Molecular Epidemiologic Evidence for Diabetogenic Effects of Dioxin Exposure in U.S. Air Force Veterans of the Vietnam War

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BACKGROUND: One of the outcomes positively associated with dioxin exposure in humans is type 2 diabetes.

OBJECTIVES: This study was conducted in order to find the molecular biological evidence for the diabetogenic action of dioxin in adipose samples from Vietnam veterans.

METHODS: We obtained 313 adipose tissue samples both from Vietnam veterans who were exposed to dioxin (Operation Ranch Hand) and from comparison veterans who served in Southeast Asia with no record of dioxin exposure. We conducted quantitative reverse-transcribed polymerase chain reaction studies on selected marker mRNAs from these samples.

RESULTS: We found the most sensitive and reliable molecular indicator of dioxin-induced diabetes to be the ratio of mRNA of glucose transporter 4 (GLUT4) and nuclear transcription factor kappa B (NFkB), a marker of inflammation. This ratio showed significant correlations to serum dioxin residues and to fasting glucose among those in the Ranch Hand group and, surprisingly, even in the comparison group, who have low levels of dioxin comparable to the general public. Such a correlation in the comparison group was particularly significant among those with known risk factors such as obesity and family history of diabetes.

CONCLUSIONS: These results show that the GLUT4:NFkB ratio is a reliable marker for the diabetogenic action of dioxin, particularly at very low exposure levels that are not much higher than those found in the general public, implying a need to address current exposure levels.

KEY WORDS: adipose tissue, Agent Orange, biological markers, diabetes, fasting glucose, glucose transporter type 4, inflammation, molecular epidemiology, NFkB, tetrachlorodibenzodioxin.

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pathway we measured mRNA expression of the genes c-Src, CEBPa, NfkB, and GLUT4 in adipose tissue. In addition we measured GAPDH, a housekeeping gene (Gorzelniak et al. 2001), for use as a normalization standard to correct for differences in cDNA synthesis efficiency.

**Materials and Methods**

The details of basic study design and subject selection have been published previously (Wolfe et al. 1990). The Air Force Health Study compares the health, mortality experience, and reproductive outcomes of ORH veterans with a comparison group of other Air Force veterans who served in Southeast Asia during the same period (1962–1971) the ORH unit was active but who were not involved with spraying herbicides. Comparison veterans were matched to ORH veterans on date of birth, race (black, nonblack), and military occupation (officer pilot, officer navigator, nonflying officer, enlisted flyer, enlisted ground crew). Periodic physical examinations and in-person interviews were conducted in 1982, 1985, 1987, 1992, 1997, and 2002–2003. The methods used in these studies were approved by the institutional review boards at the participating medical treatment facilities. Participation was voluntary, and informed consent was given by subjects at the examination sites.

Dioxin in serum collected from the veterans in 1987 and 1992 was measured by the Centers for Disease Control and Prevention using high-resolution gas chromatography/high resolution mass spectrometry (Patterson et al. 1987). The between-assay coefficient of variation at three different concentrations of dioxin ranged from 9.4% to 15.5%. For those veterans whose serum dioxin level was below the limit of detection, we assigned a level equal to the detection limit divided by the square root of 2 (Hornung and Reed 1990). Dioxin results were expressed in parts per trillion on a serum lipid weight basis.

Fasting glucose (milligrams per deciliter) was determined with Paramax equipment (model 720 ZX; Baxter Scientific Instruments, Deerfield, IL). Body mass index (BMI) was calculated with BMI (body mass index) = weight (kg)/height (m)². Dioxin results were expressed in parts per trillion on a serum lipid weight basis. Body mass index (BMI) was determined with BMI (body mass index) = weight (kg)/height (m)². Dioxin results were expressed in parts per trillion on a serum lipid weight basis.

