levels (> 15 ppt) had significantly higher insulin levels at all time points following the glucose tolerance test.

Figure 2 presents total insulin levels, estimated by the total AUC method, for the entire two-hour glucose tolerance test for the TCDD groups with > 15 ppt and < 15 ppt. Once again, the top 10% TCDD group (> 15 ppt) had a significantly higher insulin AUC than the group with TCDD less than 15 ppt ($p < 0.05$; Kruskal-Wallis one way ANOVA on ranks).

Among subjects without diabetes or impaired glucose tolerance, fasting and post oral glucose insulin levels vary considerably, and reflect the ability of the pancreas to compensate for the prevailing insulin resistance. Insulin levels at each time point (fasting and 30, 60, and 120 min after consuming a 75 g glucose challenge) were ranked in order to analyze the data in terms of the likelihood of having a high insulin level. The 90th percentiles of insulin levels were identified for each time point. Insulin levels at or above the 90th percentile were designated as high. Values determined to be high were: fasting > 4.5 μIU/ml; 30 min > 177 μIU/ml; 60 min > 228 μIU/ml; and 120 min > 97.7 μIU/ml.

Table 2 provides the odds ratio for high insulin during the glucose tolerance test. The odds of high fasting insulin were 8.5-fold greater in subjects with a high TCDD level of > 15 ppt than in subjects with a low level of TCDD (< 15 ppt). In addition, among subjects with a TCDD level > 15 ppt, the odds of high insulin at 30 min, 60 min, and 120 min were 7-fold, 12-fold, and 56-fold, respectively, greater than the odds among those subjects with a lower level of TCDD (Table 2). All excess odds ratios were statistically significant and the 95% confidence limits did not include 1 ($p < 0.05$; Fisher Exact Test).

DISCUSSION

TCDD is widespread and persistent in the environment. Most persons' intake of TCDD is via food and has remained fairly constant. Blood sera lipid TCDD levels are typically between 2 and 5 ppt (Cranmer *et al*., 1994; Michalek *et al*., 1998), although a significant number of persons have levels above 10 ppt, the level reported to be associated with an increased incidence of diabetes and hyperinsulinemia (Henriksen *et al*., 1997). TCDD is in equilibrium in the various lipid compartments of the body and is sequestered in adipose tissue. In addition, TCDD is resistant to metabolism and has a half-life in humans of 7–9 years (Geyer *et al*., 1993; Michalek *et al*., 1996. There is no known medical intervention that will speed the removal of TCDD from the human body.

TABLE 2
Insulin Levels and Odds Ratios for Subjects with High or Normal Insulin at Each Time Point

	TCDD > 15		TCDD < 15		Relative risk	
Time	High insulin	Normal insulin	High insulin	Normal insulin	Odds ratio	95% C.L. Odds ratio
Fasting[a]	3	4	5	57	8.5	1.49–49.4
30 min[b]	3	4	6	56	7	1.26–39.0
60 min[c]	4	3	6	56	12	2.23–70.1
120 min[d]	6	1	6	56	56	5.7–556

Note. Odds ratios are for high insulin for subjects with TCDD > 15 ppt as compared to persons with TCDD < 15 ppt. Statistically significant, $p < 0.05$ using the Fisher Exact Test.

[a]High insulin level, > 4.5 μIU/ml.
[b]High insulin level, > 177 μIU/ml.
[c]High insulin level, > 228 μIU/ml.
[d]High insulin level, > 97.7 μIU/ml.

Several studies of workers have demonstrated an association between TCDD exposure and diabetes (Pazderova-Vejlupkova *et al*., 1981; Sweeney *et al*., 1992; Henriksen *et al*., 1997). High TCDD levels were also positively correlated with a number of conditions characteristic of impaired glucose metabolism, including decreased time to onset of diabetes and increased hyperinsulinemia in non-diabetic veterans (Henriksen *et al*., 1997). This latter observation, from the Air Force epidemiological study, is consistent with the study reported herein.

This study was designed to compare blood levels of TCDD and insulin in healthy persons with normal glucose levels. Therefore, persons with diabetes, impaired glucose tolerance, or persons using drugs which influence insulin levels were excluded from consideration. Examination of Table 1 reveals that the high TCDD ($>$ 15 ppt) and normal TCDD subjects ($<$ 15 ppt) did not differ with respect to age, gender distribution, BMI, total lipids, fasting glucose, or glucose levels at 30, 60, and 120 minutes after a 75 g challenge. Blood levels of insulin in the $<$ and $>$ 15 ppt TCDD groups were evaluated using ANOVA to determine if differences existed. The data, summarized in Table 1, reveal that the high TCDD group exhibited a disproportionate prevalence of hyperinsulinemia at fasting and at all sampling times after administration of a glucose challenge. Since all other known confounding variables had been demonstrated to be equivalent, it was concluded that TCDD levels above 15 ppt are highly correlated with excess risk of hyperinsulinemia. Nevertheless, the 15 ppt TCDD levels should not be considered a threshold, since similar results have been reported in Vietnam veterans at 10 ppt (Longnecker *et al*., 2000).

