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Dioxin-induced acute cardiac mitochondrial oxidative damage and increased activity of ATP-sensitive potassium channels in Wistar rats

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ABSTRACT

The environmental dioxin 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is classified as a Group 1 human carcinogen and teratogenic agent. We hypothesize that TCDD-induced oxidative stress may also interfere with mitochondrial ATP-sensitive potassium channels (mitoKATP), which are known to regulate and to be regulated by mitochondrial redox state. We investigated the effects of an acute treatment of male Wistar rats with TCDD (50 μg/kg i.p.) and measured the regulation of cardiac mitoKATP. While the function of cardiac mitochondria was slightly depressed, mitoKATP activity was 52% higher in animals treated with TCDD. The same effects were not observed in liver mitochondria isolated from the same animals. Our data also shows that regulation of mitochondrial ROS production by mitoKATP activity is different in both groups. To our knowledge, this is the first report to show that TCDD increases mitoKATP activity in the heart, which may counteract the increased oxidative stress caused by the dioxin during acute exposure.

1. Introduction

The very persistent environmental toxin 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) harbors a low breakdown rate; in fact, this dioxin is omnipresent in the global environment and has an estimated half-life of 7.6 years in humans (Michalek and Tripathi, 1999). The US Environmental Protection Agency (EPA) has estimated that more than 90% of total human exposure to dioxins and dioxin-like compounds – polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/Fs) – occurs through food consumption (U.S. EPA. Dioxin Reassessment. National Academy of Sciences (NAS) Review Draft 2004. U.S. Environmental Protection Agency, 2004), although water and, to a lesser extent, inhalation and skin absorption can also be exposure sources (Mandal, 2005). PCDD/Fs can also originate from natural sources such as forest fires; however, the more significant origins of dioxins include waste incineration, pesticide manufacture, chlorine bleaching of pulp and paper, municipal and industrial incineration, and metal refining (Hays and Aylward, 2003).

Low doses of TCDD administered to rodents by different routes cause tumors at multiples sites (Knerr and Schrenk, 2006). Different mechanisms have been proposed for the carcinogenic effect of TCDD, including binding and activation of the cytosolic aromatic hydrocarbon receptor (AhR) (Ray and Swanson, 2009), a ligand-activated nuclear transcription factor. Also, TCDD increases reactive oxygen species (ROS) generation, which results in lipid peroxidation, leading to cell membrane disruption, DNA and protein damage and calcium homeostasis disruption in various rodent tissues (Aly and Khafagy, 2011; Hassoun et al., 2000; Shertzer et al., 2006). Generation of ROS such as hydroxyl radical, superoxide anion and hydrogen peroxide appear to be involved in TCCD-induced oxidative damage (Stohs et al., 1986).

Mitochondria may mediate, at least in part, TCDD-induced oxidative stress effects. Nohl et al. (1989) demonstrated that

Abbreviations: 5-HD, 5-hydroxydecanoic acid; ADP, adenosine diphosphate; AhR, aromatic hydrocarbon receptor; ATP, adenosine triphosphate; BSA, bovine serum albumin; ΔΨ, mitochondrial transmembrane electric potential; DMSO, dimethylsulphoxide; DNA, deoxyribonucleic acid; DNPH, dinitrophenylhydrazine; DZX, Diazoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRP, horseradish peroxidase type vi-a; HW/BW, heart weight/body weight; LW/BW, liver weight/body weight; mitoKATP, mitochondrial ATP-sensitive potassium channels; PCDD/Fs, polychlorinated dibenzofuran; RCR, respiratory control ratio; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TCDD, 2,3,7,8-Tetrachlorodibenzo-p-dioxin; TFA, trifluoroacetic acid; TPP^+, tetraphenylphosphonium cation.

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superoxide anion and hydrogen peroxide are produced following *in vitro* addition of TCDD to bovine heart mitochondria (Nohl et al., 1989). Exposure of C2C12 myocytes to TCDD results in inhibition of mitochondrial transcription, disruption of mitochondrial transmembrane potential ($\Delta\Psi$) and altered calcium homeostasis (Biswas et al., 2008). In addition to developing an increased resistance to apoptosis, TCDD-treated C2C12 cells also acquired increased invasive capacity, none of the effects being dependent on the AhR (Biswas et al., 2008). These findings suggest that TCDD toxicity may present a mitochondrial component. In fact, mitochondria are a plausible target for several xenobiotics and an excellent *in vitro* model to predict compound toxicity (Pereira et al., 2009).

The mitochondrial K^+ cycle consists of electrophoretic K^+ uptake and electroneutral K^+ efflux across the inner membrane (Garlid, 1996), the latter being mediated by a K^+/H^+ antiporter and influx is mediated by a mitochondrial ATP-sensitive potassium channels (mitoKATP) (Garlid, 1996). Other potassium channels are present in the inner mitochondrial membrane including a large conductance Ca^{2+}-activated potassium channel (Siemen et al., 1999), the voltage-gated Kv1.3 potassium channel (Szabo et al., 2005) and the twin-pore domain TASK-3 potassium channel (Rusznak et al., 2008). The putative functional roles of these several channels include the modulation of mitochondrial matrix volume, respiration and membrane potential (Szewczyk et al., 2009). In addition, the activity of these channels can also modulate the generation of reactive oxygen species (ROS) by mitochondria (Facundo et al., 2007). The molecular identity of mammalian mitoKATP is a matter of debate. ATP-sensitive K^+ (KATP) channels were initially identified in the plasma membrane of cardiac myocytes (Noma, 1983). These channels were further distinguished by their specific localization within the cell. Sarcolemmal (sarcKATP), nuclear (nucKATP) or mitochondrial (mitoKATP) ATP sensitive potassium channels have been identified. Although the mitoKATP was characterized (Inoue et al., 1991) and later purified and partly reconstituted (Paucek et al., 1992), their composition and nature is still debatable. Many research groups propose that the channel has a structure similar to classical KATP channels, based on the pharmacological sensitivities and immunoreactivity with specific antibodies. Also, it has been suggested that the mitoKATP from rats heart include a K^+ inward rectifier (mitoKIR, subunits Kir6.1 and Kirb 6.2) and sulfonylurea receptors (SURs, namely SUR2) (Cuong et al., 2005; Suzuki et al., 1997).