Table 1. Sample sizes and demographics.

| Group/diabetic status | Age category | Lean (n) | Obese (n) | Total (n) |
|----------------------|--------------|----------|-----------|-----------|
| Comparison           |              |          |           |           |
| Nondiabetic          | Young        | 58       | 18        | 76        |
|                      | Old          | 56       | 24        | 80        |
| Diabetic             | Young        | 3        | 7         | 10        |
|                      | Old          | 9        | 9         | 18        |
| ORH                  | Nondiabetic  | 46       | 13        | 59        |
|                      | Old          | 40       | 11        | 51        |
| Diabetic             | Young        | 4        | 4         | 8         |
|                      | Old          | 6        | 5         | 11        |
| Total                |              | 313      |           |           |

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shipped to the laboratory in dry ice, thawed on ice, washed in sterile phosphate buffer solution to remove blood, refrozen, and stored at −80°C until sample analysis. We extracted RNA from approximately 500 mg tissue from each sample using TRIzol LS (Invitrogen, Carlsbad, CA), followed by a digest with RNase-free DNase I (Roche Applied Science, Indianapolis, IN) and a repeat extraction. The yield of RNA was determined spectrophotometrically. Specimens from two individuals were used up in preliminary studies. We synthesized cDNA from 1 μg total RNA using the Omniscript RT Kit (Qiagen, Valencia, CA), modifying the kit instructions by doubling the Oligo-dT primer and 10X buffer amounts and adjusting the water amount to yield 40 μl total volume.

Using conventional Taq DNA polymerase (Qiagen), 99 cDNA samples were amplified in duplicate to reduce variation due to saturation effects. Amplified fragments were separated on a 1% agarose gel alongside a DNA ladder of increasing molecular weight. Band density was quantified using the ChemiImager 4400, version 5.5 (Alpha Innotech, San Leandro, CA). Primers used for GAPDH, c-Src, and NFκB were previously described (Ercolani et al. 1988; Kubota et al. 2001; Meyer et al. 1991), whereas we designed primers for CEBPα [5′-TTCCGGTGCTCTCTGAAAGC-3′ (sense) and 5′-ACAGCCAGATCTCTAGGTCT-3′ (antisense)] and GLUT4 [5′-CAACTGGACGAGACATTCAA-3′ (sense) and 5′-CCAGGTCCTCAATTTCTACCA-3′ (antisense)]. Amplification conditions were as follows: 2 min initial denaturation at 94°C, cycling steps of 1 min denaturation at 94°C, 1 min annealing, and 1 min elongation at 72°C, ending with 10 min final elongation at 72°C. Annealing temperatures and cycle numbers were 60°C and 25 for GAPDH and CEBPα; 55°C and 25 for c-Src; 60°C and 30 for GLUT4; and 62°C and 32 for NFκB. One sample was designated as the internal standard for polymerase chain reaction (PCR) and repeated in each run and on each gel in order to compare between runs. The remaining 212 cDNA samples were analyzed without duplication by real-time PCR on the LightCycler (Roche Applied Science) using the QuantiTect SYBR Green PCR kit (Qiagen) (Vogel et al. 2005) for GAPDH, c-Src, and GLUT4, and LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science) for CEBPα and NFκB. We designed a new GAPDH primer pair using Primer3 (Rozen and Skaltsky 2000): 5′-GAGTCAACGGATTTGGTCGT-3′ (sense) and 5′-TTGATTGGGAGGAGCTCG-3′ (antisense). PCR conditions followed kit instructions, with annealing temperatures and extension times of 54°C and 12 sec for GAPDH, 53°C and 28 sec for c-Src, 55°C and 20 sec for GLUT4, 60°C and 28 sec for CEBPα, and 62°C and 17 sec for NFκB. We increased the magnesium chloride concentration for the LightCycler kit to 4 mM, and the PCR product size was checked on a 1% agarose gel. We calculated relative sample concentrations from crossing point data using the transform 2−θ. To make LightCycler-determined values from different runs comparable to each other and to conventional values, we reran subsets of samples from each run in a common run.

