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TCDD decreases ATP levels and increases reactive oxygen production through changes in mitochondrial F_0F_1-ATP synthase and ubiquinone

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Abstract

Mitochondria generate ATP and participate in signal transduction and cellular pathology and/or cell death. TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) decreases hepatic ATP levels and generates mitochondrial oxidative DNA damage, which is exacerbated by increasing mitochondrial glutathione redox state and by inner-membrane hyperpolarization. This study identifies mitochondrial targets of TCDD that initiate and sustain reactive oxygen production and decreased ATP levels. One week after treating mice with TCDD, liver ubiquinone (Q) levels were significantly decreased, while rates of succinoxidase and Q-cytochrome *c* oxidoreductase activities were increased. However, the expected increase in Q reduction state following TCDD treatment did not occur; instead, Q was more oxidized. These results could be explained by an ATP synthase defect, a premise supported by the unusual finding that TCDD lowers ATP/O ratios without concomitant changes in respiratory control ratios. Such results suggest either a futile cycle in ATP synthesis, or hydrolysis of newly-synthesized ATP prior to release. The TCDD-mediated decrease in Q, concomitant with an increase in respiration, increases complex 3 redox-cycling. This acts in concert with glutathione to increase membrane potential and reactive oxygen production. The proposed defect in ATP synthase explains both the greater respiratory rates and the lower tissue ATP levels.

Keywords

ATP; complex 3; energy coupling; F_0F_1-ATP synthase; mouse liver; mitochondria; oxidative stress; reactive oxygen; redox-cycling; respiration; TCDD; ubiquinone; UCP2

Abbreviations

COX - Cytochrome c oxidase; G6P - Glucose-6-phosphate; HMG-CoA - 3-hydroxy-3-methylglutaryl-CoA; ISP - Iron sulfur protein; KP_i - 0.1 M potassium phosphate buffer- pH 7.25; KCl-RB - Potassium chloride respiratory buffer; Q - Ubiquinone (Coenzymes Q_9 and Q_{10}); QCR - Ubiquinol cytochrome *c* reductase; RCR - Respiratory control ratio; SCR - Succinate cytochrome *c* reductase; SDH - Succinate dehydrogenase; SOX - Succinoxidase; Succinate-dependent oxygen consumption; SQR - Succinate ubiquinol reductase; TCDD - 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

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Introduction

All living cells conduct reduction-oxidation (redox) activities that are essential to cellular function. Many such processes, such as mitochondrial respiration, mono-oxygenase and oxidase activities, activate molecular oxygen by chemical reduction, forming reactive oxygen. Rapid or chronic production of reactive oxygen can overwhelm antioxidant defenses, leading to oxidative stress-related processes that may contribute to the development of aging, as well as several diseases, including cardiovascular disease, neurodegenerative disorders, alcohol-related diseases, and cancer (Ames *et al.*, 1993; Cadenas *et al.*, 2000). Mitochondria are the major source of reactive oxygen in most mammalian cell types, as well as a major target organelle for oxidative damage (Chance *et al.*, 1979; Chomyn *et al.*, 2003). It has been estimated that 1–2% of oxygen consumed systemically is converted to reactive oxygen by mitochondria (Cadenas and Davies, 2000). Mitochondrial superoxide and H_2O_2 can cause direct damage to mitochondrial proteins (Lenaz, 1998), result in nuclear and mitochondrial genotoxicity (Shen *et al.*, 2005; Richter, 1995; Esposito *et al.*, 1999), and initiate apoptosis (Bossy-Wetzel *et al.*, 1999; Polster *et al.*, 2004; Gulbins *et al.*, 2003). In addition to mediating cytotoxicity, reactive oxygen also alters cellular signal transduction, involving homeostatic growth factors and cytokines that can modify the cell cycle and regulate mitosis (Sundaresan *et al.*, 1995; Lo *et al.*, 1996).

Since mitochondrial reactive oxygen is involved in regulating pathologic and homeostatic cellular events, it is important to understand the pathways involved in the generation of mitochondrial reactive oxygen under normal and pathological conditions, and during exposure to environmental chemicals, including the environmental toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD is a persistent and ubiquitous environmental contaminant and toxicant (Grassman *et al.*, 1998). TCDD is also a very potent tumor promoter in mice, about 10,000 times more potent than the commonly studied tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Poland *et al.*, 1982). Like TPA, TCDD generates reactive oxygen which may be important to its action as a tumor promoter. Most biological activities of TPA and TCDD are signaled through gene regulation involving DNA response elements, the TPA response element (TRE) and the aryl hydrocarbon receptor response element (AHRE), respectively.

We have previously shown that TCDD elicits sustained hepatic oxidative conditions that require several days to develop and persist for at least 8 weeks following a low dose administration of TCDD (Shertzer *et al.*, 1998; Senft *et al.*, 2002a). Reactive oxygen production is dependent on the AHR but not on the AHR monooxygenase target genes, *Cyp1a1* and *Cyp1a2* (Senft *et al.*, 2002b). Although TCDD increases microsomal reactive oxygen production (Shertzer *et al.*, 2004b; Shertzer *et al.*, 2004a), reactive oxygen derived from mitochondria (Senft *et al.*, 2002a; Senft *et al.*, 2002b) appears to be more biologically relevant, since TCDD induces preferential oxidative damage to mitochondrial DNA over nuclear DNA (Shen *et al.*, 2005). In response to TCDD-induced oxidative stress, reduced glutathione (GSH) levels increase in both cytosol and mitochondria (Senft *et al.*, 2002a; Senft *et al.*, 2002b; Shen *et al.*, 2005). GSH functions as an efficient one-electron and two-electron radical scavenger (Dalton *et al.*, 2004), and in addition reduces H_2O_2 to water via glutathione peroxidase (GPX). The *de novo* synthesis of GSH is regulated by glutamate cysteine ligase (*Gcl*), a gene under the control of electrophile response elements (Dalton *et al.*, 2004). The scavenging and metabolic functions of GSH are especially important in mitochondria which contain GPX1, since catalase, which also degrades H_2O_2, is absent in the mitochondria of most animal cells (Esworthy *et al.*, 1997). Thus, higher levels of mitochondrial GSH would result in less H_2O_2 release to the cytosol.

In addition to simply elevating levels of GSH, TCDD also produces an increase in the chemical reduction state of glutathione, as measured by the GSSG/2GSH redox couple (Shen *et al.*, 2005). Manipulation of this redox couple in isolated mitochondria increased reactive oxygen production proportional to the increase in reduction potential. The GSSG/2GSH-driven increase in reactive oxygen production by TCDD appeared to be driven by mitochondrial membrane hyperpolarization under ADP-limited state 4 conditions. It therefore appeared that TCDD initiated a positive feedback loop, where the additional reactive oxygen produced by TCDD could be explained by the change in chemical reduction potential of GSSG/2GSH and the resulting membrane hyperpolarization, and the increase in GSH could be explained by the TCDD-induced production of reactive oxygen. The argument is circular, and it remains to be determined how TCDD initiates and sustains this cycle of events.

In order to answer this question, we examined our previous observations that TCDD treatment lowered hepatic levels of ATP, and increased the consumption of O_2 in respiratory states 3 and 4 (Senft *et al.*, 2002a; Senft *et al.*, 2002b; Shen *et al.*, 2005). This paper presents new findings regarding alterations in complex 3 and in F_0F_1-ATPase (ATP synthase) that allow us to explain the changes in respiration and ATP levels, in the context of TCDD-mediated reactive oxygen production. A comprehensive model is proposed which is consistent with known results regarding the changes in liver mitochondria by TCDD.

Methods

Chemicals

TCDD was purchased from Accustandard (New Haven, CT). All other chemicals and reagents were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO) as the highest available grades.

Animals and treatment

Experiments involving mice were performed according to the National Institutes of Health standards for care and use of experimental animals and the University of Cincinnati Institutional Animal Care and Use Committee. Animals were group-housed, maintained on a 12-h light/dark cycle, and had access to standard rodent chow and water *ad libitum*. C57BL/6J inbred male mice (8–12 weeks of age) were purchased from The Jackson Laboratories (Bar Harbor, ME).

Mice were administered a single dose of TCDD (5 µg/kg body weight) in corn oil by intraperitoneal injection; controls were given equivalent volumes of corn oil. At 7 days following treatment, the mice were killed by carbon dioxide asphyxiation. The liver was excised and washed in ice-cold 0.9% NaCl. A 10% whole homogenate was prepared in 250 mM sucrose, 1 mM EDTA, and 1 mM EGTA, 0.1% defatted and recrystallized bovine serum albumin, 10 mM HEPES, pH 7.2, using a motor-driven (500 rpm) Potter-Elvehjem homogenizer. A mitochondrial fraction was prepared as described previously (Senft *et al.*, 2002b), and suspended in a potassium chloride respiratory buffer (KCl-RB), consisting of 140 mM KCl, 0.1 mM EDTA, 2.5 mM KH_2PO_4, 2.5 mM $MgCl_2$ and 0.05% bovine serum albumin, in 5 mM HEPES, pH 7.4.

