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Cell death and impairment of glucose-stimulated insulin secretion induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the β-cell line INS-1E

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Abstract

The aim of this research was to characterize 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity on the insulin-secreting β-cell line INS-1E. A sharp decline of cell survival (below 20%) was observed after 1 h exposure to TCDD concentrations between 12.5 and 25 nM. Ultrastructurally, β-cell death was characterized by extensive degranulation, appearance of autophagic vacuoles, and peripheral nuclear condensation. Cytotoxic concentrations of TCDD rapidly induced a dose-dependent increase in intracellular calcium concentration. Blocking calcium entry by EGTA significantly decreased TCDD cytotoxicity. TCDD was also able to rapidly induce mitochondrial depolarization. Interestingly, 1 h exposition of INS-1E cells to very low TCDD concentrations (0.05–1 nM) dramatically impaired glucose-stimulated but not KCl-stimulated insulin secretion. In conclusion, our results clearly show that TCDD exerts a direct β-cell cytotoxic effect at concentrations of 15–25 nM, but also markedly impairs glucose-stimulated insulin secretion at concentrations 20 times lower than these. On the basis of this latter observation we suggest that pancreatic β-cells could be considered a specific and sensitive target for dioxin toxicity.
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Keywords: Dioxin; Diabetes; INS-1 cells; Insulin secretion

Introduction

Dioxins are a group of environmental contaminants commonly found throughout the ecosystem. Polichlorinated dibenzo-*p*-dioxins, including the most toxic of these compounds 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are inadvertently produced by paper and pulp bleaching, by incineration of municipal, toxic and hospital wastes, in PCB-filled electrical transformer fires, in smelters, and during production of chlorophenoxy herbicides (Tiernan et al., 1985). Almost all human subjects have detectable body burdens of dioxin-like compounds, mostly stored in the adipose tissue (Arisawa et al., 2005). Dioxin and related halogenated aromatic hydrocarbons have received increasing attention over the past years because of their ability to induce a variety of toxic and biochemical changes (for a review see Mitrou et al., 2001), some of which occur at very low doses and are long-lasting. One of the most common symptoms seen in animal species as a result of TCDD poisoning is body weight loss or reduced weight gain, involving mostly the loss of fat and muscle tissue ("wasting syndrome") (McConnely et al., 1978; Olson et al., 1980; Tuomisto et al., 1995). The loss of adipose tissue is accompanied by hypophagia (Peterson et al., 1984), hyperlipidemia, particularly hypertrigliceridemia (Swift et al., 1981), probably related to the TCDD-induced drastic decline in adipose lipoprotein lipase activity (Brewster and Matsumura, 1984), and hypoinsulinemia (Ebner et al., 1988). These observations seem to indicate that the affected animals are unable to utilize the nutritional elements (e.g. glucose, tryglicerides, cholesterol, etc.) available in their blood. More recently, among the various metabolic derangements induced by TCDD poisoning, those related to glucose metabolism have been considered to play a crucial role in the induction of the wasting syndrome. The most relevant alteration of glucose homeostasis

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induced by TCDD has been demonstrated to be a marked decrease in glucose uptake by adipose tissue, liver, and pancreas in vivo as well as in vitro (Enan et al., 1992a, 1992b). The main cause for the reduction in glucose uptake seems to be the TCDD-induced decline in the titer of glucose transporter (Enan and Matsumura, 1994; Liu and Matsumura, 1995). Recently, we have demonstrated that a single low dose of TCDD (1 μg/kg b.w.), well below the estimated lethal dose (125 μg/kg), was able to rapidly induce a significant decrease of the pancreatic insulin content in the rat, suggesting that endocrine pancreas is a sensitive target of TCDD toxicity (Novelli et al., 2005). Furthermore, a significant impairment of glucose-stimulated insulin secretion was also observed in islets isolated from TCDD-treated rats (Novelli et al., 2005).

The aims of the present research were: a) to further characterize TCDD-induced β-cell toxicity, by directly evaluating its effects on cell viability and function in the insulin-secreting cell line INS-1E, which is considered to be a reliable β-cell model (Merglen et al., 2004); b) to provide some insights into its mechanism of action.

Materials and methods

Cell culture. INS-1E cells were cultured in a humidified atmosphere containing 5% CO_2 in complete medium composed of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. The maintenance culture was passaged once a week by gentle trypsinisation, and cells were seeded at a density of 4×10^4 cells/cm^2, i.e. 3×10^6 cells, in 75-cm^2 Falcon bottles with 10 ml complete medium.

Cytotoxicity. Cells were seeded in 96-well plates at a density of 4×10^4 cells/cm^2. After 48 h, cells were incubated with fresh medium containing different TCDD concentrations (0, 1.0, 3.1, 6.2, 12.5, 25 and 50 nM, respectively) for 1 h. Cell survival after 1 h exposition to TCDD was evaluated on the basis of the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases, by using a commercially available kit (Cell Proliferation Reagent WST-1. Roche Diagnostics, Germany). Absorbance at 440 nm was measured in a scanning multiwell spectrophotometer Victor^3 1420 (Perkin Elmer). A similar protocol was followed for the protection experiments, but the cells were preincubated for 1 h before TCDD exposure with fresh medium containing 200 μM epigallocatechin gallate (EGCG, Sigma).

Electron microscopy. After 1 h incubation in fresh medium containing different concentrations of TCDD, INS-1E cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 20 min at room temperature, washed in 0.1 M phosphate buffer pH 7.3, post-fixed in 0.1% osmium tetroxide in 0.1 M phosphate buffer pH 7.3 and dehydrated in a graded series of ethanol. In the last phase of dehydration, cells were scraped and the cell suspension was centrifuged to obtain a pellet. Centrifuged pellets were rapidly transferred to propylene oxide and embedded in PolyBed 812. Ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate and observed under a Zeiss 902 transmission electron microscope.