The modulation of mitoKATP (O'Rourke, 2004) by pro-oxidant xenobiotics with environmental relevance has received scarce attention. The putative functional roles of mitoKATP include modulation of mitochondrial matrix volume, mitochondrial respiration and membrane potential. The activity of mitoKATP can also modulate the generation of ROS by mitochondria (Szewczyk et al., 2009).

Kowaltowski and co-workers proposed mitoKATP openers to have direct effects on mitochondrial respiration, membrane potential and Ca^{2+} uptake under physiological conditions (Kowaltowski et al., 2001). MitoKATP are redox-sensitive channels with higher activity under increasingly oxidative conditions (Facundo et al., 2007; Liu et al., 2002), suggesting a role of this channel in redox mitochondrial signalling in the heart, where higher activity of this channel has been described (Kowaltowski et al., 2001).

The hypothesis for the present work is that an acute TCDD treatment causes oxidative damage to cardiac mitochondria resulting not only in mitochondrial damage, but also in an altered regulation of mitoKATP. Our hypothesis includes that increased activation of mitoKATP would counteract TCDD-induced oxidative stress and prevent further mitochondrial damage. The heart was focused in the present work since the mitoKATP are an important component of different cardiac signalling pathways that respond to alterations in the redox state of the cell. One such pathway with clinical relevance involves KATP on ischemic pre-conditioning (Queliconi et al., 2011).

2. Material and methods

2.1. Reagents

All reagents used were of the highest grade of purity commercially available (analytical grade or better), and all aqueous solutions were prepared in ultrapure (type I) water. A 99% chemical pure TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) from LGC Standards (Barcelona, Spain) was dissolved in dimethylsulphoxide (DMSO) 66.7 μg/ml. Diazoxide (DZX, in DMSO), 5-hydroxydecanoic acid (5-HD, in water) stock solutions were prepared just prior to each experiment. Amplex Red (in DMSO) and horseradish peroxidase type VI-A (HRP) provided by Sigma–Aldrich, St. Louis, MO, USA, rotenone (in DMSO), adenosine triphosphate (ATP, in water), glutamate/malate and succinate (in water) solutions were kept as frozen aliquots.

For Western blotting assays, the primary antibody used was Rabbit Anti-DNP (D9656) from Sigma (Portugal). The secondary antibody was Goat anti-rabbit IgG-AP (sc-2007) from Santa Cruz Biotechnology Inc. (Portugal). The protein molecular weight markers (Precision Plus Protein Dual Color Standards) were obtained from Bio-Rad Laboratories, Inc., CA, USA, while the ECF substrate was obtained from Amersham Biosciences, Buckinghamshire, England.

2.2. Animal care and ethical statement

All experiments involving animals were conducted in accordance with the European convention for the protection of vertebrate animal used for experimental and other scientific purposes (CETS no. 123 of 18 march 1986 and 2005 revision) and the Commission Recommendation of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes (C (2007) 2525). The authors are accredited by the Federation of Laboratory Animal Science Associations (FELASA) for animal experimentation. A limited number of animals were used in the present study based on preliminary data obtained for liver mitochondria and due to concerns in the use of a larger pool of animals with this particular environmental toxicant. Male Wistar Han IGS rats were purchased from Charles River (France). Animals were group-housed in type III-H cages (Tecniplast, Italy) with appropriated corn cob bedding and environmental enrichment. Rats were maintained in proper environmental requirements including room temperature set at 22 °C, relative humidity at 45–65%, ventilation with 15–20 changes/hour, a12 h light/dark cycle, noise level <55 dB, and *ad libitum* access to standard rodent food (4RF21 GLP certificate, Mucedola, Italy) and acidified water (at pH 2.6 with HCl, in order to prevent bacterial growth). All animals were acclimated 10–14 days prior to experiment initiation. For the current study, 10 week old rats (280–350 g) were injected with TCDD (50 μg/kg of body weight) in DMSO or with an equivalent volume of DMSO alone (controls) via intraperitoneal injection (i.p.), 24 h before the sacrifice. All animals were weighed at the beginning and end of the experimental period. This dosing protocol was chosen based on previous studies (Choi et al., 2008; Stohs et al., 1991). Non-fasted rats were euthanized by cervical dislocation between 9:00 and 10:00 AM to eliminate possible effects due to diurnal variation. Hearts (about 0.8 g) and livers (about 10.0 g) were removed, weighed and placed in chilled isolation medium containing 300 mM sucrose, 10 mM HEPES-KOH (7.2), 1 mM EGTA and 1 mg/ml fatty acid free BSA.

2.3. Isolation of the cardiac mitochondrial fraction

Heart mitochondria were isolated from male Wistar rats (10 weeks old) as previously described (Oliveira et al., 2001). After weighing, the hearts were immediately excised and finely minced in an ice-cold isolation medium, with blood removed. Minced blood-free tissue was then resuspended in 40 ml of isolation medium supplied with 0.2 mg protease Type VIII (Sigma n° P-5390) per gram of tissue and homogenized with a tightly-fitted homogenizer (Teflon pestle:glass homogenizer) for 2–3 min in order to minimize loss of mitochondrial membrane integrity. The suspension was incubated for 1 min (4 °C) and then re-homogenized. The protease was then removed from the homogenate by centrifugation at 14,400× g for 5 min at 4 °C. The supernatant was decanted and the pellet, essentially free of protease, was gently homogenized to its original volume with a loose-fitting homogenizer. The suspension was centrifuged at 750× *g* for 10 min and the resulting supernatant was centrifuged at 12,000× *g* for 10 min with washing medium (300 mM sucrose, 10 mM HEPES-KOH (7.2)). The pellet was resuspended manually using a paintbrush and washed twice at 12,000× *g* for 10 min, being then resuspended in 500 μL of washing medium. Mitochondrial protein content was determined by the Biuret method (Gornall et al., 1949) using BSA as standard. Mitochondrial yield was also calculated as the mitochondrial protein obtained divided by heart weight (×100) and expressed in percentage (%). Samples were kept on ice and used within 1 h of isolation to ensure mitoKATP activity and pharmacological regulation.