Mitochondrial respiration and energy coupling

Mitochondrial oxygen consumption was measured polarographically with a computer-controlled Clark-type oxygen electrode (Hansatech Instruments; Norfolk, England). The respiratory mixture consisting of 0.5 ml of KCl-RB, 20 mM glucose, 100 U hexokinase and 100 µg of mitochondrial protein was equilibrated at 37°C with stirring. Succinate-stimulated state 4 respiration (ADP-limited) was determined after adding 6 mM succinate. The rate of

state 3 respiration was then determined following the addition of 0.25 mM ADP. The respiratory control ratio (RCR) was calculated as the ratio of state 3 to state 4 respiration. In order to determine ATP synthesis, a 400 μl aliquot of respiratory mixture was mixed with 400 μl ethanol containing 15 μl concentrated formic acid. After centrifugation, the supernatants were dried under a stream of argon gas. ATP production was determined as glucose-6-phosphate (G6P) generated in the respiratory mixture by the ATP-dependent hexokinase reaction. Following the G6P-dependent reduction of $NADP^+$ by G6P dehydrogenase, NADPH was quantified using a luciferase-based luminescence assay (Jungling *et al.*, 1996). Standards for G6P were produced by adding known amounts of ATP directly to the respiratory mixture.

Reactive oxygen assays

H_2O_2 production was monitored in freshly-prepared mitochondria as catalase-inhibited luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) chemiluminescence (Senft *et al.*, 2002a; Senft *et al.*, 2002b). The reaction mixture consisted of 5 μM luminol, 2.5 U/ml horseradish peroxidase, 50 μg of mitochondrial protein and KCl-RB, in a final volume of 1.0 ml, and the reaction was initiated by the addition of 6 mM sodium succinate, and monitored at 37°C. The H_2O_2 luminescence signal was completely quenched by 500 U catalase/ml.

Mitochondrial membrane potential and membrane fluidity

The mitochondrial inner membrane potential was quantified using the cationic lipophilic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (Reers *et al.*, 1995; Cossarizza *et al.*, 1993). Mitochondrial protein (10 μg) was incubated with 0.05 nmol JC-1 in 1.0 ml KCl-RB. Fluorescence ratios were determined at 37°C, using our experimentally determined wavelength pairs, as the ratio of red (Ex = 488 nm; Em = 595 minus 620 nm) to green fluorescence (Ex = 488 nm; Em = 535 minus 510 nm). The fluorescence ratios were standardized to membrane potential by applying known concentration gradients of potassium in the presence of valinomycin across the membrane and applying the Nernst equation to calculate potassium diffusion potentials, as previously described (Shen *et al.*, 2005).

The fluorescence probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) were used to probe membrane fluidity at the interior and surface of mitochondrial membranes, respectively (Azzi, 1974; Shinitzky *et al.*, 1978). Briefly, 1 μM final probe concentration was added to 1 mg mitochondrial protein/ml, and fluorescence intensity measurements were determined at 25°C with excitation (357 nm) and emission (428 nm) polarization filters in the parallel and perpendicular orientations. Fluorescence polarization anisotropy was calculated as an r value (Shertzer *et al.*, 1991), where lower values correspond to greater membrane fluidity.

Western blot for UCP2

Western blot analysis for uncoupling protein-2 (UCP2) was performed by separating 40 μg of mitochondrial protein on a denaturing 10% polyacrylamide gel and transferring proteins to nitrocellulose. After verification of equivalent loading using Ponceau S, the membrane was blocked with 3% bovine serum albumin in 10 mM phosphate buffer (pH 7.4) containing 0.9% saline and 0.1% Tween 20 (PBST). Primary anti-UCP2 antibody (Alpha Diagnostic International, San Antonio, TX) was applied at 1:500 dilution in PBST at 4°C overnight. After washes in PBST, horseradish peroxidase-conjugated anti-rabbit secondary was applied at a dilution of 1:1000 for 30 min at room temperature. After PBST washes, the membrane was incubated in enhanced chemiluminescence reagent (ECL; Amersham) and exposed to x-ray film.

Ubiquinones

Reduced and oxidized Q_9 (ubiquinol 9/ubiquinone 9; coenzyme Q_9) and Q_{10} (ubiquinol 10/ubiquinone 10; coenzyme Q_{10}) were extracted and quantified by a modification of the described HPLC procedure (Takada *et al.*, 1984; Takada, 1989). A freshly prepared mitochondrial suspension (2.5 mg in 500 μl) was extracted with 3.5 ml of ethanol:hexane (2:5), which had been flushed with argon. After centrifuging at 1000*g* for 3 min, the upper hexane layer was transferred to a clean borosilicate glass test tube. The extraction was repeated three times, flushing with argon after each extraction, and the combined hexane layer was dried under a stream of argon. The resulting residue was dissolved in 100 μl of argon-flushed ethanol for HPLC analysis. A Waters 3.9 mm X 150 mm (4 Å) NovaPak C18 reverse phase column was used with isocratic conditions with solvents A:B (40:60). Solvent A was 95% methanol. Solvent B was ethanol:methanol:water:trifluoroacetic acid (55:40:4.9:0.1). The detection wavelength was 283 nm, a wavelength that yields about 85% maximal absorbance for both reduced Q_9 and reduced Q_{10}. The sample injection volume was 30 μl with a solvent flow rate of 1.0 ml/min. Chemically reduced Q_9 and Q_{10} standards were produced by treating oxidized Q with $NaBH_4$ (Takada, 1989). The time from preparation of mitochondria to completion of HPLC was under 6 h.

Other assays

Cytochrome *c* oxidase (COX) (Schneider *et al.*, 1972), succinic dehydrogenase (SDH) (Ackrell *et al.*, 1978), and ubiquinol-cytochrome *c* reductase (QCR) (Gutierrez-Cirlos *et al.*, 2002) were assayed as described. Succinate-cytochrome *c* reductase (SCR) was measured in 20 mM Tris-SO_4 buffer (pH 7.5) containing 10 μM ferricytochrome *c*, 0.5 mM KCN and 150 μg mitochondrial protein. The reaction was initiated by the addition of 40 mM sodium succinate, maintained at 37°C, monitored at 550 nm, and quantified using $E_M^{550 \text{ nm}} = 18{,}500 \text{ M}^{-1}\text{cm}^{-1}$ (Trumpower *et al.*, 1979). Succinate-ubiquinol reductase (SQR) was assayed under conditions similar to SCR, but without cytochrome *c* in the reaction buffer. After adding succinate, the reaction was monitored at 283 nm. The experimentally-determined extinction coefficient, assuming equal reduction of Q_9 and Q_{10} is $E_M^{283 \text{ nm}} = 10{,}400 \text{ M}^{-1}\text{cm}^{-1}$. Protein was measured by the bicinchoninic acid method (Pierce Chemical Co.; Rockford, IL), according to details provided by the manufacturer.

Statistics

Statistical significance of the differences between group sample mean values were determined as indicated in the legends to the tables and figures. Statistics were performed using SigmaStat Statistical Analysis software (SPSS Inc., Chicago, IL).

Biohazard precaution

TCDD is highly toxic and a likely human carcinogen. All personnel were instructed in safe handling procedures. Lab coats, gloves and masks were worn at all times, and contaminated materials were collected separately for disposal by the Hazardous Waste Unit or by independent contractors. TCDD-treated mice were housed separately, and their carcasses were treated as contaminated biological materials.

Results

We showed previously that mitochondria isolated from livers of mice exposed to low dosages of TCDD showed an increase in succinate-generated mitochondrial membrane potential, an effect apparently exacerbated by GSH-mediated closure of the mitochondrial permeability transition pore (MPTP) (Shen *et al.*, 2005). We have not previously considered the likely possibility that a decrease in uncoupling protein 2 (UCP2, the sole UCP in liver), or modified

UCP2 function, might contribute to the increased membrane potential following TCDD exposure. The Western immunoblots (Fig. 1) do not show differences in levels of UCP2 protein between mice treated with TCDD and vehicle control mice. We also determined the functional activity of UCP2 on H_2O_2 production and membrane potential (parameters of UCP2 function). We found that the UCP2 allosteric fatty acid activator oleic acid decreased H_2O_2 production and membrane potential in a dose-dependent manner in mitochondria from both control and from TCDD-treated mice (Fig. 1). In addition, the UCP2 allosteric nucleotide phosphate inhibitor GDP increased H_2O_2 production and membrane potential in a dose-dependent manner in mitochondria from both control and from TCDD-treated mice. A two-way ANOVA revealed that treatment with TCDD modified H_2O_2 production and membrane potential in a manner that was dependent on the concentration of oleate (in the absence of GDP), but not dependent on the concentration of GDP (in the absence of oleate). That is, there was a statistically significant interaction between TCDD and oleate, at the $P<0.001$ level. Such results should be expected, since the hyperpolarized mitochondrial membrane generated by TCDD would be depolarized by oleate, which in turn would minimize the ability of mitochondria to generate H_2O_2. A three-way ANOVA was then used to evaluate the combined effects of oleate (0, 25, 50, and 200 μM) and GDP (0, 25, 50, and 200 μM) on the effects of TCDD treatment on H_2O_2 production and membrane potential (data shown in part in Fig. 1). The results showed that the level of GDP did not alter the effect of TCDD on H_2O_2 production or membrane potential at any level of oleate. Stating this in mechanistic terms, the ability of oleate to increase membrane permeability (open the permeability transition pore and uncouple) is dominant over the ability of GDP to decrease membrane permeability (closing the permeability transition pore), both with and without TCDD treatment. We conclude that there are no major changes in the amount of protein or the allosteric regulation of UCP2 in TCDD-treated mice.