We performed general linear model statistical analysis using SAS software, version 9.1 for Windows (SAS Institute, Cary, NC). We considered and dismissed main effects models of PCR values in terms of dioxin with adjustment for age, body fat, diabetic status, and exposure group because none of these yielded significant results. Subsequently, interaction models involving the product of dioxin with age, body fat, and group were considered; many of these indicated significant interactions, motivating stratification. PCR values were transformed for analysis using various log and power transforms as needed to remove skewness. For purpose of analysis, body fat categories of lean and obese were based on 1997 measurements. All statistical testing was two-sided, with a significance level of p < 0.05.

Results

Raw mRNA values for expression of the four genes studied, before GAPDH normalization, had interquartile ranges of 2.2-fold for CEBPα, 3.3-fold for NFκB, 3.4-fold for GLUT4, and 3.6-fold for c-Src. By comparison, the interquartile range for GAPDH was > 6-fold. GAPDH-normalized gene expression values for c-Src, NFκB, and CEBPα, after transformation, were significantly negatively correlated with transformed GAPDH values (p < 0.001).

One of the most statistically significant correlations of mRNA expressions or their ratios to change in PBF was the ratio of GLUT4 to NFκB (GLUT4:NFκB) among combined nondiabetic subjects at p < 0.001 (Figure 1A). The ratio of GLUT4 to C/EBPα (p = 0.002, data not shown) and the ratio of Src to NFκB (p = 0.031, data not shown) also showed significant correlations to PBF in the same population. In all cases the value of the ratio tended to drop with increasing PBF among nondiabetic subjects, as shown in Figure 1A compared with the corresponding diabetic subjects, which showed no relationship (e.g., Figure 2B). The differences in these ratios between the nondiabetic and diabetic populations were significant in all three cases. To increase the sensitivity of detection of this physiologic change, we adopted the GLUT4:NFκB ratio as the main marker. Further analyses of nondiabetic subgroups using the same GLUT4:NFκB ratio
showed that, among nondiabetic individuals in the comparison group without family history of diabetes, there was actually no negative or positive correlation to body weight change (Figure 1C). In contrast, among the corresponding subgroup (nondiabetic with no family history of diabetes) in the ORH group (Figure 1D), we found a significant negative correlation ($p = 0.003$). Even among nondiabetic comparison subjects, those with a family history of diabetes showed a negative slope (Figure 1E; $p = 0.03$), which was also statistically different ($p = 0.05$) from the slope in Figure 1C.

To study the effect of dioxin on the expression of each marker, we subdivided each group into quartiles according to measured levels of serum dioxin residues and compared gene expression ratios among nondiabetic individuals of these subgroups. GLUT4:GAPDH and NFκB:GAPDH ratios showed significant differences among quartiles (Figure 2A,B). A similar result was obtained when GLUT4 was compared to C/EBPα instead of GAPDH (data not shown). However, the ratio that gave us the clearest trend with dioxin was again GLUT4:NFκB (Figure 2C). The most conspicuous difference between the two service groups was the direction of slopes. Veterans of the first quartile of the comparison group had a significantly higher GLUT4:NFκB ratio than those of the second and third dioxin quartiles, exhibiting a negative relationship to dioxin residues; in contrast, Agent Orange-exposed ORH subjects clearly showed a positive trend. Among comparison subjects, only the highest dioxin residue quartile contradicted the downward trend.

In view of this finding, we further analyzed the relationships between the GLUT4:NFκB ratio and dioxin exposure in the full cohort of veterans (Figure 3A vs. Figure 3B). In general, dioxin was associated with an increase in the GLUT4:NFκB ratio in the ORH group, whereas there was no trend in the comparison group. Further analyses of several subgroups within each service group revealed that the GLUT4:NFκB ratio tended to decline when serum dioxin increased among nondiabetic individuals in the comparison group who are obese and have a family history of diabetes (Figure 3C). Other subgroups that showed significantly different GLUT4:NFκB responses to increasing serum dioxin were the combined (comparison + ORH) subgroup consisting of obese subjects with a family history of diabetes (Figure 3D) compared with the combined subgroup of lean subjects with a family history of diabetes (Figure 3E).