2.4. Detection of mitochondrial carbonyl groups

The protein reactive carbonyls in mitochondrial fraction were analyzed according to previously published methods (Robinson et al., 1999) with slight modifications. A determined volume of mitochondrial suspension (V) containing

50 μg of protein was derivatized with dinitrophenylhydrazine (DNPH). For this purpose, a volume (1V) of 12% sodium dodecyl sulfate (SDS) and two volumes (2V) of 20 mM DNPH prepared in 10% TFA was added to each sample, followed by a 20 min incubation in the dark. After this period, a neutralization reaction with 1.5 volumes (1.5 V) of 2M Tris and 18% of β-mercaptoethanol was performed. A negative control with 20 mM $NaBH_4$, which reduces carbonyl groups to alcohols, was simultaneously prepared for each sample. After dilution in Tris buffer solution to obtain a final concentration of 0.1 μg/μl, 5 μg mitochondrial protein were loaded into a 12% polyacrylamide gel using SDS-PAGE electrophoresis and probed against protein carbonyl groups (anti-DNP, 1:2500 overnight at 4 °C).

2.5. Mitochondrial respiratory activity

Oxygen consumption of isolated mitochondria was monitored polarographically with a Clark-type oxygen electrode connected to a suitable recorder. The suspension was under controlled temperature (37 °C) and constant stirring in 1 mL of reaction medium (130 mM sucrose, 50 mM KCl, 5 mM KH_2PO_4, 100 μM EGTA, 5 mM HEPES-KOH pH 7.4) in a thermostatic water-injected closed chamber. Mitochondria (0.5 mg) were incubated for 1 min and energized with either 2 mM of glutamate/malate or 2 mM of succinate plus 3 μM rotenone. After reaching steady-state oxygen consumption, 100 nmol ADP was added to initiate state 3 respiration.

The respiratory control ratio (RCR), an indication of mitochondrial integrity, and the ADP/O ratio, a measure of the efficiency of the phosphorylation system, were calculated as described previously (Chance and Williams, 1955).

2.6. Determination of mitochondrial transmembrane potential ($\Delta\Psi$)

The $\Delta\Psi$ was indirectly estimated by evaluating the transmembrane distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) using a TPP^+ selective electrode in combination with an Ag/AgCl saturated reference electrode as previously described (Oliveira et al., 2000). Both the TPP^+ electrode and the reference electrode were inserted into a chamber at 37 °C with magnetic stirring in 1 mL of medium (130 mM sucrose, 50 mM KCl, 5 mM KH_2PO_4, 100 μM EGTA, 5 mM HEPES-KOH pH 7.4) containing 3 μM TPP-Cl. The $\Delta\Psi$ (in mV) was estimated without any binding correction, according to Kamo et al. (Kamo et al., 1979) and as previously described (Sardao et al., 2002). Mitochondria (0.5 mg) were incubated for 1 min and energized with 2 mM of glutamate/malate or 2 mM of succinate plus 3 μM rotenone. After reaching a steady-state distribution of TPP^+, 100 nmol ADP was added and $\Delta\Psi$ fluctuations were recorded. To confirm state 3 respiration results, the phosphorylative lag-phase was also calculated as the time in seconds taken by the mitochondrial suspension to phosphorylate the added ADP and restore the basal $\Delta\Psi$ values.

2.7. Measurement of mitochondrial matrix volume changes modulating the mitoKATP activity

Light-scattering changes due to K^+ uptake and swelling in mitochondrial suspensions (Kowaltowski et al., 2001; Facundo et al., 2007) were followed over time using a Perkin Elmer LS-55B Fluorescence Spectrometer operating with excitation and emission wavelengths both set at 520 nm and slit widths of 2.5 nm with continuous stirring at 37 °C. Mitochondria (0.25 mg protein/ml) were suspended in a buffered salt medium containing 130 mM KCl, 2 mM KH_2PO_4, 2.5 mM $MgCl_2$ and 10 mM HEPES-KOH pH 7.2 or in media in which all K^+ salts were substituted by Na^+. Except for basal assays, mitochondria were energized with 2 mM succinate and 1 μg/ml oligomycin was included to inhibit $\Delta\Psi$ variations resulting from ATP synthesis by the F_oF_1-ATP synthase, as well as regulation of mitochondrial ROS production by this enzyme (Shertzer et al., 2006; Veenman et al., 2010). In some experiments, 3 μM rotenone was added to inhibit reverse electron transfer from complex succinate-reduced II to complex I. Additionally, 1 mM ATP, 150 μM 5-HD and/or 30 μM DZX were also added to the reaction buffer (please see table and figure legends for further details). When used, DZX was added to the suspension 2 s after mitochondria to ensure an even media distribution. It should also be noted that DZX is ineffective unless ATP is already present, because a channel opener will not act on an already open channel (Facundo et al., 2007). Similarly, 5-HD is ineffective unless ATP and a channel opener, such as DZX, are already present because 5-HD alone does not block the channel, but rather prevents the effect of diazoxide (Jaburek et al., 1998). Light-scattering recordings were initiated by the addition of mitochondria and followed for more than 200 s. Data was analyzed using the software FL WinLab provided by the manufacturer (PerkinElmer Life and Analytical Sciences, Boston, MA).