We next began experiments designed to explain how TCDD increased respiration and decreased ATP synthesis, in the context of the increase in reactive oxygen production. We have reported previously the absence of major changes in cytochromes b, $c+c_1$ or $a+a_3$ following TCDD exposure (Senft *et al.*, 2002a). In order to localize the site(s) responsible for TCDD-stimulated increased succinate-dependent respiration activity, we assayed activities of specific regions within succinoxidase (SOX, complexes 2, 3 and 4). We assayed activities of succinic dehydrogenase (SDH, complex 2), succinate ubiquinol reductase (SQR, complex 2), succinate-cytochrome c reductase (SCR, complexes 2 and 3), ubiquinone cytochrome c reductase (QCR, complex 3), and cytochrome c oxidase (COX, complex 4). While SDH and SQR activities were not changed by TCDD treatment, and COX was slightly decreased, SCR and QCR activities were higher, suggesting that complex 3 is rate limiting for state 4 succinate-dependent respiration (Fig 2).

A major component of complex 3 is ubiquinol/ubiquinone (Q; 2,3-dimethoxy-5-methyl-6-polyprenyl-1,4-benzoquininone), where the polyprenylated side chain is about 85% 9 units long (Q_9) in rodents, with the rest 10 units long (Q_{10}) (Huertas *et al.*, 1991). Since Q is involved in both respiration via the proton-motive Q cycle, and reactive oxygen production via redox-cycling, we assayed for mitochondrial levels of reduced and oxidized Q_9 and Q_{10}. We found that the concentrations of both forms of Q to be substantially diminished in liver mitochondria following TCDD treatment (Fig. 3). If Q_9 and Q_{10} were a homogeneous pool, then the reduction states for both forms should be similar, and a diminution of Q levels should increase the reduction state of Q. This was not the case. First, Q_9 and Q_{10} had very different reduction states, with Q_9 being primarily oxidized and Q_{10} primarily reduced. The greater reduction state of Q_{10} over Q_9 has been observed previously (Takada *et al.*, 1984), although to a lesser extent. Second, Q_9 and Q_{10} behaved as separate pools, with Q_{10} essentially unchanged in reduction state, while Q_9 was significantly less reduced following TCDD treatment.

Although lower levels of Q in association with greater oxygen consumption would produce higher rates of Q redox-cycling and higher levels of reactive oxygen production, the forces driving the increase in respiration under both state 3 and state 4 conditions remained obscure. We speculated that Q could cycle more rapidly if Q diffusion rate within the mitochondrial membrane were to increase, such as in an environment of greater membrane fluidity. Such fluidity changes would be expected under conditions of reactive oxygen production and resulting lipid peroxidation that would decrease the chain length of fatty acyl moieties. Fig. 4 shows that TCDD treatment leads to an increase in membrane fluidity (decrease in fluorescence polarization anisotropy, or r value), both in the more hydrophobic interior (using the fluorescence probe DPH), as well as at the membrane surface (using the fluorescence probe TMA-DPH). A greater increase in fluidity occurred at the membrane surface, which would be expected as a result of greater exposure of the membrane surface to reactive oxygen.

Although an increase in membrane fluidity would be sufficient to stimulate respiration, it could not explain an increase in Q oxidation state. We reasoned that Q could redox cycle more rapidly and also increase in oxidation state, if a site rate-limiting for respiration were to be relieved downstream. Since the rate constant for COX decreases following TCDD treatment, it is not likely that COX activity changes could explain the increase in respiration and the increase in Q oxidation state. We therefore considered a second candidate site, the F_0F_1-ATP synthase, complex 5. Under state 4 conditions, complex 5 is the major rate-limiting factor in respiration, and even under state 3 conditions, respiratory uncouplers such as carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) or calcium stimulate O_2 consumption. It should be remembered that states 4 and 3 represent extreme conditions of external ADP deficiency and saturation, respectively. *In vivo*, mitochondria spend most of their time somewhere between the two extremes.

We examined the possibility of a defect in complex 5 under state 3 (not ADP-limited) conditions (Table 1). The rate of ATP production in mitochondria from TCDD-treated mice was about 60% of control rates, while mitochondrial O_2 consumption nearly doubled. As a result, TCDD treatment generated ATP/O ratios that were only a third of control values. The resulting deficit in mitochondrial ATP production is consistent with a tissue phenotype of significantly diminished levels of ATP (Table 1). It should be noted that when limiting amounts of ADP were added to state 4 (ADP-limited) mitochondria from either control or TCDD-treated mice, there was no burst of increased respiration, and following the utilization of ADP in state 3, mitochondria returned to state 4 respiratory rates. Such a result indicates that the F_0F_1-ATP synthase was not simply catalyzing a rapid futile cycle of ATP synthesis and hydrolysis, supported by trace levels of adenine nucleotides.

Under state 3 conditions, H_2O_2 production is low as expected (Table 1), since the membrane potential is at a low steady-state level, with positive pressure due to proton translocation linked to electron transport, and proton-motive force collapse due to proton flux through complex 5 linked to ATP synthesis. The extreme dependence of H_2O_2 production on membrane potential is shown in Fig. 5. As mitochondria become more uncoupled, H_2O_2 production decreases much more rapidly than RCR or membrane potential, such that a reduction in state 4 succinate-dependent membrane potential by 50% nearly abolishes the production of H_2O_2.

We (Senft *et al.*, 2002a) and others (Nohl *et al.*, 1989) have shown that high concentrations of TCDD, in the range of 1000 pmol TCDD/mg mitochondrial protein, can affect respiration directly. We therefore further examined the potential for TCDD to have a direct effect in lowering ATP/O ratios. At a low concentration of 100 pmol/mg protein, TCDD stimulated respiration, but did not alter the RCR (Table 2). However, ATP synthesis was significantly decreased by TCDD, with a resulting decrease in the ATP/O ratio to less than half the control value. At a high concentration of 1000 pmol/mg protein, TCDD inhibited respiration, but did

not affect the RCR. ATP production was more severely inhibited than at the low concentration of TCDD, due presumably to inhibition of respiration at the level of complex 3 (Senft *et al.*, 2002a; Nohl *et al.*, 1989). Clearly, different mechanisms are in play at low or at high concentrations of TCDD. We do not consider the results obtained using the high concentration as toxicologically relevant, and focus the discussion on the results obtained using the lower concentration.

Discussion

One week after TCDD treatment of mice, a number of hepatic alterations are observed, including a decrease in tissue ATP, and increases in mitochondrial succinate-dependent respiration, reactive oxygen production, GSH levels and membrane potential (Shertzer *et al.*, 1998; Senft *et al.*, 2002a; Senft *et al.*, 2002b; Shen *et al.*, 2005). The mitochondrial electrochemical membrane potential is generated primarily via proton separation across the inner membrane at coupling sites in complexes 1, 3 and 4. We previously showed that in mice treated with 5 μg TCDD/kg body weight (the same dosage used in the present study), TCDD generates an increase in mitochondrial reactive oxygen production and membrane potential though a mechanism involving an elevated reduction state of glutathione. GSH then prevents mitochondrial permeability transition pore (MPTP) opening and diminishes MPTP flicker (Shen *et al.*, 2005). Most importantly, TCDD generated 2-fold and 4-fold increases in formamidopyrimidine DNA *N*-glycosylase (FPG)-sensitive cleavage sites in hepatic nuclear and mitochondrial DNA, respectively (Shen *et al.*, 2005). These results suggest strongly that TCDD not only increases the production of reactive oxygen, but actually generates an oxidative stress response, in that the antioxidant defense systems of the cell have been sufficiently overwhelmed to allow oxidative DNA damage.