Insulin secretion. The secretory responses to glucose and other secretagogues were tested in INS-1E cells between passages 54 and 65. Cells were seeded in 24-well plates at a density of 4×10^4 cells/cm^2. After 48 h, cells were preincubated with fresh medium containing different TCDD concentrations (0, 0.05, 0.5 and 1.0 nM, respectively) for 1 h. At the end of the preincubation time, cells were washed twice with Krebs–Ringer bicarbonate (KRBH) buffer supplemented with 0.5% bovine serum albumin (fraction V, Sigma) and 2.8 mM glucose, and then incubated for 1 h at 37 °C in 1 ml fresh KRBH buffer containing different glucose concentrations (2.8, 5.6, 8.3 and 11.2 mM, respectively). At the end of this period, the buffer was collected for insulin determination. Finally, 1 ml of cold acidified ethanol (150:47:3, v/v, absolute ethanol/H_2O/concentrated HCl) was added to the cells in order to extract their insulin content. In order to evaluate KCl-stimulated insulin secretion, after 1 h preincubation in medium containing the above mentioned TCDD concentrations, cells were washed twice with Krebs–Ringer bicarbonate (KRBH) buffer supplemented with 0.5% bovine serum albumin (fraction V, Sigma) and 2.8 mM glucose, and then incubated for 1 h at 37 °C in 1 ml fresh modified KRBH buffer containing 30 mM KCl. Insulin was measured by radioimmunoassay according to Herbert et al. (1965), using rat insulin as a standard. The sensitivity and the coefficient of variation of the radioimmunoassay were as follows: detection limit 0.13 ng/ml, intra-assay variation 3.3%, inter-assay variation 10.5%.

Calcium uptake. Cells cultivated in 24-well plates were loaded according to standard procedures with 4 μM fluo-3/AM dissolved in KRBH buffer supplemented with 0.5% bovine serum albumin at 37 °C for 30 min. This membrane permeant, non-fluorescent acetoxymethyl ester is converted to fluorescent form by intracellular esterases and exhibits a 40-fold increase fluorescence intensity upon Ca^{2+} binding. Control experiments indicated that the probe concentration and dye loading were optimal and that no residual unconjugated dye was present when cells were analysed. Following loading, cells were washed twice with KRBH buffer, pH 7.4 and then kept in this buffer containing 100 μM sulfinpyrazone to minimize leakage of trapped fluo-3. Fluorescence was measured in a Perkin Elmer scanning multiwell spectrophotometer Victor^3 1420 (excitation and emission wavelengths, 488 and 525 nm, respectively). At the end of each incubation, digitonin (50 μg/ml) and EGTA (as a solution of 0.5 M EGTA in 3.0 M Tris, pH 9.0, to have a final concentration of 20 mM) were added in order to measure maximal (F_{max}) and minimal (F_{min}) fluorescence values, respectively. Values of [Ca^{2+}] were calculated according to the formula:

$$[Ca^{2+}] = K_d[(F - F_{min})/(F_{max} - F)]$$

The K_d for the Ca^{2+}-fluo-3 complex was assumed to be 325 nM at 22 °C.

Mitochondrial membrane potential. To measure the mitochondrial membrane potential ($\Delta\psi_m$) in INS-1 cells, we employed, as recommended by Jayaraman (2005), the fluorescent dye tetramethylrhodamine methyl ester (TMRE, Molecular Probes, Eugene, OR, USA), that accumulates in the inner mitochondrial membrane according to the $\Delta\psi_m$. A loss of $\Delta\psi_m$ can be monitored by flow cytometry as a decrease in FL-2, expressed in a log scale. Briefly, INS-1E cells, were gently detached by cold PBS, resuspended (1×10^6 cells/ml) in KRBH buffer supplemented with 2.8 mM glucose and preincubated with 25 nM TMRE for 15 min at 37 °C before being exposed to TCDD. The $\Delta\psi_m$ was measured before and 10 min after TCDD exposure with a Becton-Dickinson Facscan equipped with a Cell Quest software.

Statistical analysis. All results are expressed as mean ± S.E.M. Statistical significance was evaluated by using analysis of variance (ANOVA) or Student's *t*-test, when appropriate.

Results

Characterization of TCDD toxicity

To characterize dioxin toxicity, we initially evaluated the effects of 1 h incubation with increasing concentrations of TCDD on the viability of INS-1E cells. Fig. 1 shows that the presence of TCDD in the culture medium caused a progressive, dose-related decrease of cell survival with respect to the untreated controls. In particular, we observed a sharp decline in the viability of cells exposed to 25 nM TCDD: at this concentration cell survival decreased to 16.6 ± 3.43%, whereas it was 70.9 ± 5.44% with a TCDD concentration of 12.5 nM.

The cytotoxic effect of TCDD on INS-1E cells was confirmed by ultrastructural observations (Fig. 2). In particular, after 1 h

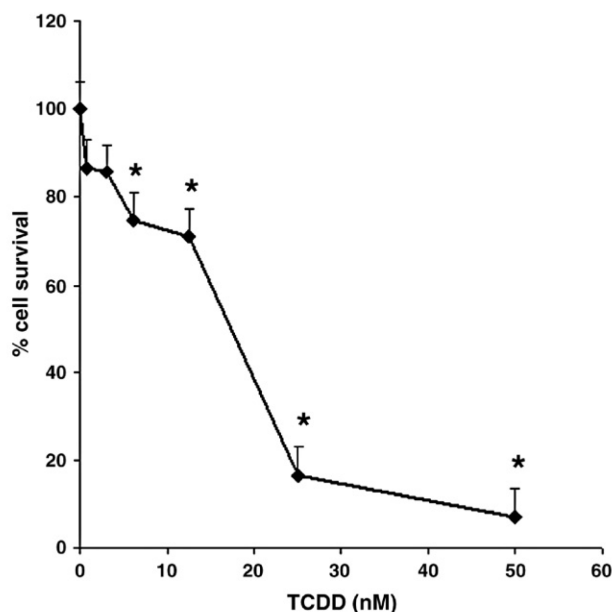

Fig. 1. INS-1E cell survival after 1 h exposition to different concentrations of TCDD. Data are expressed as mean ± S.E.M. of four different experiments. *$p < 0.01$ vs. control untreated cells (Student's t-test).