To test the validity of using the GLUT4:NFκB ratio as a molecular parameter to assess diabetogenic conditions, we analyzed its relationship to the high levels of serum glucose after fasting (fasting glucose). As seen in Figure 4A, the fasting glucose levels stayed within a narrow range of values among all (comparison + ORH) nondiabetic study subjects over the wide range of GLUT4:NFκB ratios, as expected. In contrast, among all diabetic subjects (Figure 4B), those with lower GLUT4:NFκB ratios exhibited higher fasting glucose levels. We further checked the relationship between PBF and fasting glucose levels in all nondiabetic subjects (Figure 4C) compared with all diabetic subjects (Figure 4D). We found a significant relationship in nondiabetic subjects ($r = 0.27, p < 0.001$) but not in diabetic subjects ($r = -0.05, p = 0.73$).

Next, we studied the relationship between the levels of fasting glucose and serum dioxin to further test our premise that dioxin acts as a diabetogenic agent. When both nondiabetic and diabetic subjects were combined within each service group, we found a positive correlation between fasting glucose and serum dioxin levels in the comparison group (Figure 4E; $p = 0.02$). In contrast, these two parameters were not correlated in the corresponding ORH subgroup (Figure 4F).

**Discussion**

Initially, we conducted a preliminary survey of the expressions of the proposed markers in all samples to see which markers or their combinations would give a reliable indication of physiologic conditions leading to diabetes. To aid in this process, we formulated a working hypothesis that the diabetogenic action of TCDD could be phenotypically similar to that of obesity. Therefore, we studied the relationship between mRNA expression of those selected markers and PBF gain over the last 5 years among veterans. Of all of the molecular markers and their combinations examined, the most readily recognizable gene expression effects were those of the GLUT4:NFκB ratio...
in response to the presence of dioxin, as assessed in the serum of the veterans. This observation agrees well with the generally accepted view that in the case of obesity-induced type 2 diabetes TNF-α plays the central role through its action to activate NFκB, which down regulates GLUT4 (Ruan et al. 2002). Theoretically, if the TNF-α→NFκB pathway is activated, a rise in NFκB expression will lead to a drop in GLUT4 expression; thus, the ratio is expected to be more responsive than either individual gene normalized by a housekeeping gene. The trends for GLUT4/C/EBPα and Src/NFκB may represent merely weaker responses of ratios that contain the GLUT4 or NFκB component of the GLUT4:NFκB ratio.

This GLUT4:NFκB ratio marker approach was most effective in detecting the effect of dioxin at low-to-medium levels of exposure, corresponding to the lower three quartiles in the comparison group (Figure 1). This range of dioxin levels corresponds to 1–5.3 ppt. In fact the highest dioxin level for the entire comparison group is 16.3 ppt. The level of dioxin in serum lipids among U.S. workers that Sweeney et al. (1997–1998) found in their comparison group was 0–20 ppt, indicating that the range we found in the comparison group in the present study was not much different from that of the general public in the United States. In this regard, it is important to point out that we found significant signs of dioxin-correlated diabetogenic tendency among comparison group subjects with low levels of dioxin, particularly in those with genetic (family history of diabetes) and physiologic (obesity) risk factors (Figure 3C).

Furthermore, dioxin, even at this low concentration range, definitely has an effect on the levels of fasting glucose (Figure 4E). The GLUT4:NFκB response to dioxin exposure levels found in the lower three quartiles of the comparison group also agrees well with the data obtained from cell models (Ruan et al. 2002) and animal models (Dunlap et al. 2002; Liu and Matsumura 1995).

The observation that the GLUT4:NFκB ratio declined with weight gain, independent of exposure history (Figure 1A) and with higher serum dioxin residues among comparison individuals (Figure 2C), particularly obese individuals with family history of diabetes (Figure 3C), suggests that dioxin works synergistically with known diabetes risk factors to alter glucose metabolism in a way that resembles the inflammation mechanism of weight gain. The GLUT4:NFκB ratio appears to be a useful biomarker for the detection of the diabetogenic action of these factors. The inflammation mechanism also seems to operate at low background levels of dioxin, as seen in the three lowest serum dioxin quartiles (i.e., those with ≤ 5.3 ppt) of the larger group of background-exposed veterans (Figure 2C). Such an observation alone would not constitute a proof for the identical action mechanism of dioxin to diabetogenic action of obesity, but it allows us to formulate a hypothesis along this line of logic to help our future studies.