Besides the MPTP, a major pathway for regulating membrane potential involves mitochondrial uncoupling proteins (UCPs). Although UCP2 is expressed in almost all tissues (Tu *et al.*, 1999; Yamada *et al.*, 1998), it is the major UCP expressed in liver, where levels can be modified by physiological conditions and inducing agents (Grav *et al.*, 2003; Ribeiro *et al.*, 2004). In addition, UCP2 is regulated allosterically by metabolic intermediates, including fatty acid activators such as oleate, and the purine nucleotide inhibitors such as GDP (Jaburek *et al.*, 1999). Since UCP2 has been shown to be induced under conditions of oxidative stress (Pecqueur *et al.*, 2001), it was a surprise to find that TCDD did not alter the expression levels of UCP2 protein. Neither did TCDD modify the inhibition by oleate or stimulation by GDP on either mitochondrial membrane potential or the succinate-dependent production of H_2O_2. Thus, UCP2 is not of major importance in the TCDD-induced mitochondrial oxidative stress response. The lack of UCP2 induction under oxidative stress conditions may be the result of the increased mitochondrial thiol reduction state (Shen *et al.*, 2005), which may serve to suppress transcriptional activation of UCP2.

Since the TCDD-induced mitochondrial membrane hyperpolarization is not relieved through UCP2 or the MPTP, then thermodynamic considerations predict an increase in the energy required to generate a proton electrochemical gradient. This would increase the reduction state of components of complex 3, thereby increasing redox-cycling and reactive oxygen production (Senft *et al.*, 2002b). Contrary to these expectations, we found that hepatic mitochondrial Q was in a lower reduction state following TCDD treatment. It was important to explain this finding in relationship to our previously reported TCDD-mediated increase in oxygen consumption without a change in RCR (Senft *et al.*, 2002a; Senft *et al.*, 2002b).

This study showed that by adding TCDD to liver mitochondria from untreated mice, we could produce a respiratory phenotype similar to that observed in mitochondria from TCDD-treated mice. Thus, TCDD may have a direct effect *in vivo* in uncoupling respiration from net ATP

synthesis, by generating a futile cycle at the level of F_1. Although this is uncoupling in the truest sense of the term (Skulachev, 1998), the RCR is not changed by TCDD. RCR is often used as an indicator of the degree of mitochondrial coupling; in fact, it is only valid to the extent that it represents the dependency of respiration on the presence of ADP. It does not necessarily, however, represent the dependency of respiration on ATP synthesis and release. Our data shows that following TCDD treatment, respiration remains dependent on ADP (no change in RCR), but ATP synthesis sharply decreases (decrease ATP/O ratio). A precedent for such effects may occur with the inhibitor BMS-199264, which inhibits ATP hydrolysis without affecting ATP synthesis (Grover *et al.*, 2004). BMS-199264 is a 5-ring heterocycle with some structural resemblance to TCDD. The direct effect of TCDD in decreasing ATP/O ratio without a change in RCR may be explained by a direct interaction of TCDD within complex 5, specifically with the β subunit of the mitochondrial F_0F_1-ATPase (ATP synthase) (Papa *et al.*, 2000), or with inhibitor protein IF-1 that is bound to the β subunit (Ichikawa *et al.*, 2005). The current notion of ATP synthesis at the inner membrane involves a highly hydrophobic proton channel (F_0) that binds a proton-driven rotor, OSCP and several other proteins (Devenish *et al.*, 2000), which in turn rotates within the α and β subunits of F_1 (Papa *et al.*, 2000). The β subunits are the sites of ATP synthesis, and can exist in 3, or possibly 4, major configurations, depending on its orientation relative to the rotating shaft (Fig. 6) (Bianchet *et al.*, 2000). Since there is no change in RCR, the dependency of respiration on ADP is unchanged by TCDD, suggesting that TCDD is not acting on the open configuration or the loose (ADP + Pi)-binding configuration. TCDD, a highly hydrophobic molecule, is proposed to act at the level of the nucleotide tight-binding site, which is shielded from the medium. There the effective ATP concentration is low, and ADP + Pi concentrations are high, resulting in ATP formation, driven by a negative free energy of formation due to a low mass action ratio of $[ATP][H_2O]/[ADP][Pi]$. TCDD may change the conformation of the tight-binding site, such that shielding from the medium is less complete and the mass action ratio increases to make the free energy of ATP formation less negative. Such a situation would reduce the probability of ATP formation with each turn of the rotating shaft, such that net ATP formation is reduced, but not eliminated.

An alternative mechanism to explain the decrease in ATP/O ratio observed in liver mitochondria from TCDD-treated mice is a decrease in the number of protons pumped per O consumed. The argument against this mechanism is the expectation that a decrease in protons pumped per O would result in a lower membrane potential under state 3 conditions, whereas we observed no change in membrane potential. Therefore, we consider this a viable but minor contributing mechanism. Another possible mechanism for the decrease in ATP/O ratios is a rapid futile cycling of ATP synthesis and hydrolysis, catalyzed by F_0F_1-ATP synthase and supported by trace levels of adenine nucleotides. If this were the case, then it would be expected that trace or limiting amounts of ADP added to mitochondria under state 4 conditions would produce a burst of increased respiration, and mitochondria would not return to state 4 respiration following the depletion of ADP. In fact, neither of these events occurred.

A second striking data set resulting from these studies is effects of TCDD on liver mitochondrial Q. Although Q_9 and Q_{10} are both decreased in concentration following TCDD treatment, Q_9 appears to exhibit a greater degree of decrease, as well as a diminution in chemical reduction state. Q_{10} does not show such a decrease in chemical reduction state following TCDD treatment, but is present at a much higher level of chemical reduction than is Q_9. These data suggest that Q_9 and Q_{10} are distinct, yet probably overlapping, functional pools of Q in the mitochondria.

Q appears to have multiple functions in the mitochondrial inner membrane. Q is the only lipid-soluble chain-breaking antioxidant that is synthesized endogenously, being formed from intermediates in the cholesterol metabolic pathway (James *et al.*, 2004; Genova *et al.*, 2003).

Q also has antiapoptotic properties, but these seem to be independent of its ability to act as an antioxidant (Papucci *et al.*, 2003). Most importantly in the context of the current study, Q is an integral component of respiration as a major constituent in the proton-motive Q cycle (Mitchell, 1975). Furthermore, Q is involved in regulating the MPTP by preventing MPTP opening, since it was shown in a recent study that Q_{10} is able to counteract mitochondrial membrane depolarization, ATP depletion, cytochrome *c* release, caspase-9 activation and DNA fragmentation, in keratinocytes upon apoptotic stimuli (Turunen *et al.*, 2004; Papucci *et al.*, 2003). This mechanism does not appear to be operative for TCDD-induced oxidative stress, since Q level decreases while membrane potential increases following TCDD treatment. It has also been reported that an association with oxidized Q may be required for activation of UCPs to enhance proton transport, an association that would tend to dissociate this Q pool from those participating in the proton-motive Q cycle or from Q acting as antioxidant (Echtay *et al.*, 2000). However, this property of Q in acting as a cofactor for UCP has been called into question (Jaburek *et al.*, 2003; Esteves *et al.*, 2004).

With such diverse functions, it seems improbable that Q exists as a single homogeneous pool of metabolite. Although Q is synthesized in all mammalian tissues at a rate sufficient to supply its diverse cellular needs (Turunen *et al.*, 2004; Dallner *et al.*, 2003), the regulation of Q metabolite pool levels is not fully understood. Biosynthesis of the isoprenoid moiety of Q (Szkopinska, 2000) is regulated in part by nuclear receptors, including the 9-*cis*-retinoic acid receptor (RXRα) and the peroxisome proliferator-activated receptor (PPARα). In RXRα-deficient mice, the hepatic Q content was only half that of wild-type liver, a decrease related to a constitutive lower rate of biosynthesis (Bentinger *et al.*, 2003). RXR is a transcriptional dimerization partner with a number of other receptors, such as all-*trans*-retinoic acid receptor (RAR), liver X receptor (LXR), and farnesoid X receptor (FXR). Both LXR and FXR are related to lipid metabolism associated with the mevalonate pathway, a biosynthetic pathway from acetyl CoA to farnesyl pyrophosphate, the substrate for the biosynthesis of Q, cholesterol, dolichol and isoprenylated proteins (Dallner *et al.*, 2003; Turunen *et al.*, 2002). The ligand-activated PPAR-RXR heterodimeric transcription factor binds the peroxisome proliferator response element (PPRE), which in turn upregulates the downstream mitochondrial HMG-CoA synthase gene (Rodriguez *et al.*, 1994; Hegardt, 1999). Under *in vivo* conditions, the different nuclear receptors compete for binding with RXR, bringing about a mechanism for cross-talk. TCDD is clearly involved in these retinoid signaling pathways, since all-*trans*-retinoic acid (a ligand for RAR) is increased, while 9-*cis*-retinoic acid (a ligand for RXRα) is decreased, as a result of exposure to TCDD (Schmidt *et al.*, 2003). In addition, data from AHR null mice suggest the AHR is directly involved in the retinoid signaling pathway by TCDD (Andreola *et al.*, 1997; Puga *et al.*, 2005). Enzymes involved in such signaling include retinaldehyde dehydrogenase, CYP1A1, CYP1A2, CYP2S1 and CYP2C39 (Saarikoski *et al.*, 2005; Puga *et al.*, 2005). Moreover, the amount of Q in liver is regulated by dietary vitamin A (retinol), with vitamin A deficiency increasing Q, and vitamin A administration decreasing Q (Sohlenius-Sternbeck *et al.*, 2000). Vitamin A is the precursor of all-*trans*-retinoic acid. So, it is likely that reduction of the amount of Q in liver mitochondria after TCDD treatment is mediated by increased levels of all-*trans*-retinoic acid, and concomitant decreases in 9-*cis*-retinoic acid, regulated by TCDD through the AHR. These pathways are depicted in Fig. 7.