incubation with 6.25 nM TCDD (Fig. 2B), the cellular ultrastructure was generally well preserved, but several mitochondria with irregular shape and dilated cristae as well as detached ribosomes were often observed. The rough endoplasmic reticulum appeared abundant and dilated Golgi cisternae were present. Numerous secretory granules (arrows) appeared close to the plasma membrane, where several coated pits also formed. After 1 h exposure to 12.5 nM TCDD (Fig. 2C), INS-1E cells showed nuclei characterized by dispersed chromatine. Many mitochondria were markedly enlarged, round-shaped, with dispersed matrix and dilated cristae. In all the cells, we observed several large autophagic vacuoles (arrow), containing identifiable organules, such as mitochondria or degraded membranes which appeared as mielinic figures. Secretory granules were still numerous and disposed close to the plasma membrane. Upon 1 h exposure to 25 nM TCDD (Fig. 2, panels D and E), a significant proportion of the observed cells appeared dead, as they showed highly condensed nuclei, swelled cytoplasmic organelles, and several vacuoles containing membrane residues (arrows). Furthermore, plasma membrane integrity was lost. Other cells, apparently still alive, showed nuclei with chromatine condensation and evident nucleoli. In the cytoplasm, we observed several large autophagic vacuoles, containing identifiable organelles (mitochondria and rough reticulum) and membrane residues. Mitochondria were usually swelled with dispersed matrix and fragmented cristae. The rough endoplasmic reticulum appeared distributed in all the cytoplasm with elongated cisternae forming round figures. Secretory granules were markedly decreased. After 1 h incubation with 50 nM TCDD (Fig. 2F), the majority of INS-1E cells appeared dead. Surviving cells showed nuclei characterized by dispersed chromatine and well evident electrondense nucleoli. Cytoplasmic matrix appeared dispersed and the organelles were very rare. The few swelled mitochondria contained dispersed matrix and dilated cristae. RER was dispersed through the cytoplasm with cisternae forming circular structures. Several ribosomes were dispersed in the cytoplasm. No secretory granules were found. Frequently, short tracts of plasma membrane appeared disrupted, with residual organelles protruding from the cell.

Mechanisms of TCDD cytotoxicity

In order to investigate potential mechanisms involved in the toxic action of TCDD on INS-1E cells, we initially evaluated the ability of TCDD to increase the intracellular calcium concentration. Fig. 3A shows that in INS-1E cells TCDD was able to induce a rapid, sustained and dose-dependent increase in the intracellular calcium concentration. This significant TCDD-induced increase in cellular calcium is attributable both to a transient and quantitatively modest calcium release from intracellular stores and to a more relevant and persistent entry of extracellular calcium into the cell (Fig. 3B). To assess the role of this elevation of cellular calcium in TCDD-induced cytotoxicity, we evaluated cell survival after 1 h exposition to 25 nM TCDD of INS-1E cells incubated in RPMI medium supplemented with 0.2 mM EGTA in order to block the entry of extracellular calcium into the cells (Fig 3C). In these conditions, the survival of INS-1 cells nearly doubled after 1 h incubation with TCDD, indicating that the increase in cellular calcium is involved in TCDD toxicity. However, incubation of INS-1E cells with the EGTA-supplemented medium (that will block calcium entry into the cells but not calcium release from intracellular stores) did not completely abolish the TCDD-induced decrease in cell viability, thus indicating that other mechanisms could be also involved in the cytotoxicity of TCDD. In order to ascertain whether the TCDD-induced sharp increase in the intracellular calcium concentration could be detrimental for mitochondrial function, we assayed the mitochondrial membrane potential in INS-1E cells after TCDD exposure by cytofluorimetric analysis. After 10 min exposure to different concentrations of TCDD we observed (Fig. 4) a dose-related mitochondrial depolarization, measured as a decrease in FL-2, in INS-1E cells. In particular, cytofluorimetric analysis showed that mitochondria were completely depolarized in INS-1E cells incubated with 25 and 50 nM TCDD.

Recently it has been reported that the green tea polyphenol epigallocatechin 3-gallate indirectly inhibits the Aryl Hydrocarbon Receptor (AhR) (Palermo et al., 2005), a ligand-activated transcription factor known to mediate the toxic effects of numerous environmental contaminants, including dioxins and polycyclic aromatic hydrocarbons (Bock and Köhle, 2006). Thus, we decided to explore the possibility to prevent TCDD cytotoxicity by pre-incubating INS-1 cells for 1 h before TCDD exposure with 200 mM EGCG. The obtained results (Fig. 5) showed that EGCG was able to significantly increase the survival of INS-1 after 1 h incubation with TCDD. This protective effect of EGCG was evident, and quantitatively remarkable for the higher TCDD doses (cell survival was 2.5- and 6-fold higher in ECGC-pretreated cells for 25 and 50 nM TCDD, respectively). To verify whether the protective effect of EGCG