Odds ratios were used for members of the $<$ and $>$ 15 ppt TCDD groups to compare the relative risk of being hyperinsulinemic. Table 2 provides a summary. The excess odds of hyperinsulinemia for the high TCDD group vary from 7 to 56 over the 4 time periods. The lower 95% confidence limits for all sampling points exceed unity (1). The odds ratio statistics support the conclusion that the excess risk of hyperinsulinemia represents a significant health risk for persons with TCDD over 15 ppt.

This study has several limitations. The subjects with high TCDD demonstrated hyperinsulinemia while maintaining normal glucose levels. This study did not directly measure insulin resistance; moreover, the methods used cannot distinguish subtle degrees of insulin resistance. However, hyperinsulinemia in the presence of normal glucose levels strongly suggests that insulin resistance is the underlying cause. In addition, the number of subjects in this study and the necessarily retrospective study design limits our ability to generalize about the precise nature of TCDD-mediated insulin resistance.

Although the mechanism by which TCDD may produce insulin resistance is unclear, there are several possibilities. TCDD is highly soluble in adipose tissue (Geyer *et al*., 1993) and binds to a cytosolic, high-affinity receptor known as the aryl hydrocarbon (Ah) receptor (Hankinson, 1995). TCDD has multiple effects in adipose and other tissues that may be important in glucose metabolism. For example, TCDD decreases expression of the insulin-responsive glucose transporter Glut 4 (Hauner *et al*., 1995; Stephens and Pekala, 1991), and several animal studies have demonstrated a TCDD-mediated decrease in glucose transport *in vitro* and *in vivo* (Enan *et al*., 1992; Enan *et al*., 1996; Liu and Matsumura, 1995; Olsen *et al*., 1994).

TCDD is also known to increase tumor necrosis factor-α (TNFα) expression in several different cell types (Dohr *et al*., 1994; Vogel and Abel, 1995. For example, administration of an anti-TNFα antibody resulted in less TCDD-induced oxidative stress in hepatic nuclei (Alsharif *et al*., 1994). Anti-TNFα antibodies have also been found to reduce dioxin-mediated mortality in mice (Taylor *et al*., 1992). The stimulation of TNFα by TCDD is relevant to insulin resistance and diabetes because of the association between increased adipose tissue TNFα expression and insulin resistance (Hotamisligil *et al*., 1993; Hotamisligil *et al*., 1995; Kern *et al*., 1995). Indeed, recent studies have demonstrated that TNFα knockout mice do not become insulin resistant when fed a high fat meal, whereas control mice do become hyperinsulinemic (Uysal *et al*., 1997). Thus, it is possible that the concentration of TCDD in adipose tissue leads to increased adipose TNFα expression, which could lead to insulin resistance.

It is well recognized that insulin resistance and hyperinsulinemia may precede the development of impaired glucose tolerance and type 2 diabetes by many years (Mitchell *et al*., 1992). Insulin resistance contributes to the risk of coronary artery disease (Mykkänen *et al*., 1994), and prolonged exposure to elevated insulin levels may predispose an individual to accelerated atherosclerosis and cardiovascular disease, even without the development of diabetes (DeFronzo, 1992; Kahn *et al*., 1995; Katz *et al*., 1996. A recent study from Seveso, Italy, where 45,000 residents had varying levels of exposure to TCDD after an industrial accident caused widespread pollution, revealed significant increases in the incidence of death from coronary artery disease and diabetes in exposed subjects, when compared to a reference group (Pesatori *et al*., 1998).

Environmental exposure to TCDD may disproportionately affect vulnerable members of the population. For example, continual environmental exposure to TCDD coupled with a 7–9 year half-life leads to the expectation that higher levels of TCDD should be found in older segments of the population. This, in fact, has been observed (Orban *et al*., 1994; Patterson *et al*., 1986). The risk of diabetes also increases with age (Harris *et al*., 1998). Thus, accumulated tissue levels of TCDD may place the elderly at increased risk for the development of insulin resistance, hyperinsulinemia, glucose intolerance, and diabetes. Another group of subjects at risk is children. The solubility of TCDD in fat results in increased rates of exposure for nursing infants through milk fat. Exposures during nursing may be disproportionately important because they occur during

sensitive development periods. Since TCDD has a very long half-life, early exposures persist and may contribute to insulin resistance in children for decades.

In conclusion, TCDD blood sera lipid levels > 15 ppt have been demonstrated to be associated with excess risk of hyperinsulinemia and probably insulin resistance. Further study is needed to confirm these findings in other TCDD-exposed subjects. Future studies are also needed to elucidate mechanisms that may lead to strategies to prevent hyperinsulinemia, insulin resistance, and diabetes in subjects at risk due to industrial and environmental exposure to TCDD and similar acting chemical substances.

ACKNOWLEDGMENTS

This work was sponsored by the Arkansas Department of Health and partially supported by funds from the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) trust fund H75/ATH698389–01 from the U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry. P. A. Kern was supported by a VA Merit Grant and NIH grant DK 39176.

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