An alternative mechanism for the reduction in mitochondrial Q levels is based on our previous observation that plasma cholesterol levels increased following TCDD treatment (Dalton *et al.*, 2001). If farnesyl pyrophosphate is rate-limiting in concentration and represents a regulated branch point in metabolism (Szkopinska, 2000), then it might be expected that TCDD increases the synthesis of steroids at the expense of Q. This possibility is speculative, especially since inhibition of squalene synthase by squalestatin did not increase the synthesis of Q, suggesting that the pathway to Q synthesis is saturated with respect to farnesyl pyrophosphate (Hartmann *et al.*, 2000).

In addition to the decrease in the content of mitochondrial Q, our data indicate a marked difference in chemical reduction state between Q_{10} (75–80% reduced) and Q_9 (15–20% reduced). Such data suggests the presence of an oxidation state crossover point, reflective of one or more rate-limiting sites located between the more oxidized and the more reduced redox couples in the pathway. In the case of complex 3, the rate-limiting steps are likely to be the electron transfers between Rieske iron-sulfur proteins (ISPs) and Q, determined primarily by the differences between the midpoint potentials of the ISP clusters and the Q/QH_2 redox couple (Denke *et al.*, 1998). Fig. 6 shows a model where Q_{10} is located more proximate to substrate than Q_9, and would be the primary form of Q to accept electrons from complexes 1 and 2. By this model, Q_{10} would transfer electrons to Q_9 which in turn would enter into the proton-motive Q cycle. This argument is thermodynamically reasonable, to the extent that Q_9 has a more positive standard midpoint reduction potential than Q_{10} by approximately 35 mV (Tang *et al.*, 2004). However, in order to postulate that complexes 1 and 2 do not reduce Q_9 directly, kinetic or structural (as opposed to thermodynamic) restrictions must apply. We propose that Q_9 and Q_{10} can remain as separate pools if Q_9 is the essential semi-bound ubiquinone component of complex 3, and the ability of Q_9 to accept electrons from complexes 1 or 3 is thereby limited. In this respect Q_9 would be expected to be somewhat better than Q_{10} in membrane diffusion, an essential requirement for proton-motive Q cycle function (Hunte *et al.*, 2003). In contrast, Q_{10} is the primary electron acceptor from complexes 1 and 2 (Fig. 6). Although Q is depicted as the component that redox cycles with O_2 to generate superoxide, it is difficult to distinguish Q from ISPs in this regard. For example, in Q-depleted bovine heart mitochondria, the redox-active centers supporting NADH-dependent superoxide production are ISPs in complex 1, rather than Q (Genova *et al.*, 2001). The scheme presented in Fig. 6 is also applicable to NADH-supported respiration, since even in the presence of complex 1 substrates, complex 3 appears to be the major site for reactive oxygen production (Chen *et al.*, 2003).

A second possibility for the different reduction states for Q_9 and Q_{10} is that reduction state reflects different primary functions. For example, a portion of the Q_9 pool may serve more as an antioxidant, absorbing oxidative hits that would render it in a relatively more oxidized state. Q_{10} may exist in a more chemically reduced state by virtue of its relatively greater role in electron transport, relative to electron scavenging (antioxidation). Such differences in function of Q_9 and Q_{10} are suggested by recent studies regarding the synthesis of endogenous Q. In eukaryotes, the nuclear gene products for Q synthesis are transported to the inner mitochondrial membrane, the site of Q biosynthesis (Johnson *et al.*, 2005). Although synthesized and dietary Q may be utilized for supporting complex 3 respiration (Santos-Ocana *et al.*, 2002), they are not identical metabolite pools. Recent studies indicate that enzymes involved in Q biosynthesis may exist as a structural complex which may be stabilized by a Q synthetic intermediate (Gin *et al.*, 2005). We speculate that such an endogenously synthesized Q, consisting in mouse of primarily Q_9, would be a different functional pool than that derived from dietary Q, consisting primarily of Q_{10}.

Regardless of the rationale, it is clear that Q_9 and Q_{10} exist as separate pools with perhaps overlapping functions. Analogous distinct pools of Q are likely to exist in humans, but would be difficult to discern because almost all of the ubiquinone exists as Q_{10} (Takada *et al.*, 1984). Q is unlikely to exist as a single pool dispersed in a homogeneous manner throughout the membrane, as sometimes depicted [Model A in (Lenaz, 2001)]. Rather, functional evidence supports the notion of a respirasome, or non-random assembly of respiratory components, including Q [Model B in (Lenaz, 2001)]. A relatively nonmobile Q pool (primarily Q_{10} in mice) may accept electrons from the iron-sulfur clusters of complexes 1 and 2, then transfer the electrons to either oxygen (redox-cycling) or a separate pool of Q associated with complex 3 (primarily Q_9 in mice, and also able to redox cycle). The binding of Q to respiratory proteins may be an important factor determining superoxide production (Lass *et al.*, 1999). An aging-

related impaired binding of Q to complex 3 in rat heart mitochondria is associated with an electron leak and the production of more reactive oxygen (Moghaddas *et al.*, 2003).

Recent studies suggest that the content of Q may not be rate-limiting for respiration (Lenaz, 2001). Even a large decrease of the Q content by 60–70% in *C. elegans* does not reduce the rate of mitochondrial electron transport (Asencio *et al.*, 2003). These observations are consistent with our findings that even though complex 3 is often rate-limiting for respiration, factors other than Q concentration regulate the rate of electron flow, including membrane fluidity and functional activities of complex 4 (COX) and complex 5 (F_0F_1-ATP synthase). Furthermore, the TCDD-mediated decrease in Q concomitant with an increase in respiration would result in an increase in complex 3 Q redox-cycling, which may explain the TCDD-mediated increase in membrane potential and the associated increase in reactive oxygen production.

In summary, the mitochondrial alterations generated by TCDD that elicit the oxidative stress response are complicated. The primary changes consist of an increase in reduction state of glutathione that permits inner membrane state 4 hyperpolarization, a decrease in Q associated with complex 3, and a defect in ATP synthesis that increases respiration but decreases tissue ATP levels. The resulting increase in Q redox-cycling drives reactive oxygen synthesis that increases mitochondrial membrane fluidity and mitochondrial DNA damage.