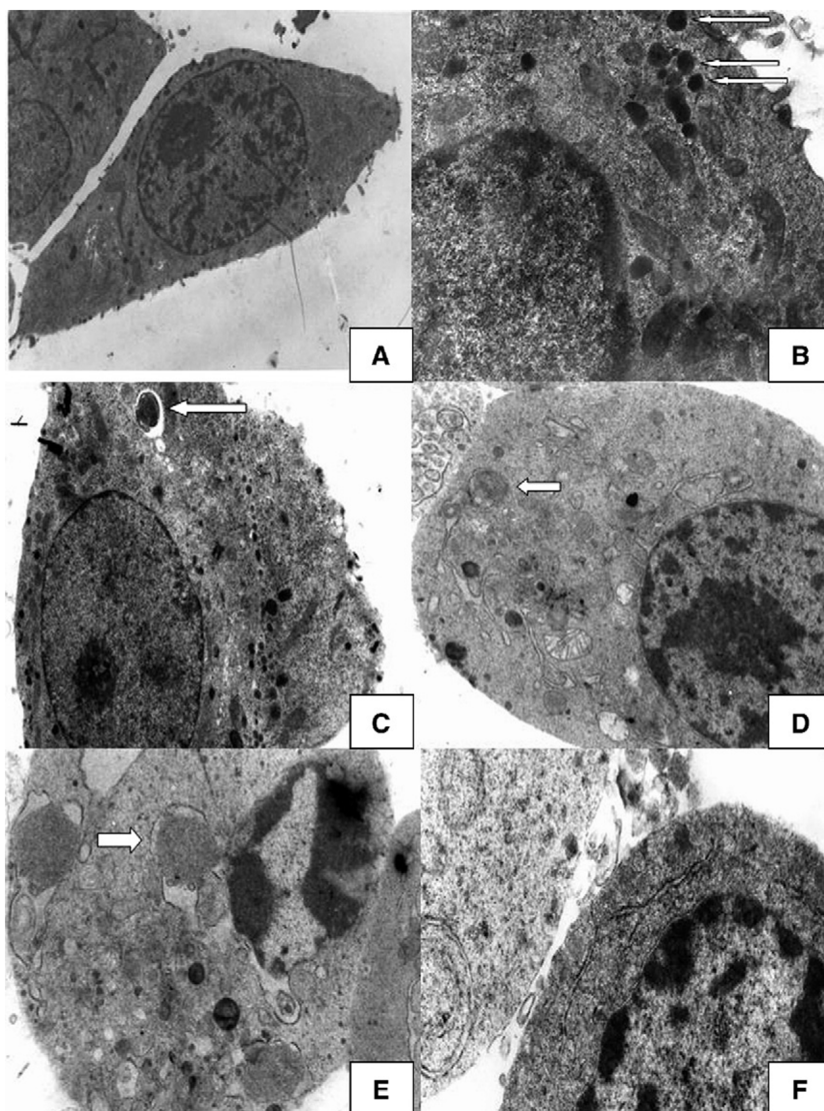

Fig. 2. Electron microscopy of INS-1E cells after 1 h exposition to different concentrations of TCDD. A: control (×7000); B: 6.25 nM TCDD (×28,000); C: 12.5 nM TCDD (×10,000); D: 25 nM TCDD (×16,000); E: 25 nM TCDD (×16,000); F: 50 nM TCDD (×28,000). In panels B, arrows indicate insulin granules; in panels C, D and E, arrows indicate autophagic vacuoles.

could be attributable to an inhibition of the TCDD-induced increase of cellular calcium, we assayed the effect of TCDD stimulation on cellular calcium concentration in the presence of 200 mM EGCG. No difference was found in the TCDD-induced increase in cell calcium in INS-1 incubated with or without EGCG (data not shown).

Impairment of glucose-stimulated insulin secretion

To verify whether TCDD could cause a functional impairment of INS-1E cells, we evaluated glucose-stimulated insulin secretion after 1 h incubation with three different (non-cytotoxic) concentrations of TCDD (0.05, 0.5 and 1.0 nM, respectively). While no difference was found in the insulin content between control and TCDD-treated cells (data not shown), our results (Fig. 6A) showed that glucose-stimulated insulin secretion was significantly decreased in INS-1E incubated with TCDD with respect to control cells. In particular, unlike control cells, TCDD-treated-INS-1E cells did not show any significant difference in insulin secretion at 5.6 and 8.3 mM glucose with respect to the basal secretion at 2.8 mM glucose. At 11.2 mM glucose, insulin secretion increased in TCDD-treated INS-1E cells, but was significantly less than in control cells, and the impairing effect of TCDD was clearly dose-related. Cellular insulin content was not significantly different between TCDD-treated and untreated cells (not shown).

Interestingly, when TCDD-treated INS-1E cells were stimulated with a depolarizing concentration of KCl (30 mM) insulin secretion stimulated by the KCl-evoked cell depolarization was unaltered (Fig. 6B), suggesting that the mitochondrion rather than the exocytotic process was the sensitive site. Finally, we evaluated the effect of 1 h incubation with 1 nM TCDD on the changes in the intracellular calcium levels induced by 11.2 mM glucose in INS-1E cells. Our results (Fig. 6C) showed