2.8. Measurement of hydrogen peroxide production

The production of H_2O_2 by the respiratory chain and modulation by mitoKATP was evaluated fluorometrically using the fluorescent probe Amplex Red, as described previously (Facundo et al., 2007; Zhou et al., 1997) with some modifications. Briefly, Amplex Red (Sigma–Aldrich, St. Louis, MO) was dissolved in DMSO to achieve a concentration of 10 mM; horseradish peroxidase type VI-A (HRP, Sigma–Aldrich, St. Louis, MO) was prepared in reaction buffer (130 mM KCl, 2 mM KH_2PO_4, 2.5 mM $MgCl_2$ and 10 mM HEPES-KOH pH 7.2 or in media in which all K^+ salts were substituted by Na^+) to a final concentration of 1550 U/ml, and were kept at 4 °C through the end of the experiments. All experiments were conducted in duplicate using a Costar 96 wells black clear-bottom microplate (Corning, NY) with 100 μg protein, 5 μM Amplex Red, 1 U/L peroxidase, 1ug/mL oligomycin and supplemented with 200 μL buffer per well. Depending on the assay, 2 mM succinate, 1 mM ATP, 150 μM 5-HD and/or 30 μM DZX were added to the reaction medium. Sample fluorescence was then followed for 10 min in intervals of 25 s at 37 °C in a SpectraMax Gemini EM Fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) with 563 nm excitation and 587 nm emission wavelengths (570 nm cut-off). The rates of H_2O_2 production by rat heart mitochondria were obtained from the initial slopes determined using software provided by the manufacturer (SoftMax Pro version 5.0) and are plotted as fluorescence arbitrary units per second (A.U./s).

2.9. Data analyses and statistics

Data are presented as mean ± SEM of three to five experiments conducted with different and independents mitochondrial preparations. Comparisons between control and treated were conducted using a Student's *t*-test (one variable) or using one-way ANOVA followed by specifics Bonferroni multiple comparison tests. Differences were considered significant if: $^*p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$. Statistical analyses were performed using Graph Pad Prism version 5.

3. Results

3.1. Body and organs weight did not change in response of TCDD

After a single dose of 50 μg TCDD/kg body weight, no alterations were observed in male Wistar rats body weight 24 h post-treatment (Supplementary Table 1). The heart and liver weights of treated animals did not differ from control animals which resulted in unchanged organ weight/body weight (HW/BW and LW/BW). Blood glucose was measured prior to the injections and right before animal sacrifice. No alterations were found between groups (Supplementary Table 1). Data also shows that acute treatment with TCDD did not alter cardiac mitochondrial isolation yield.

3.2. Effects of TCDD on cardiac and hepatic mitochondrial bioenergetics

Oxygen consumption data on glutamate/malate- and succinate-energized cardiac and liver mitochondria from control and treated-animals are presented in Table 1. In the heart, a decrease in the RCR value with glutamate-malate as mitochondrial substrate was observed as a result of TCDD treatment (3.8 ± 0.4 *vs* 2.7 ± 0.4, $p = 0.03$), although no differences were found with succinate as substrate (2.3 ± 0.2 *vs* 1.8 ± 0.1, $p = 0.14$). Statistically significant differences were also found in the ADP/O ratio in heart mitochondria with the TCDD group having a lower value, but only when succinate was used as substrate (2.1 ± 0.2 nmolADP/natmsO *vs* 1.7 ± 0.3 nmolADP/natmsO, $p = 0.04$).

Interestingly, as opposed to the slight deterioration of cardiac mitochondrial respiration, an apparent improvement in liver mitochondrial function after TCDD was found (Table 1), characterized by a significant increase in both state 3 (succinate, 205.0 ± 46.7 natms O/min/mg prot *vs* 320.8 ± 22.9 natms O/min/mg prot, $p = 0.04$) and RCR (both substrates, for glutamate 4.6 ± 0.3 *vs* 6.6 ± 0.3 $p = 0.006$ and for succinate 4.9 ± 0.5 *vs* 6.1 ± 0.2, $p = 0.03$). Also, decreased state 2 respiration was found in liver mitochondria from TCDD-treated animals, when glutamate-malate was used as substrate (43.1 ± 5.1 natms O/min/mg prot *vs* 34.8 ± 3.6 natms O/min/mg prot, $p = 0.03$).

Cardiac mitochondria from DMSO-treated animals developed a $\Delta\Psi$ around 220 mV (negative inside) after energization (Fig. 1). TCDD treatment decreased maximal $\Delta\Psi$ generated by cardiac mitochondria and caused an overall increase in liver mitochondria. Nevertheless, the differences did not reach statistical significance

Table 1
Respiratory parameters in energized cardiac and hepatic rat mitochondria in the different experimental groups.

	Glutamate/malate				Succinate			
	Heart		Liver		Heart		Liver	
	Control	TCDD	Control	TCDD	Control	TCDD	Control	TCDD
State 2 (natms O/min/mg prot)	76.2 ± 15.6	67.0 ± 11.3	43.1 ± 5.1	34.8 ± 3.6*	152.0 ± 28.1	123.0 ± 15.0	42.2 ± 8.1	47.9 ± 2.9
State 3 (natms O/min/mg prot)	243.4 ± 9.9	189.5 ± 24.1	194.9 ± 17.8	216.5 ± 22.5	354.5 ± 39.5	322.5 ± 33.6	205.0 ± 46.7	320.8 ± 22.9*
State 4 (natms O/min/mg prot)	72.6 ± 17.4	73.3 ± 13.9	43.2 ± 7.2	33.0 ± 4.6	167.7 ± 37.2	184.1 ± 23.3	43.9 ± 4.6	53.0 ± 3.3
RCR	3.8 ± 0.4	2.7 ± 0.4*	4.6 ± 0.3	6.6 ± 0.3**	2.3 ± 0.2	1.8 ± 0.1	4.9 ± 0.5	6.1 ± 0.2*
ADP/O (nmol ADP/natoms O)	2.7 ± 0.3	2.6 ± 0.4	2.3 ± 0.2	2.4 ± 0.1	2.1 ± 0.2	1.7 ± 0.3*	2.1 ± 0.3	1.9 ± 0.3

Respiratory activities were measured polarographically with a Clark-type oxygen and in the presence of glutamate/malate or succinate. ADP (100 nM) was added to induce state 3 respiration. The values are means ± SEM of 3 (liver) and 4–5 (heart) independent experiments and were compared using paired Student's *t*-test (control *vs* TCDD, $^{*}p < 0.05$; $^{**}p < 0.01$).

with the exception of the TCDD-induced decreased $\Delta\Psi$ in heart mitochondria (Fig. 1A, −224.4 ± 2.7 mV *vs* -214.3 ± 3.2 mV, $p = 0.001$). The phosphorylative lag phase (Fig. 1B) was also altered by TCDD, causing an increase in the heart and a decrease in the liver, although again statistical significance was only observed in liver mitochondria for glutamate-malate (37.0 ± 3.6 s *vs* 27.0 ± 1.7 s, $p = 0.04$). Finally, ADP-induced depolarization was unaltered by TCDD treatment in the heart and significantly increased after treatment of liver mitochondria energized by glutamate-malate (24.4 ± 1.0 mV *vs* 27.0 ± 0.3 mV, $p = 0.02$).