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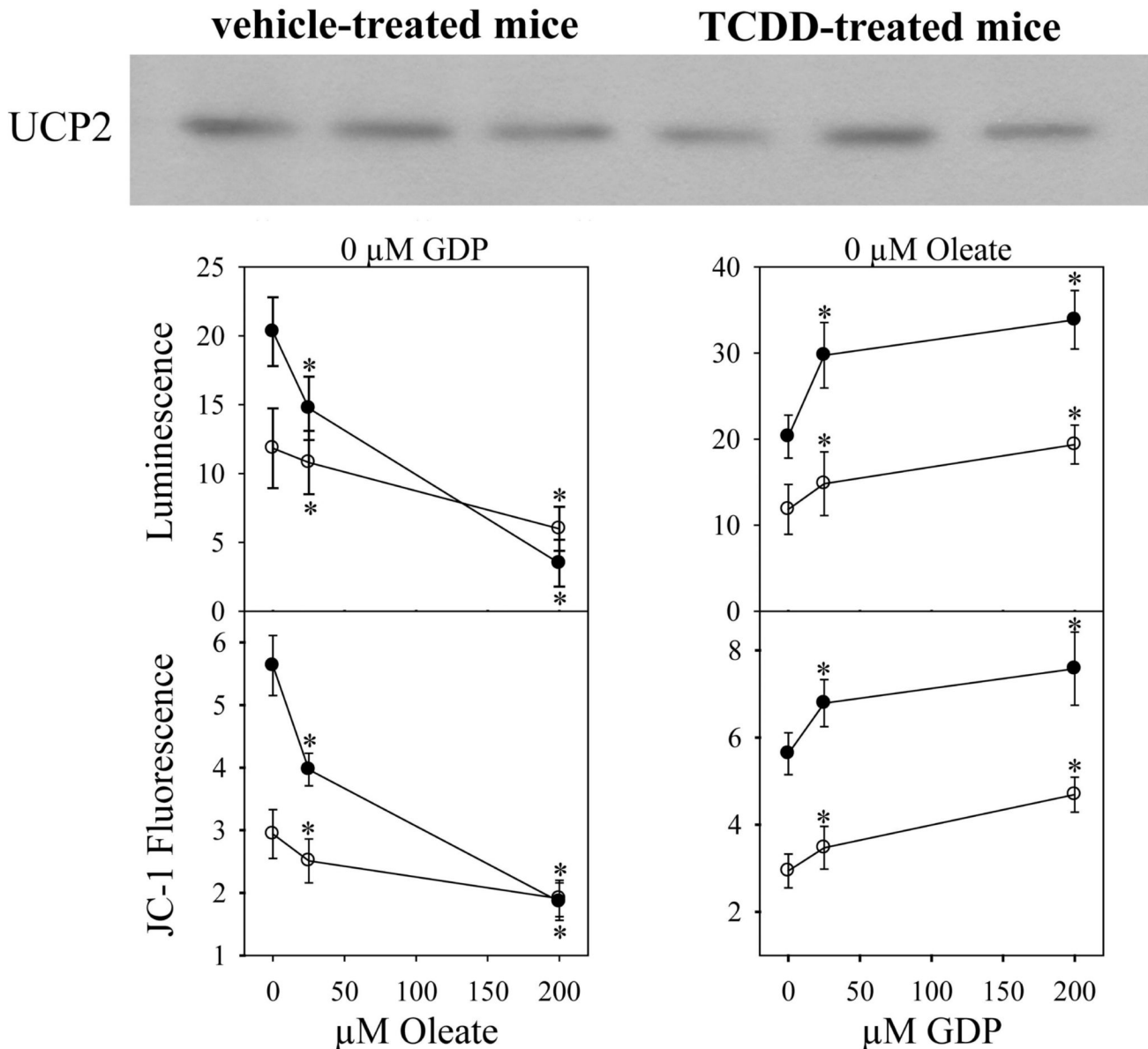
Fig. 1. UCP2 expression and function

Mitochondria were prepared from mouse liver, 7 days after treatment with corn oil (open circles) or TCDD (closed circles). A Western immunoblot using a UCP2 antibody was performed using mitochondrial protein from 3 corn oil-treated mice (the left 3 blots) and from 3 TCDD-treated mice (the right 3 blots). The influence of effectors of UCP2 on H_2O_2 production was estimated using luminol chemiluminescence (center row, expressed as luminescence units X 10^{-3} min^{-1}mg^{-1}). The fatty acid oleate activates UCP2, while the purine nucleotide GDP inhibits. The influence of effectors of UCP2 on mitochondrial membrane potential was determined using JC-1 fluorescence ratios (lower row). A fluorescence ratio of 4 is approximately the equivalent of –180 mV.

Data are presented as the mean value ± S.E. (n = 3). Data were evaluated statistically using a two-way ANOVA, with the factors being TCDD and GDP concentration (left panels), and TCDD and oleate concentration (right panels). A three-way ANOVA was also performed and

discussed in the *Results* section, with factors being oleate concentration, GDP concentration and TCDD treatment.

*P<0.05 versus 0 μM oleate (left panels), and versus 0 μM GDP (right panels), using Student-Newman-Keuls test for pairwise comparison.
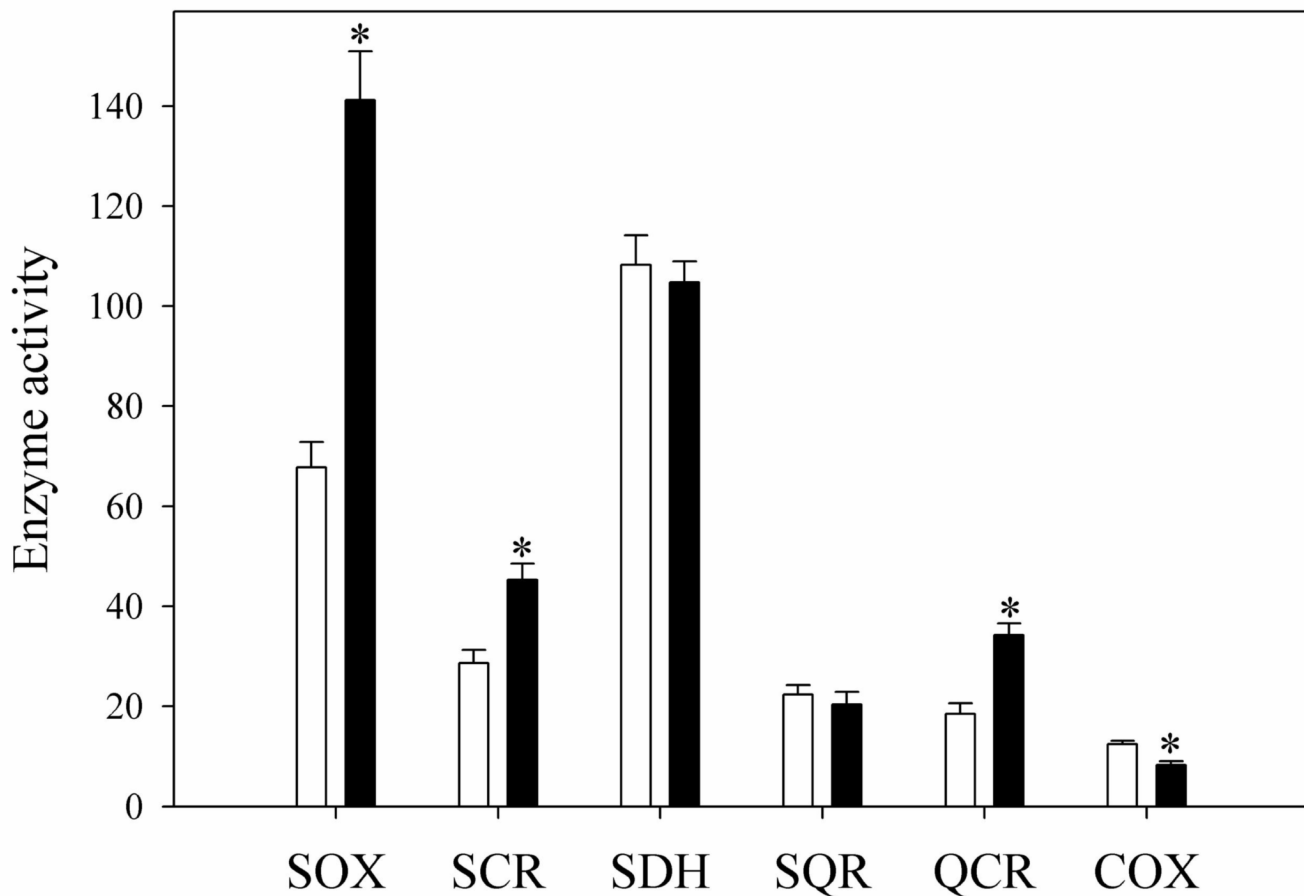
Fig. 2. Mitochondrial enzyme activities after TCDD treatment
Electron transport activities for different regions of the respiratory chain were determined in liver mitochondria prepared from corn oil-treated (open bars) or TCDD-treated (closed bars) mice. Activities are expressed as nmol min^{-1} mg protein^{-1} for SOX (succinoxidase), SCR (succinate cytochrome c reductase), SDH (succinate dehydrogenase), SQR (succinate ubiquinol reductase) and QCR (ubiquinol cytochrome c reductase). Activity is expressed as a first order rate constant [s^{-1} mg protein^{-1}] for COX (cytochrome oxidase). Data are presented as the mean value ± S.E. (n = 6). A t-test was used to evaluate statistical significance of differences between group sample mean values.
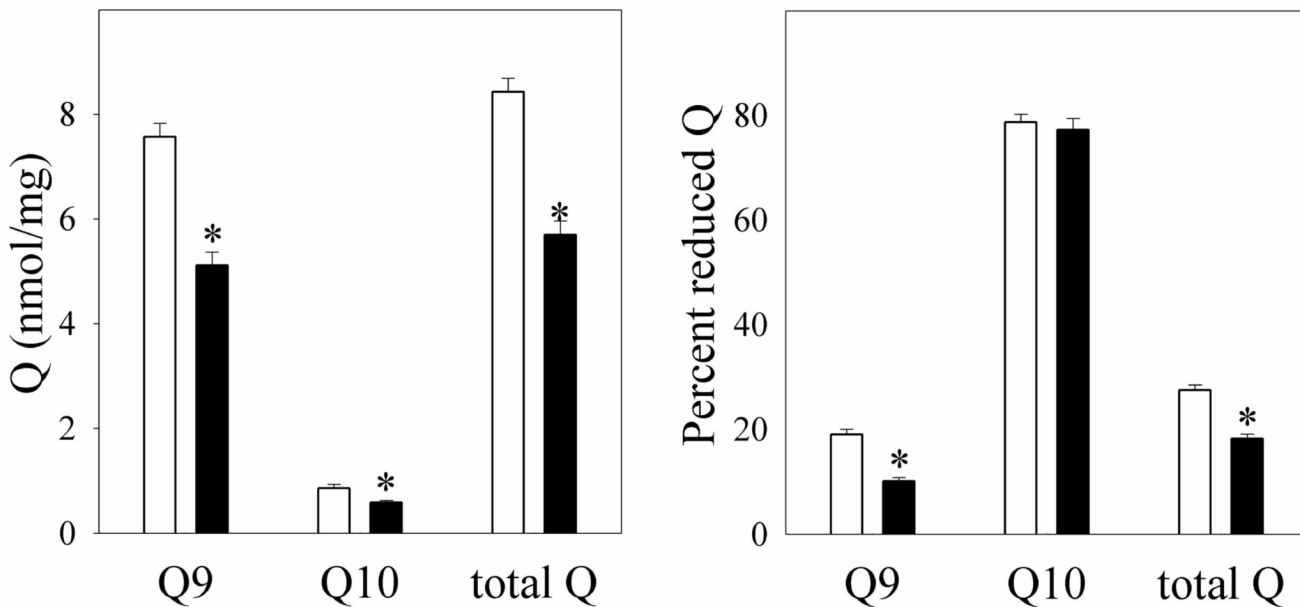*P<0.05 versus vehicle-treated mice.