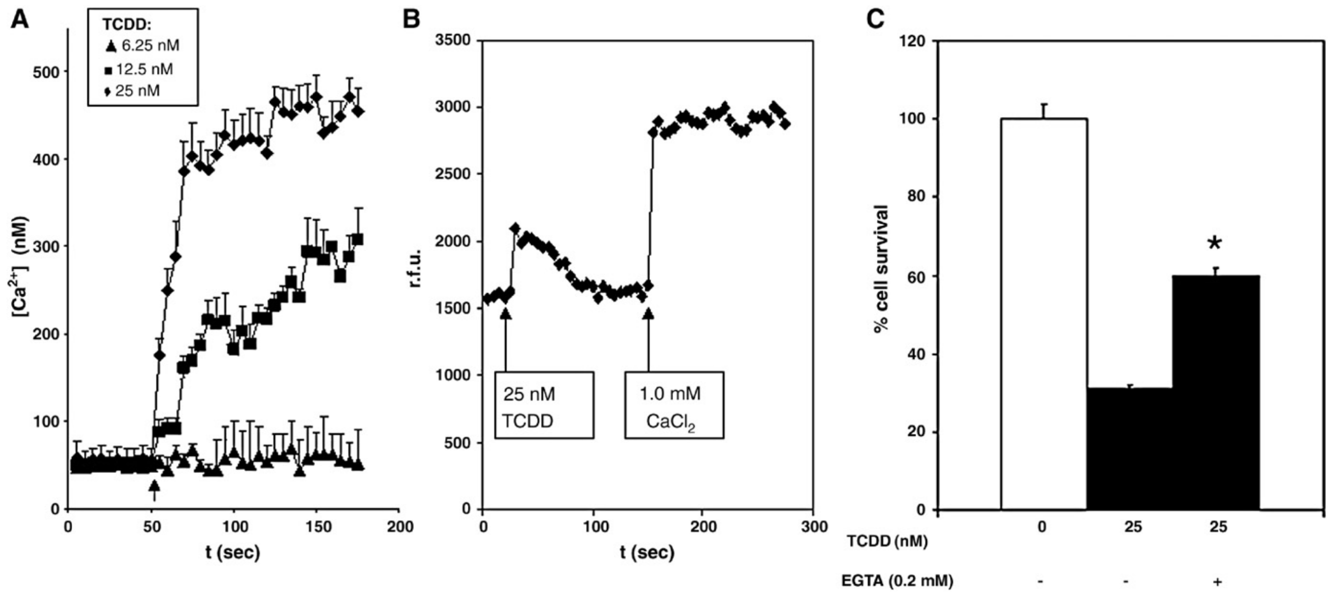

Fig. 3. A: Variations of the intracellular Ca^{2+} concentration in INS-1E cells after the addition (arrow) of three different TCDD concentrations. Data are expressed as mean ± S.E.M. of four different experiments. B: Fluo-3 fluorescence changes in relative fluorescence units (r.f.u.) in INS-1E cells incubated in a calcium-free buffer with 0.2 mM EGTA after the addition of 25 nM TCDD and 1.0 mM $CaCl_2$. C: INS-1 cell survival after 1 h exposition to 25 nM TCDD in the presence or the absence of 0.2 mM EGTA in the culture medium. Data are the mean ± S.E.M. of four different experiments. $*p < 0.01$ vs. the corresponding TCDD-treated cells without EGTA (Student's *t*-test).

that this exposure to low TCDD dose completely abolished the glucose-induced rise of intracellular calcium, consistent with the impairing effect of TCDD on glucose-stimulated insulin secretion.

Discussion

The present research was aimed at characterizing the acute toxicity of TCDD on the β-cell-derived INS-1E cell line from the point of view of cell function and survival. Our results clearly demonstrate that TCDD is highly toxic for INS-1E cells. Indeed, 1 h exposition to 25–50 nM TCDD induces the death of nearly all cells. Such "in vitro" concentrations could appear quite elevated when compared with the mean body burden for TCDD and other less toxic related cogeners in the general population (estimated to be around 13 ng TEQ/kg body weight), but it should be considered that in particular conditions (such after industrial accidents) the body burden can increase to remarkably higher levels (up to 7000 ng TEQ/kg b.w.). (Mitrou et al., 2001; Arisawa et al., 2005). Thus, at least in such conditions of high environmental exposure, pancreatic β-cells are likely to undergo relevant damage. On the other hand, for comparison, we remind that well known β-cytotoxic agents such as alloxan can produce similar effects on INS-1 cell via-

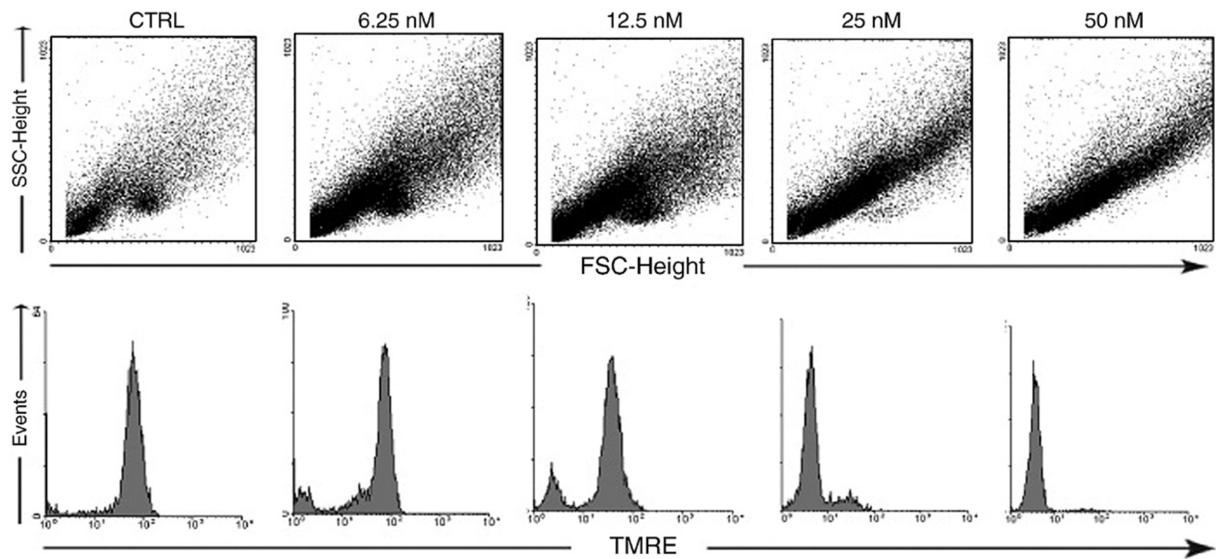

Fig. 4. Cytofluorimetric analysis of mitochondrial membrane potential in INS-1E cells after 10 min exposition to different concentrations of TCDD.

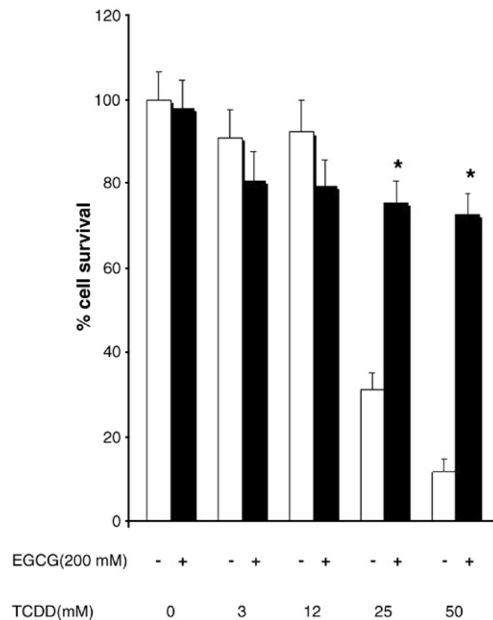

Fig. 5. Effect of 1 h preincubation with 200 μM epigallocatechin gallate (EGCG) on INS-1 cell survival after 1 h exposition to different concentrations of TCDD. Data are the mean ± S.E.M. of four different experiments. *$p<0.01$ vs. the corresponding TCDD dose without EGCG (Student's *t*-test).

bility only upon prolonged exposure to much higher concentrations (0.5 mM for 24 h) (Sakurai et al., 2001). The high toxicity of TCDD is confirmed by the ultrastructural observations, clearly indicating the presence of severe and widespread alterations of the exposed cells. Our observations indicate that TCDD-induced INS-1E cell death shared only partially the morphological features of apoptosis, whereas several aspects rather reminded the recently described new type of cell death, the "autophagic cell death" (Levine and Yuan, 2005). Indeed, numerous and large autophagic vacuoles were observed in the cytoplasm of cells treated with 12.5 and 25 nM TCDD. Of particular interest was the early and widespread involvement of mitochondria, whose morphology was altered even in the presence of the lowest TCDD concentrations and that were often recognizable inside the autophagic vacuoles.