3.3. Cardiac mitochondrial carbonyl groups increases with TCDD treatment

The second objective of the present study was to investigate whether TCDD treatment would result in increased oxidative stress in addition to altering cardiac mitochondrial function. A significant increase in protein carbonyls content was found in cardiac mitochondria from TCDD-treated rats (Fig. 2, 29120 ± 1334 *vs* 33580 ± 781, $p = 0.006$). The presence of oxidized proteins in both groups was confirmed by the specificity of DNPH, confirming that TCDD causes cardiac mitochondrial oxidative damage. When carbonyl groups were reduced to alcohols by using $NaBH_4$, the signal was significantly decreased, confirming once again the specificity of the method.

3.4. MitoKATP activity is enhanced in the hearts of TCDD-treated rats

To directly assess if mitoKATP activity was altered in TCDD-treated rats, light scattering variations were measured in mitochondrial fractions from both experimental groups as previously described (Facundo et al., 2007). Experiments were performed with succinate alone since these experimental conditions are required to optimally follow mitoKATP regulation by endogenous mitochondrial ROS (Facundo et al., 2007). The traces in Fig. 3 are representative of volume alterations due to the opening and closing of mitoKATP in heart mitochondria from control (Fig. 3A, CH) and treated rats (Fig. 3B, TH). Succinate-energized mitochondria rapidly accumulate K^+, leading to osmotic water entry and to a new steady-state volume, a process requiring around 120 s at 37 °C to be completed (Fig. 3). In the absence of ATP (Fig. 3, control trace), mitoKATP are open and mitochondria accumulate K^+ and water to achieve a higher steady-state volume than the one observed in the presence of ATP (Fig. 3, ATP trace), which is explained by the inhibition of mitoKATP-dependent K^+ uptake. Concomitant addition of diazoxide (Fig. 3, ATP + DZX trace) reverses ATP inhibition. As expected, no effects are observed when DZX is added to mitochondria in the absence of ATP (data not show), as mitoKATP are already in the open state (Facundo et al., 2007). Effects of DZX are reversed by the addition of the mitoKATP inhibitor 5-hydroxydecanoate (Fig. 3, ATP + DZX + 5HD trace). Importantly, only 30 μM DZX was used in our experimental protocol, a concentration that does not inhibit complex II activity (Dzeja et al., 2003; Kowaltowski et al., 2001). Also, no differences in mitochondrial swelling were observed in media without K^+ (Fig. 3A and B).

Swelling rate in cardiac mitochondria from TCDD-treated animals was significantly increased under control conditions, since TH swelling rate was approximately 52% higher than the one observed in the CH group (see Fig. 3A and B for typical traces, Fig. 4A for average swelling rates, 0.86 ± 0.06 −dAbs/*s vs* 1.31 ± 0.16 −dAbs/s, $p < 0.01$). However, DZX was more effective in re-opening mitoKATP after ATP inhibition in TCDD-treated rats than in the control group (0.72 ± 0.12 −dAbs/*s vs* 1.12 ± 0.25 −dAbs/s, $p < 0.05$). The data suggests that mitoKATP activity is enhanced in the hearts of TCDD-treated rats.

The activity of the channels was also measured as the total variation in swelling amplitude, which was determined as the difference between the value of light scattering detected at 120 s (end of assay) and 62 s (2 s after the addition of mitochondrial suspension). In general, swelling amplitude data reflected the same trend as the data obtained from swelling rates, although the latter appears to be more sensitive in detecting differences between experimental groups (see Fig. 4A and B). We think that the apparent lower sensitivity is due to the fact that CH and TH mitochondria reach a very similar steady-state volume, although the kinetics of volume alterations is different. When liver mitochondria from the same animals were assayed for the same parameters, not only was a minimal activity of mitoKATP in both control and treated groups measured, but also no differences were found between the two experimental groups (data not shown).

3.5. Mitochondrial H_2O_2 release due to mitoKATP channels is decreased in the hearts of TCDD-treated rats

MitoKATP are regulated by the mitochondrial redox state, which result in an alteration of their channel activity, contributing to a secondary regulation of mitochondrial ROS production. Because of the decreased cellular antioxidant networks in the heart (as compared with other tissues), heart mitochondria may be particularly susceptible to oxidative stress (Facundo et al., 2007), and thus mitoKATP regulation of ROS production can be an extra protective feature. We next aimed to correlate altered mitoKATP activity and ROS production by mitochondria in cardiac mitochondria from both control and TCDD-treated rats.

Under control conditions, respiratory chain H_2O_2 release rates were lower than those observed in the presence of ATP, a potential mitoKATP inhibitor (Fig. 5, CH control *vs* CH ATP: 5.59 ± 0.92 *vs* 9.48 ± 0.71, $p < 0.001$). The presence of ATP caused an increase in H_2O_2 release by the respiratory chain, which was higher in DMSO-treated than in TCDD-treated rats (Figs. 5, 69% *vs* 43% and CH ATP *vs*