Fig. 3. Effects of TCDD treatment on mitochondrial ubiquinones Q_9 and Q_{10}
Liver mitochondria were prepared from corn oil-treated (open bars) or TCDD-treated (closed bars) mice. The levels of mitochondrial oxidized and reduced Q_9 and Q_{10} were determined by HPLC (left panel), and the percent reduced Q (right panel) were calculated. Data are presented as the mean value ± S.E. (n = 6). A *t*-test was used to evaluate statistical significance of differences between group sample mean values.
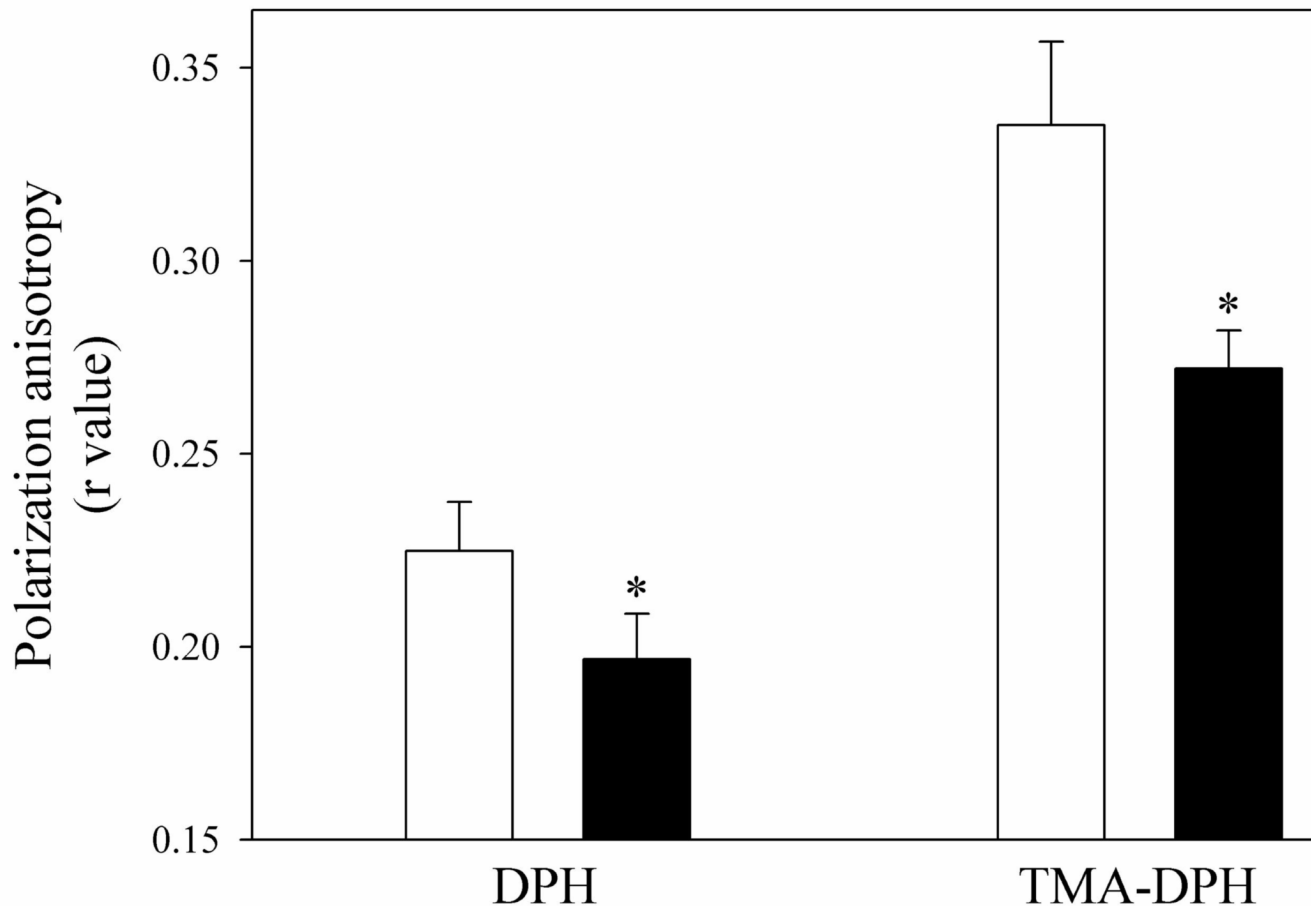*P<0.05 versus vehicle-treated mice.