Another interesting aspect of dioxin toxicity on INS-1E cells was its ability to rapidly induce an increase in intracellular calcium levels, as described in other cell types (Canga et al., 1988; Hanneman et al., 1996; Puga et al., 1997). As it is well known that perturbation in cellular calcium may be associated with early development of cell injury (for a review see e.g. Orrenius et al., 2003), this TCDD-induced increase of intracellular Ca^{2+} concentration might play an important role in the mechanism of dioxin toxicity in INS-1E cells. Indeed, a significant reduction of cell death was observed when INS-1E cells were exposed to TCDD in the presence of the calcium-chelating agent EGTA. The mechanism of the TCDD-induced rise in the cytoplasmic calcium concentration has not been so far clarified. A primary route for Ca^{2+} influx is through "store-operated channels" in the cell membrane (originally termed capacitative Ca^{2+} entry), probably activated by a fall in Ca^{2+} within the endoplasmic reticulum (Parekh and Putney, 2005). Our data are consistent with such a mechanism: indeed, we demonstrated that the significant TCDD-induced increase in cellular calcium is attributable both to a transient and quantitatively modest calcium release from intracellular stores and to a more relevant and prolonged entry of extracellular calcium into the cell. On the basis of our results concerning the TCDD-induced mitochondrial depolarization, it seems reasonable to hypothesize that the main target of calcium-mediated toxicity in INS-1E cells might be the mitochondria. In β-cells, mitochondria are crucial for the physiological stimulus-secretion coupling. In the consensus model of the glucose-stimulated insulin secretion, mitochondrial

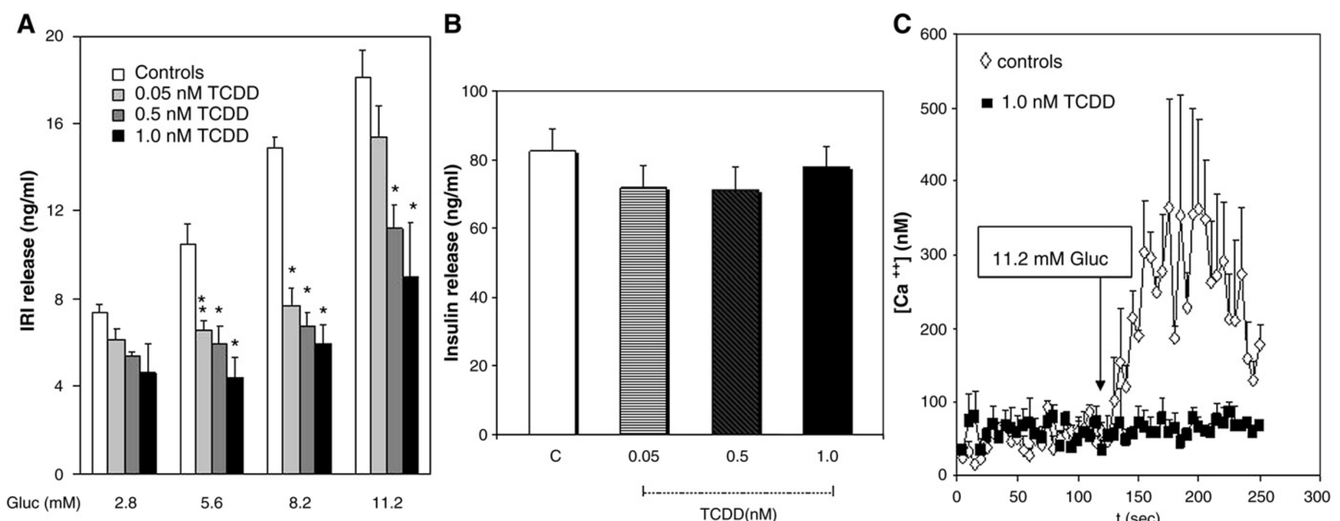

Fig. 6. A: Glucose-stimulated insulin secretion of INS-1E cells after 1 h exposition to 0, 0.05, 0.5 and 1.0 nM TCDD. Results are the mean ± S.E.M. of three different experiments. Statistical analysis (ANOVA) showed that the effects of either treatment (TCDD vs. untreated controls) and different TCDD concentrations were significant ($p<0.01$). *$p<0.01$ (vs. control cells at the same glucose concentration, Student's *t*-test). B: 30 mM KCl-stimulated insulin secretion of INS-1E cells after 1 h exposition to different TCDD concentrations. Results are the mean ± S.E.M. of three different experiments. C: Glucose-induced variations of the intracellular Ca^{2+} concentration in INS-1E cells incubated for 1 h in the presence or in the absence of 1.0 nM TCDD. Results are the mean ± S.E.M. of three different experiments.