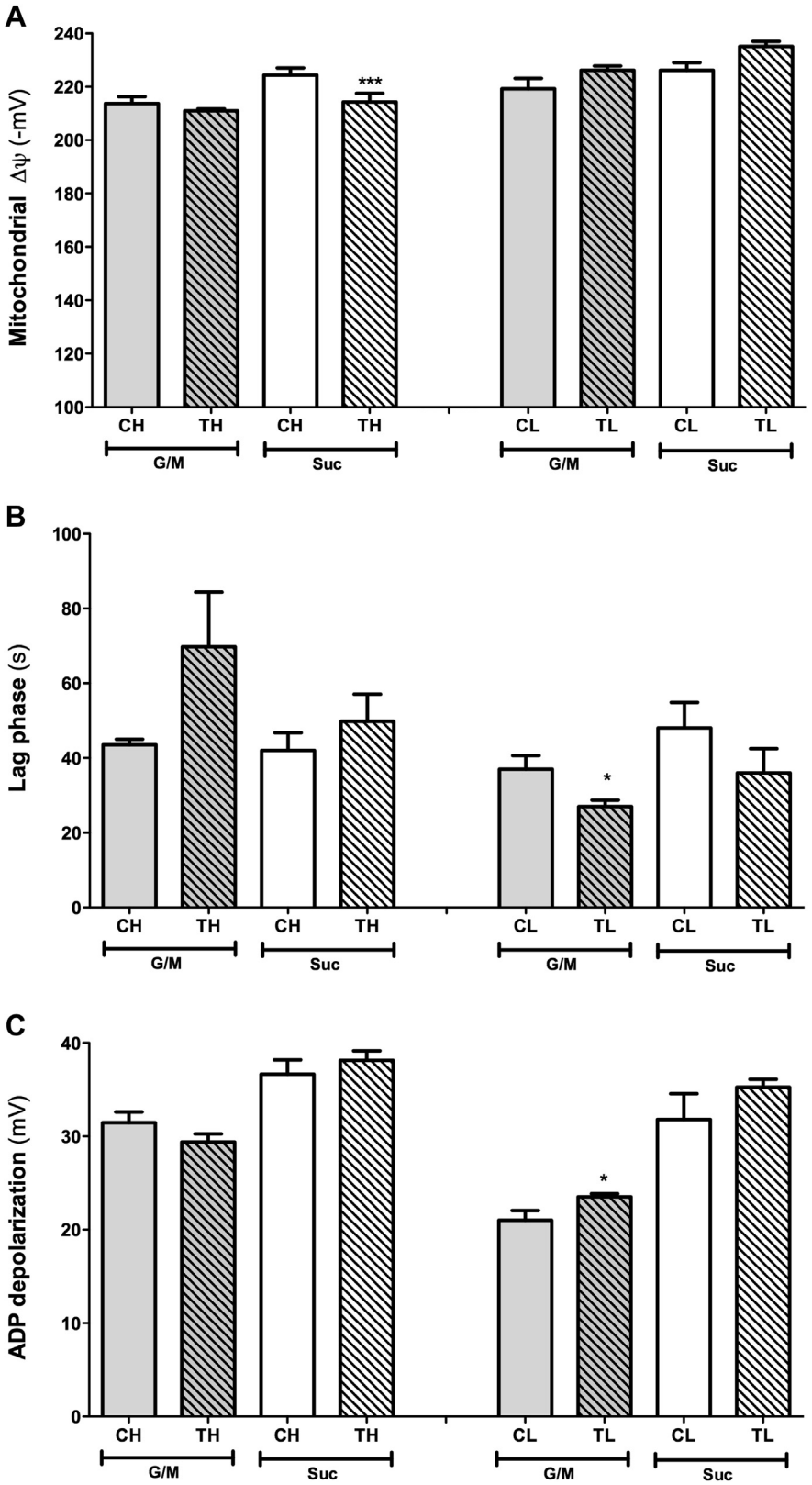

Fig. 1. Effects of TCDD on mitochondrial membrane potential fluctuations: (A) mitochondrial maximal transmembrane electric potential developed ($\Delta\Psi$, − mV), (B) Phosphorylative lag phase duration (s) and (C) ADP-induced depolarization (mV) assessed by a TPP^+-selective electrode in cardiac (H) and liver (L) mitochondria from control (CH, CL) and TCDD-treated animals (TH, TL). Mitochondria were energized with glutamate/malate or succinate. The values are means ± SEM of 3 (liver) and 4–5 (heart) independent preparations and were compared using paired Student's *t*-test (control *vs* TCDD, $^*p < 0.05$; $^{***}p < 0.001$).

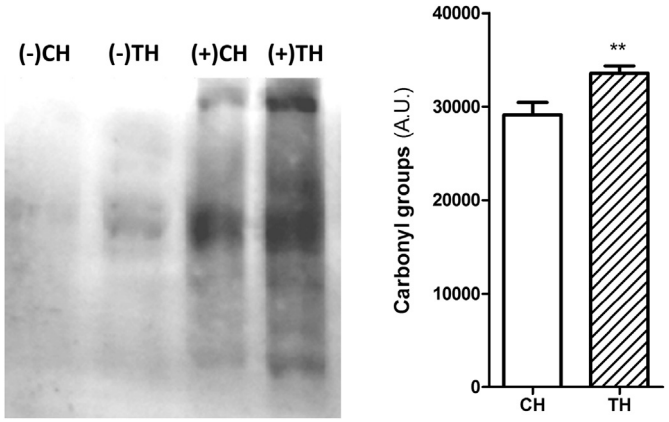

Fig. 2. Detection of carbonyl groups on mitochondria from hearts from control (CH) and TCDD-treated animals (TH). The carbonyl content was determined by immunoblotting. Left panel, representative result with (−) and (+) meaning that the reaction was made in the presence and absence of DNPH as described in materials and methods. TH, treated heart; CH, control heart. Right panel, band density intensity of the (+) lanes. The values are means ± SEM of 4 independently experiments and were compared using paired Student's *t*-test (control *vs* TCDD, $^{**}p < 0.01$).

TH ATP, 9.48 ± 0.71 *vs* 7.06 ± 0.33, $p < 0.01$). DZX decreased the production of H_2O_2 to control levels (Fig. 5, for example, CH ATP + DZX *vs* CH control, 7.23 ± 0.50 *vs* 5.59 ± 0.92, $p > 0.05$), although the amount of H_2O_2 released by cardiac mitochondria from TCDD-treated rats was lower (CH ATP + DZX *vs* TH ATP + DZX, 7.23 ± 0.50 *vs* 5.16 ± 0.49, $p < 0.05$). The agonist effect of DZX was reversed by the addition of 5-HD. In this case, the release of H_2O_2 increased to values close to those found when ATP was present (for example, CH ATP + DZX+5HD *vs* CH ATP, 8.71 ± 0.72 *vs* 9.48 ± 0.71, $p > 0.05$). Confirming the hypothesis that mitoKATP activity contributes to a decrease of H_2O_2 release from the respiratory chain, mitochondria incubated in K^+-free, Na^+-rich media presented higher H_2O_2 release rates (data not shown).