Fig. 4. Effect of TCDD treatment on mitochondrial membrane fluidity
Mitochondria were prepared from mouse liver 7 days after treatment with corn oil (open bars) or TCDD (closed bars). Mitochondrial membrane fluidity was calculated as the fluorescence polarization anisotropy, determined using the fluorescence probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl- 1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH), to probe the hydrophobic lipid interior, and the membrane surface, respectively. Data are presented as the mean value ± S.E. (n = 6). A *t*-test was used to evaluate statistical significance of differences between group sample mean values. *P<0.05 versus vehicle-treated mice.
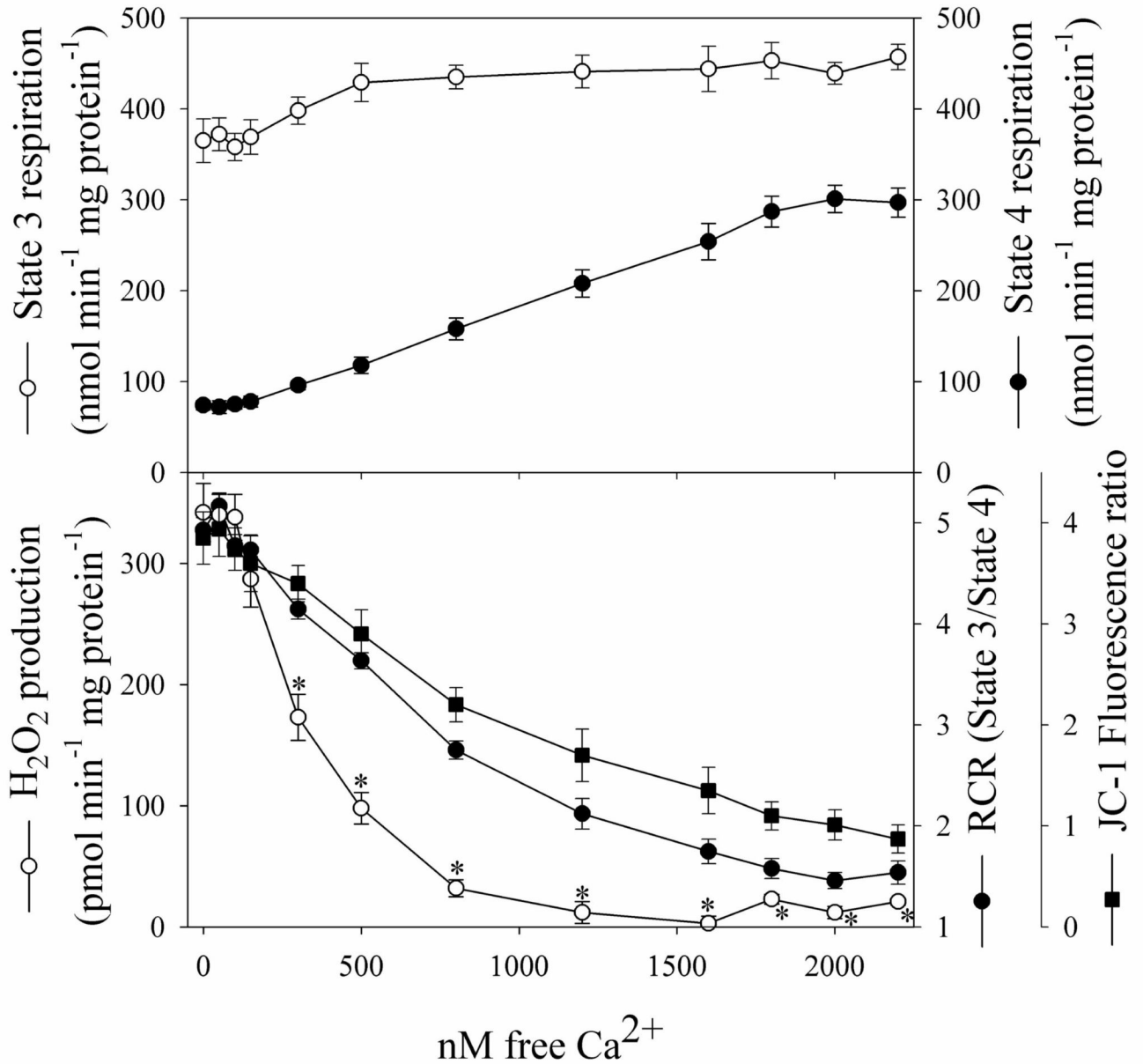
Fig. 5. Relationship between mitochondrial respiration, membrane potential and H$_2$O$_2$ production
Mitochondria were partially uncoupled by exposure to increasing concentrations of free
Ca^{++}. The concentration of free Ca^{++} was controlled using EGTA buffer in respiratory buffer
(KCl-RB), and free Ca^{++} was calculated assuming a Ca^{++}-EGTA K$_d$ = 327 nM.
Data were evaluated statistically using two-way ANOVAs, with the factors being [Ca^{++}] and
JC-1 fluorescence, and [Ca^{++}] and RCR.
*P<0.05 versus either JC-1 fluorescence or RCR at the same [Ca^{++}], using Student-Newman-
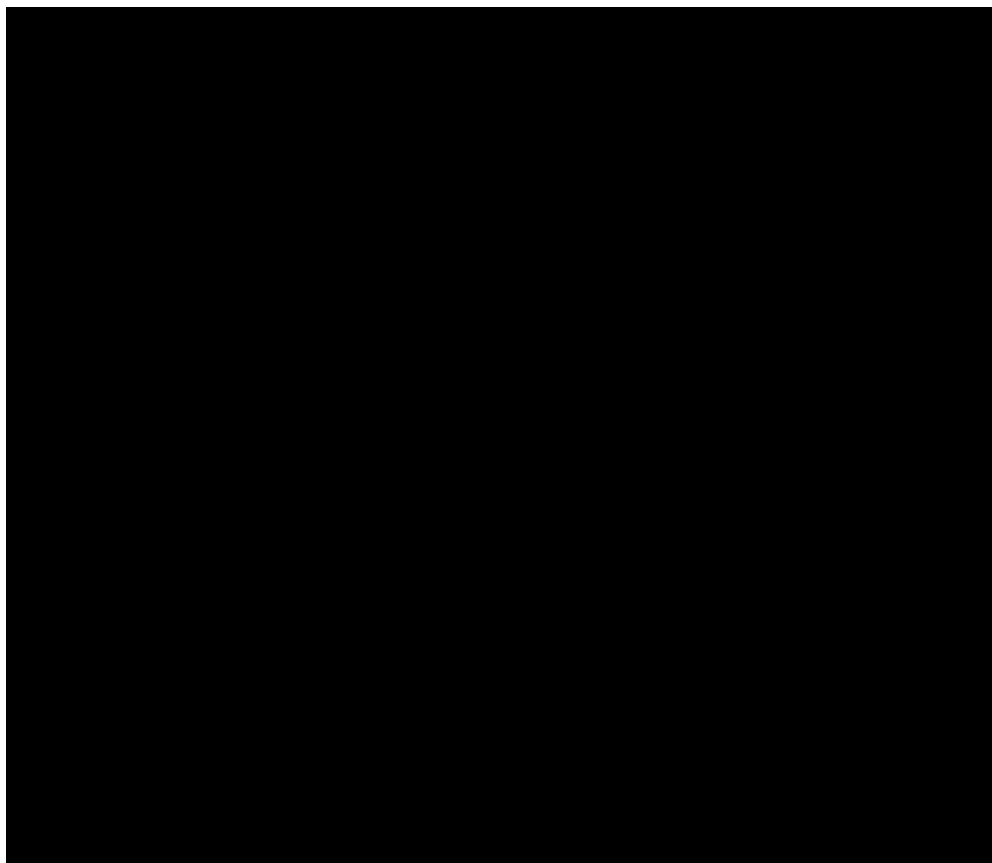Keuls test for pairwise comparison.

Fig. 6. Models for proposed electron transfer and F_0F_1-ATP synthase pathways
TCDD is proposed to decrease the efficiency of ATP formation catalyzed by the β subunit of F_1 within the F_0F_1-ATP synthase complex. Respiration would still depend on ADP, and RCR would remain unchanged. However, ATP/O ratio would decrease due to uncoupling. Electrons from complex 2 feed into complex 3, primarily utilizing Q_{10}, from which electrons flow to Q_9 and enter the proton-motive Q cycle. An oxidation state crossover point exists within complex 3, somewhere between Q_{10} and Q_9.
Abbreviations: AA, antimycin-A; b_L and b_H, respectively, low energy and high energy forms of cytochrome *b*; ISPs, iron-sulfur proteins; SDH, succinate dehydrogenase.

Fig. 7. Proposed mechanism for the TCDD-mediated decrease in Q levels
TCDD regulates the expression level for several genes encoding enzymes involved in retinoic acid (RA) metabolism. The resultant increase in *trans*-RA and decrease in *cis*-RA activate the *trans*-retinoic acid receptor (RAR) and inhibit activation of the *cis*-retinoic acid receptor (RXR), resulting in the inhibition of the rate of synthesis for HMG-CoA, a precursor for Q.

Table 1

Parameters related to mitochondrial ATP production (state 3 conditions).

Parameter	Animal treatment	
	Corn oil	*TCDD*
H$_2$O$_2$ production	57 ± 12	37 ± 18
O$_2$ utilization	324 ± 27	586 ± 45[*]
RCR	4.3 ± 0.4	3.9 ± 0.5
Membrane potential	1.7 ± 0.2	1.6 ± 0.1
ATP production	469 ± 37	281.3 ± 18[*]
ATP/O ratio	1.45 ± 0.15	0.48 ± 0.11[*]
Hepatic ATP	6.2 ± 0.4	3.4 ± 0.3[*]

Liver mitochondria were prepared one week after treating male C57BL/6J mice with one dose of TCDD (5 µg/kg) or with corn oil vehicle. The units are: H$_2$O$_2$ production, pmol min^{-1} mg protein^{-1}; O$_2$ utilization, nmol min^{-1} mg protein^{-1}; RCR (respiratory control ratio), unitless; Membrane potential (JC-1 fluorescence ratio), unitless; ATP production, nmol min^{-1} mg protein^{-1}; Hepatic ATP, nmol mg tissue^{-1}.

Data are presented as mean values ± S.E. (n = 6). A *t*-test was used to evaluate data.

[*] P<0.05 versus vehicle-treated mice.

Table 2

Direct effect of TCDD on the function of liver mitochondria from untreated mice.

| Parameter | pmol TCDD/mg mitochondrial protein | | |
	0	100	1000
state 4 respiration	82 ± 15	146 ± 12[*]	48 ± 13[*]
state 3 respiration	303 ± 25	572 ± 41[*]	187 ± 21[*]
RCR	3.70 ± 0.4	3.92 ± 0.5	3.9 ± 0.4
ATP production	436 ± 38	381 ± 34	114 ± 13[*]
ATP/O ratio	1.44 ± 0.16	0.67 ± 0.08[*]	0.61 ± 0.09[*]

Mitochondria were prepared from C57BL/6J untreated mice. To 0.6 ml KCl-RB containing 0.2 mg mitochondrial protein was added 2 μl DMSO or TCDD in DMSO at a concentration of 10 or 100 μM (to produce final concentrations of 0, 100, or 1000 pmol TCDD/mg mitochondrial protein, respectively).

The units are: Respiratory rates, nmol min^{-1} mg $protein^{-1}$; ATP production, nmol min^{-1} mg $protein^{-1}$.

Data are presented as mean values ± S.E. (n = 6). Data were analyzed by a one-way ANOVA, followed by the Student-Newman-Kuels test for pair-wise comparison of means.

[*] P<0.05 versus vehicle-treated mice.