metabolism of pyruvate, glycolitically derived from glucose, generates ATP, which promotes the closure of ATP-sensitive K^+ channels and consequent cell depolarization. This leads to Ca^{2+} influx through voltage-gated Ca^{2+} channels and increased cytosolic Ca^{2+} concentrations triggering insulin exocytosis (Maechler et al., 2006). Besides provision of ATP, mitochondria play important roles in other aspects of normal physiology and pathology. In particular, it is known that by accumulating calcium when cytosolic calcium levels are high mitochondria can play subtle roles in coordinating the complexities of intracellular calcium-signaling pathways (Brookes et al., 2004). On the other hand, it is also known that, despite its crucial role in regulating mitochondrial functions, calcium can also become a pathological stimulus eventually leading to apoptotic cell death (Brookes et al., 2004). To explain this apparent paradox (how Ca^{2+} can be both a physiological and pathological effector of mitochondrial function) it has been proposed that Ca^{2+} could modulate mitochondrial ROS production by several mechanisms (stimulation of TCA cycle and oxidative phosphorylation; stimulation of nitric oxide synthase; perturbation of mitochondrial antioxidant status) (Brookes et al., 2004). This interpretation could be of particular interest in the case of pancreatic β-cells that are considered an easy target of oxidative stress mediated-tissue damage because of their weak antioxidant defence mechanisms (Kaneto et al., 2006). On the other hand, it is known that dioxin can increase the mitochondrial ROS production (Senft et al., 2002), probably through a modification of the mitochondrial GSSG/GSH ratio (Shen et al., 2005), but whether this would be the case also in INS-1 cells will deserve further investigation. On the other hand, our results on the protective effects of EGCG against dioxin toxicity could be interpreted into the frame of this general hypothesis. Indeed, it is likely that EGCG can carry out its protective effect through its well known antioxidant properties (Surh et al., 2005).

Another major result of our research was the inhibition of glucose-induced insulin secretion induced by TCDD concentrations in the nanomolar range or below, indicating that dioxin can induce a significant perturbation of β-cells physiology early and at very low doses. At our knowledge, this is the first demonstration of a direct negative effect of very low doses of dioxin on the secretory capabilities of pancreatic β-cells, that could provide a new and sound biological basis for the epidemiological evidence of a correlation between accidental or occupational exposure to high doses of TCDD and glucose metabolism disorders (Longnecker and Daniels, 2001; Fujiyoshi et al., 2006). The biological basis of this correlation is still unclear, although a mechanism based on the opposing biochemical actions of the activated aryl hydrocarbon receptor and the peroxisome proliferator activated receptors (PPARs) has been proposed (Remillard and Bunce, 2002). It may be interesting to note that several factors which are currently under investigation for their pathogenetic role in the development of type 2 diabetes (e.g. the inhibition of PEPKC activity, the induction of fatty acid synthesis, and the effects on IL-1β and TNF-α) have been previously described in TCDD-treated rats (Weber et al., 1991; Stahl et al., 1993; Fan et al., 1997).

The observed dioxin-induced impairment of glucose-stimulated insulin secretion of INS-1E cells confirms our previous results showing a significant decrease of glucose-stimulated insulin secretion in islets isolated from TCDD-treated rats (Novelli et al., 2005). The mechanism by which TCDD can negatively influence the glucose-stimulated insulin secretion in INS-1E cells is not known. Our results demonstrate that 1 nM TCDD was able to inhibit glucose-stimulated insulin release while completely abolishing glucose-induced Ca^{2+} influx, that, according to the consensus model, is the ultimate trigger for insulin exocytosis. On the other hand, our results on the preserved KCl-stimulated insulin secretion (independent on glucose metabolism) strongly indicate that also mitochondrial alterations might play a crucial role in the inhibitory effect of TCDD on the secretory capabilities of INS-1E cells.

In conclusion, our results clearly demonstrate that TCDD is highly toxic for INS-1 cells, even at very low doses, thus providing a biological basis for the observed correlation between dioxin exposure and glucose metabolism disorders.

References

Arisawa, K., Takeda, H., Mikasa, H., 2005. Background exposure to PCDDs/PCDFs/PCBs and its potential health effects: a review of epidemiologic studies. J. Med. Invest. 52, 10–21.

Bock, K.W., Köhle, C., 2006. Ah receptor: dioxin-mediated toxic responses as hints to deregulated physiologic functions. Biochem. Pharmacol. 72, 393–404.

Brewster, D.W., Matsumura, F., 1984. TCDD reduces lipoprotein lipase activity in the adipose tissue of the guinea pig. Biochem. Biophys. Res. Commun. 122, 810–817.

Brookes, P.S., Yoon, Y., Robotham, J.L., Anders, M.W., Sheu, S-S., 2004. Calcium, ATP, and ROS: a mitochondrial love–hate triangle. Am. J. Physiol., Cell Physiol. 287, C817–C833.

Canga, L., Levi, R., Rifkin, A.B., 1988. Heart as a target organ in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity: Decreased β-adrenergic responsiveness and evidence of increased intracellular calcium. Proc. Natl. Acad. Sci. U.S.A. 85, 905–909.

Ebner, K., Brewste, D.W., Matsumura, F., 1988. Effect of 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin on serum insulin and glucose levels in the rabbit. J. Environ. Sci. Health, B Pestic. Food Contam. Agric. Wastes 23, 427–438.

Enan, E., Matsumura, F., 1994. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)-induced changes in glucose transporting activity in guinea pigs, mice and rats in vivo and in vitro. J. Biochem. Toxicol. 9, 97–106.

Enan, E., Liu, P.C.C., Matsumura, F., 1992a. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin causes reduction of glucose transporting activities in the plasma membranes of adipose tissue and pancreas from the guinea pig. J. Biol. Chem. 267, 19785–19791.

Enan, E., Liu, P.C.C., Matsumura, F., 1992b. TCDD causes reduction in glucose uptake through glucose transporters on the plasma membranes of the guinea pig adipocyte. J. Environ. Sci. Health B27, 495–510.

Fan, F., Yan, B., Wood, G., Viluksela, M., Rozman, K.K., 1997. Cytokines (IL-1β and TNF-α) in relation to biochemical and immunological effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in rats. Toxicology 116, 9–16.

Fujiyoshi, P.T., Michalek, J.E., Matsumura, F., 2006. Molecular epidemiologic evidence for diabetogenic effects of dioxin exposure in U.S. Air Force veterans of the Vietnam war. Environ. Health Perspect. 114, 1677–1683.

Hanneman, W.H., Legare, M.E., Barhoumi, R., Burghardt, R.C., Safe, S., Tiffany-Castiglioni, E., 1996. Stimulation of calcium uptake in cultured rat hippocampal neurons by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicology 112, 19–28.