We found the GLUT4:NFκB ratio to be a reliable parameter in assessing the state of diabetes. Diabetic subjects with lowered GLUT4:NFκB were less able to regulate blood glucose, whereas nondiabetic subjects maintained fasting glucose levels within a narrow range of values, independent of GLUT4:NFκB (Figure 4A,B). However, our finding regarding this effect of GLUT4:NFκB was somewhat surprising in view of the lack of correlation of GLUT4:NFκB with weight gain among diabetic subjects (Figure 1B); this finding suggests that once the study subjects develop diabetes, the GLUT4:NFκB ratio does not work well as a biomarker for obesity. However, the GLUT4:NFκB ratio does work as a biomarker for elevated fasting glucose (Figure 4B). The important message derived from Figure 4B is that the workable range of the usefulness of the GLUT4:NFκB ratio as a biomarker depends largely on the factor against which it is being regressed. Thus, one should not assume automatically that the state of disease makes it impossible to use this biomarker on all diabetes-related cellular changes.

Among nondiabetic veterans without a family history of diabetes (i.e., healthy subpopulation) in the comparison group, we found no detectable effect of obesity on the GLUT4:NFκB ratio (Figure 1C), indicating the existence of normal homeostatic control. In contrast, the corresponding subgroup from the ORH group clearly showed the effect of obesity (Figure 1D) as though they already had the genetic risk factor, as in the case of

![Figure 4. Effects of GLUT4:NFκB ratio (A, B), PBF (C, D), and serum dioxin (E, F) on fasting blood glucose.](image)
Minokoshi et al. (2003) found that tissue- 
Agent Orange–exposed veterans.
among subjects in the comparison group who
observation that fasting glucose is higher 
diabetic subjects (Figure 4D). In addition, the 
diabetes but not at the later stage, as seen in 
diabetic subjects (Figure 4D). In addition, the 
observation that fasting glucose is higher
levels of dioxin in a larger cohort of 
Agent Orange–exposed veterans.

The decrease in the expression of GLUT4 
in adipose tissue has been shown to be associ-
ated with non–insulin-dependent diabetes. 
Minokoshi et al. (2003) found that tissue-
specific ablation of GLUT4 and insulin recep-
tor in adipose tissue or muscle led to 
insulin resistance and diabetes in the mice lacking adipose GLUT4 expression but not in 
those missing GLUT4 only in muscles. In 
humans, Garvey et al. (1991) found that a 
80% decrease in GLUT4 protein per cell in 
the adipocytes of diabetic subjects compared 
with lean nondiabetic controls was associated with 
a 56% decrease in maximally insulin-
stimulated glucose uptake. The level of 
GLUT4 mRNA was correlated with the 
amount of GLUT4 protein (r = 0.77) in their 
controls but not in their diabetic subjects.

There are also precedents indicating that 
the change in NFKB is correlated to diabetes. 
NFKB is a nuclear transcription factor that is 
known to be activated by inflammatory signal-
ating of several agents, including TNF-α, and to 
transmit their messages into the nucleus. 
Although we did not include TNF-α in the 
present study, its involvement in the toxic 
action of dioxin is well known (Alsharif et al. 
1994; Taylor et al. 1992). TNF-α is one of 
the major mediators of dioxin-induced cell 
inflammatory reactions (Matsumura 2003). 
Furthermore, the role of TNF-α in the 
development of insulin resistance and type 2 
diabetes, particularly in the case of obesity-
duced diabetes, is now becoming well recog-
nized (Das 1999). Indeed, obesity induces 
increased expression of TNF-α and NFKB, 
leading to down-regulation of insulin receptor 
and decreasing expression of GLUT4 (Halle et al. 1998). Our observation in the present 
study that the GLUT4:NFKB ratio dramatically 
decreases among nondiabetic veterans who 
 Experienced a relatively recent increase in body 
fat attests to the correctness of this diagnosis.