In liver mitochondria from the same experimental animals, H_2O_2 release rates were higher in the presence of ATP, although no differences existed between the control and TCDD-treated animals (data not shown).

4. Discussion

TCDD toxicity through AhR-independent mechanisms may involve direct effects on mitochondria, thus leading to cellular bioenergetic deficits. The major hypothesis of the current study was that TCDD causes mitochondrial dysfunction and oxidative stress in rat heart, leading to an altered regulation of mitochondrial ATP-sensitive K^+ channels (mitoKATP), which would become more active and contribute to decrease the generation of free radicals by the mitochondrial respiratory chain. Previous studies have shown that lipid peroxidation and DNA damage can occur within 24 h of treatment with TCDD (Stohs et al., 1986, 1991, 1990), even after a single treatment of 50 μg TCDD/Kg body weight. For the present study we used a high dosage of TCDD for a short interval as a model for acute TCDD high exposure. The highest dosage ever recorded in humans was 144,000 pg TCDD/g blood fat, which corresponds to a calculated body burden of 1.6 mg TCDD and a dosage of 25 μg/kg body weight (Geusau et al., 2001). Higher dosages used in rats in the present and other studies (Bagchi et al., 1993; Choi et al., 2008; Stohs et al., 1990) are justified by altered metabolism and clearance of TCDD in rats *vs* humans, as previously described (Foster et al., 2010). We should thus take in consideration that the results from the present and above studies apply toward the effects of large acute doses of TCDD and may not be so relevant toward the normal environmental exposures at lower doses.

In our study, male Wistar rats injected with a single dose of 50 μg TCDD/Kg body weight did not exhibit changes in body and organs weight 24 h after treatment. However, Stohs et al. (1991) reported altered liver weight after a single oral dose of 100 μg TCDD/Kg body weight at 3 days post treatment and a significant decrease was reported in body weight on day 5 (Stohs et al., 1991). The differences may be related with the time elapsed post-treatment and with the dosage used.

The present work demonstrates that acute TCDD toxicity results in some measurable alterations of cardiac mitochondrial function,

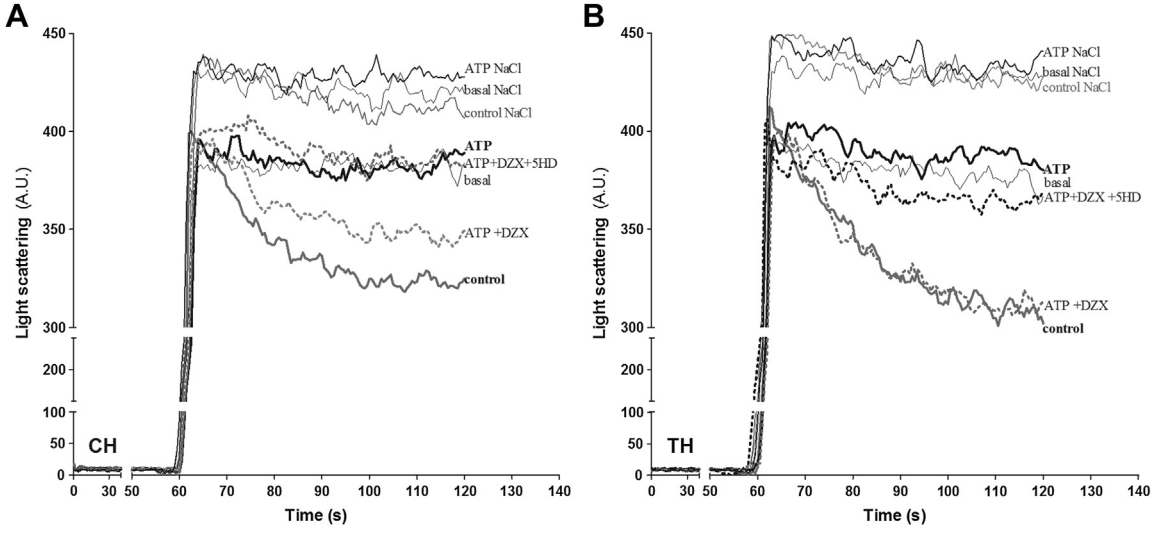

Fig. 3. Mitochondrial ATP-sensitive K^+ transport. Isolated mitochondria (0.25 mg/ml) were added to a reaction medium at 37 °C containing 130 mM KCl, 2 mM KH_2PO_4, 2.5 mM $MgCl_2$ and 10 mM HEPES-KOH pH 7.2. In all experiments, the media contained also 2 mM succinate (except in basal experiments) and 1 μg/ml oligomycin. For basal experiments, 3 μM rotenone was added. Decreases in light scattering due to mitochondrial K^+ and water uptake were measured. (A) Representative traces for heart mitochondria from control animals (CH); (B) representative traces for heart mitochondria from TCDD-treated rats (TH). One mM ATP, 30 μM diazoxide (DZX), 150 μM 5-hydroxydecanoate (5-HD) was added as shown. For heart preparations, K^+ salts in the media were substituted with Na^+, as indicated in the corresponding traces, for the basal (basal NaCl), control (control NaCl) and ATP (ATP NaCl) conditions.