Herbert, V., Lau, K.S., Gottlied, C.W., Bleicher, S.J., 1965. Coated charcoal immunoassay of insulin. J. Clin. Endocrinol. 25, 1375–1384.

Jayaraman, S., 2005. Flow cytometric determination of mitochondrial

membrane potential changes during apoptosis of T lymphocytic and pancreatic beta cell lines: Comparison of tetramethylrhodamine ethylester (TMRE), chloromethyl-X-rosamina (H2-CMX-Ros) and Mito Tracker Red 580 (MTR580). J. Immunol. Methods 306, 68–79.

Kaneto, H., Nakatani, Y., Kawamori, D., Miyatsuka, T., Matsuoka, T.A., Matsuhisa, M., Yamasaki, Y., 2006. Role of oxidative stress, endoplasmic reticulum stress, and c.Jun N-terminal kinase in pancreatic β-cell dysfunction and insulin resistance. Int. J. Biochem. Cell Biol. 38, 782–793.

Levine, B., Yuan, J., 2005. Autophagy in cell death: an innocent convict? J. Clin. Invest. 115, 2679–2688.

Liu, P.C., Matsumura, F., 1995. Differential effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the “adipose-type” and “brain-type” glucose-transporters in mice. Mol. Pharmacol. 47, 65–73.

Longnecker, M.P., Daniels, J.L., 2001. Environmental contaminants as etiological factors for diabetes. Environ. Health Perspect. 109 (Suppl. 6), 871–876.

Maechler, P., Carobbio, S., Rubi, B., 2006. In beta-cells, mitochondria integrate and generate metabolic signals controlling insulin secretion. Int. J. Biochem. Cell Biol. 38, 696–709.

McConnely, E.E., Moore, J.A., Dalgald, D.W., 1978. Toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rhesus monkeys (*Macaca mulata*) following a single oral dose. Toxicol. Appl. Pharmacol. 43, 175–187.

Merglen, A., Theander, S., Rubi, B., Chaffard, G., Wollheim, C.B., Maechler, P., 2004. Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. Endocrinology 145, 667–678.

Mitrou, P.I., Dimitriadis, G., Raptis, S.A., 2001. Toxic effects of 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin and related compounds. Eur. J. Int. Med. 12, 406–411.

Novelli, M., Piaggi, S., De Tata, V., 2005. 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin-induced impairment of glucose-stimulated insulin secretion in isolated rat pancreatic islets. Toxicol. Lett. 156, 307–314.

Olson, J.R., Holscher, M.A., Neal, R.A., 1980. Toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the golden Syrian hamster. Toxicol. Appl. Pharmacol. 55, 67–78.

Orrenius, S., Zhivotovsky, B., Nicotera, P., 2003. Regulation of cell death: the calcium-apoptosis link. Nat. Rev., Mol. Cell Biol. 4, 552–565.

Palermo, C.M., Westlake, C.A., Gasiewicz, T.A., 2005. Epigallocatechin gallate inhibits aryl hydrocarbon receptor gene transcription trough an indirect mechanism involving binding to a 90 kDa heat shock protein. Biochemistry 44, 5041–5052.

Parekh, A.B., Putney Jr., J.W., 2005. Store-operated calcium channels. Physiol. Rev. 85, 757–810.

Peterson, R.E., Seefeld, M.D., Christian, B.J., Potter, C.L., Kelling, C.K., Keesey, R.E., 1984. The wasting syndrome in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity: Basic features and their interpretation. In: Poland, A., Kimbrough, K.D. (Eds.), Biological Mechanism of Dioxin Action, Banbury Report, vol. 18. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 291–308.

Puga, A., Hoffer, A., Zhou, S., Bohm, J.M., Leikauf, G.D., Shertzer, H.G., 1997. Sustained increase in intracellular free calcium and activation of cyclooxigenase-2 expression in mouse hepatoma cells treated with dioxin. Biochem. Pharmacol. 54, 1287–1296.

Remillard, R.B., Bunce, N.J., 2002. Linking dioxins to diabetes: epidemiology and biologic plausibility. Environ. Health Perspect. 110, 853–858.

Sakurai, K., Katoh, M., Someno, K., Fujimoto, Y., 2001. Apoptosis and mitochondrial damage in INS-1 cells treated with alloxan. Biol. Pharm. Bull. 24, 876–882.

Senft, A.P., Dalton, T.P., Nebert, D.W., Genter, M.B., Hutchinson, R.J., Shertzer, H.G., 2002. Dioxin increases reactive oxygen production in mouse liver mitochondria. Toxicol. Appl. Pharmacol. 185, 74–75.

Shen, D., Dalton, T.P., Nerbert, D.W., Shertzer, H.G., 2005. Glutathione redox state regulates mitochondrial reactive oxygen production. J. Biol. Chem. 280, 25305–25312.

Stahl, B.U., Beer, D.G., Weber, L.W.D., Rozman, K.K., 1993. 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.

Surh, Y.J., Kundu, J.K., Na, H.K., Lee, J.S., 2005. Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals. J. Nutr. 135, 2993S–3001S (Suppl).

Swift, L.L., Gasiewicz, T.A., Dunn, G.D., Soule, P.D., Neal, R.A., 1981. Characterization of the hyperlipidemia in guinea pigs induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicol. Appl. Pharmacol. 59, 489–499.

Tiernan, T.O., Taylor, M.L., Garret, J.H., VanNess, G.F., Solch, J.G., Wagel, D.J., Ferguson, G.L., Schecter, A., 1985. Sources and fate of polychlorinated dibenzodioxins, dibenzofurans and related compounds in human environments. Environ. Health Perspect. 59, 145–158.

Tuomisto, J.T., Pohjanvirta, R., Unkila, M., Tuomisto, J., 1995. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced anorexia and wasting sindrome in rats: aggravation after ventromedial hypothalamic lesion. Eur. J. Pharmacol. 293, 309–317.

Weber, L.W.D., Lebofsky, M., Greim, H., Rozman, K., 1991. Key enzymes of gluconeogenesis are dose-dependently reduced in 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats. Arch. Toxicol. 65, 114–118.