We did not include measurements of 
other compounds with dioxin-like activity 
because these were not available in this popu-
lation until well after the period of this study. 
Sweeney et al. (1997–1998) found that use of 
toxic equivalents (TEQ; the body burden of 
TCDD-equivalent activity from all compo-
unds) led to a narrowing of the differences 
between acute- and background-exposed 
populations. Furthermore, the phenoxyl herbi-
cides in the Agent Orange formulation are 
known peroxisome proliferators and thus may 
have antioxidant action (Remillard and Bunce 
2002). With such confounding factors acting 
to obscure the effects of TCDD, the results we 
did find are all the more noteworthy.

One major question that remains unan-
wsered is why the overall relationship between 
the GLUT4:NFKB ratio and serum dioxin 
levels show the “V” shape (Figure 2C), indi-
cating a reversal of dioxin’s effect at levels 
higher than the background range. This is 
puzzling because, in all other cases, either the 
GLUT4:NFKB ratio or dioxin levels showed 
straightforward relationships to other parame-
ters analyzed. One possibility is that veterans 
with dioxin residue levels > 5.3 ppt are experi-
encting the effects of cachexia, a typical effect 
of dioxin exposure. It involves massive loss of 
adipose tissues in most animals studied, 
including humans (Matsumura 2003). In this 
regard, it is interesting to note the similarities 
in the direction of slopes between Figure 3A 
and Figure 3D, and between Figure 3B and 
Figure 3E, indicating that as a whole, com-
parison subjects are similar to combined 
(comparison + ORH) nondiabetic obese sub-
jects with a family history of diabetes with 
respect to their GLUT4:NFKB response to 
dioxin. In the same manner, ORH subjects 
are similar to combined (comparison + ORH) 
nondiabetic lean subjects with family history 
 of diabetes, despite the fact that there is no 
difference in the frequency of obesity between 
comparison and ORH subjects. Such an 
observation favors the view that wasting 
syndrome is already taking place among those 
exposed to high levels of dioxin and that 
ORH subjects as a whole are responding to dioxin as though they were lean. Another 
possibility is that in human adipose tissues, 
unlike the case of mice, chronic exposure to 
 high concentrations of dioxin could trigger a 
strong negative feedback reaction through 
activation of major “negative regulators” to 
counteract excessive inflammatory insults. 
The observation that three of the parameters 
we studied, NFKB, C/EBPα, and GLUT4 
expression, show this phenomenon of “revers-
al” at high dioxin concentrations > 5.3 ppt 
supports this interpretation. Nevertheless, 
much more work is needed to prove or dis-
prove these hypotheses. It is also important to 
point out that, despite the above “reversal” 
 phenomenon in the relationship between 
GLUT4:NFKB ratio and dioxin, all non-
diabetic individuals with significant dioxin 
residues, including those with residue levels
> 5.3 ppt, still show the clear sign of the obe-
sity-related risk of diabetes, judging by the 
results of experiments shown in Figure 4C.

Conclusions

In conclusion, by using this molecular epi-
demiologic approach we found definitive evi-
dence indicating that a diabetogenic shift 
occurred in the biochemistry of adipose tis-
sues from Vietnam veterans who were 
exposed to dioxin-containing Agent Orange 
herbicide preparations. Such a diabetogenic 
effect of dioxin was observed even among comparison group veterans, particularly those 
with diabetes risk factors such as obesity 
and/or a family history of diabetes, despite the 
 fact that their levels of exposure are not really 
different from those of the general public in 
the United States. The major implication of 
the present study is, therefore, that the poten-
tial health hazard of dioxin and active dioxin-
type chemicals, even at the current level of 
public exposure, must be taken seriously. 
Further research is needed to fully elucidate 
the precise mechanism through which dioxin 
promotes type 2 diabetes in humans.

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