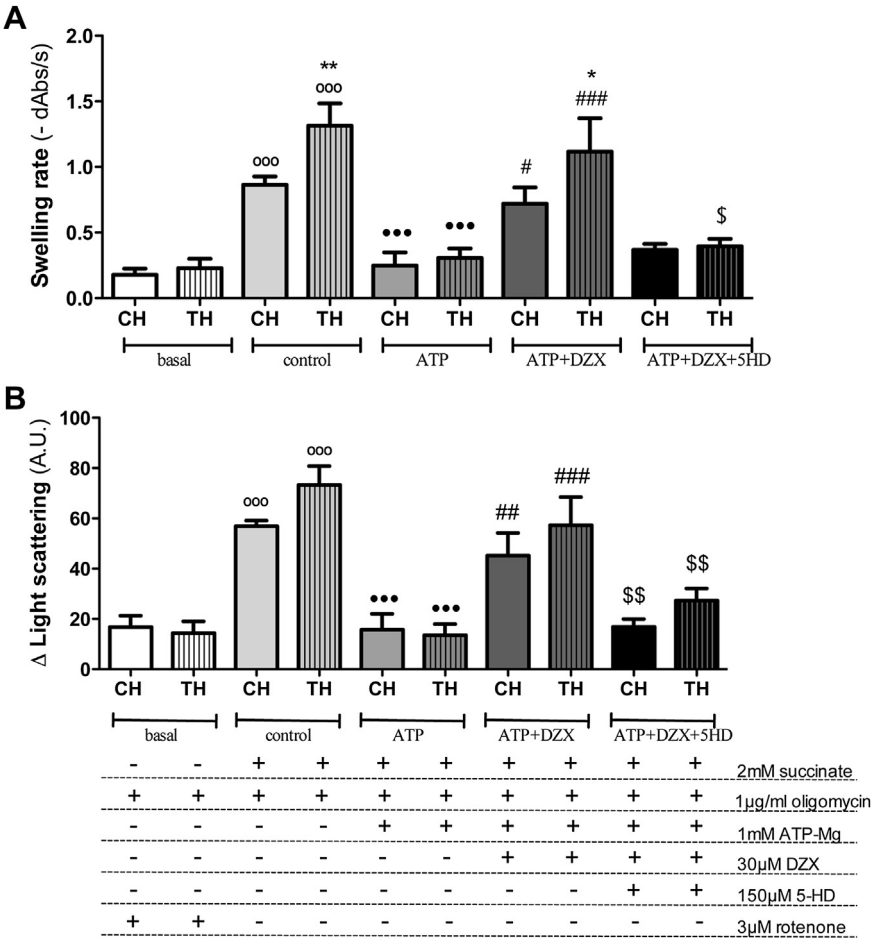

Fig. 4. Effects of TCDD on heart mitoKATP channel activity. (A) swelling rate (−dAbs/s) and (B) magnitude of light scattering variation in heart mitochondria (0.25 mg/ml) from control (CH) and TCDD-treated (TH) rats, as followed in reaction medium (130 mM KCl, 2 mM KH_2PO_4, 2.5 mM $MgCl_2$ and 10 mM HEPES-KOH pH 7.2). Where indicated, 2 mM succinate, 1 µg/ml oligomycin, 1 mM ATP, 30 µM DXZ, or 150 µM 5-HD were present in the media. Swelling rate (A) was determined as the decrease in absorbance occurred after 2 s incubation and during the next 60 s in K^+ media supplied with the compounds described under the graph (+). Control swelling rates in the presence of succinate were 0.86 ± 0.04 for CH and 1.31 ± 0.16 -dAbs/s for TH. The magnitude of swelling variation (B) was determined as the difference between the value of light scattering detected at 120 s (end of experiment) and 62 s (2 s after the addition of mitochondrial suspension). Swelling performed with succinate resulted in a variation of 56.9 ± 2.2 for CH and TH 73.3 ± 7.5 arbitrary units (A.U.). Comparisons were performed using one-way ANOVA, followed by Bonferroni multiple comparison, for the following paired observations inside the group of CH and TH: basal *vs* control (○), control *vs* ATP (●), ATP *vs* ATP + DXZ (#), ATP + DZX *vs* ATP + DZX+5HD ($) (one symbol $p < 0.05$; two symbols $p < 0.01$; three symbols $p < 0.001$) and between groups for the same condition: CH basal *vs* TH basal, CH control *vs* TH control, CH ATP *vs* TH ATP, CH ATP + DZX *vs* TH ATP + DZX, CH ATP + DZX + 5HD *vs* TH ATP + DZX + 5HD ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

which appears to be multi-factorial involving both the respiratory chain and phosphorylative system. An end result of TCDD-induced cardiac mitochondrial alterations may be decreased energy production and inability to respond to stressful events by increased energy output if normal function is not restored. Alterations in cardiac mitochondrial function are accompanied by increased formation of protein carbonyls (Fig. 2), a marker for oxidative stress, which confirms that TCDD increases mitochondrial oxidative stress and damage (Senft et al., 2002).

The next step was to investigate whether increased mitochondrial oxidative stress, besides contributing to cardiac mitochondrial degeneration, can also alter the regulation of the cardiac mitoKATP. Most studies characterizing mitoKATP activity and regulation have been conducted using heart mitochondria due to their higher activity (Facundo et al., 2007). Despite the fact the majority of reports link increased mitoKATP activity to a decrease in oxidative stress (Facundo et al., 2005), others have pointed out that channel activity may actually result into increased mitochondrial free radical production (Andrukhiv et al., 2006). The fact that mitoKATP are regulated by and regulate free radical production by the respiratory chain may explain the controversy.

The most used mitoKATP opener is diazoxide (DZX), although this compound can also inhibit succinate dehydrogenase (CII) when used at concentrations higher than 100 µM, i.e. above the concentration needed to open mitoKATP in isolated mitochondria (Dzeja et al., 2003; Facundo et al., 2007). MitoKATP opening can be inhibited by 5-hydroxydecanoic acid (5-HD), although some authors report that 5-HD can also affect mitochondrial respiration (Lim et al., 2002) and inhibit fatty acid oxidation (Hanley et al., 2005). The effects of openers and blockers of mitoKATP appear to be modulated by the metabolic state and by the thiol redox status of mitochondria (Facundo et al., 2007; Garlid, 1996). Modulation of mitoKATP opening and closure contributes to regulate mitochondrial function. The channels are involved in the regulation of K^+ transport across the inner mitochondrial membrane, controlling mitochondrial transmembrane potential, matrix osmotic pressure and volume (Facundo et al., 2005). Opening of mitoKATP dissipates the inner mitochondrial membrane potential (Holmuhamedov et al., 2004), and increases matrix volume under physiological conditions or when respiration is inhibited (Kowaltowski et al., 2001).

MitoKATP opening prevents the generation of mitochondrial ROS (Forbes et al., 2001), although this idea still raises some debate.

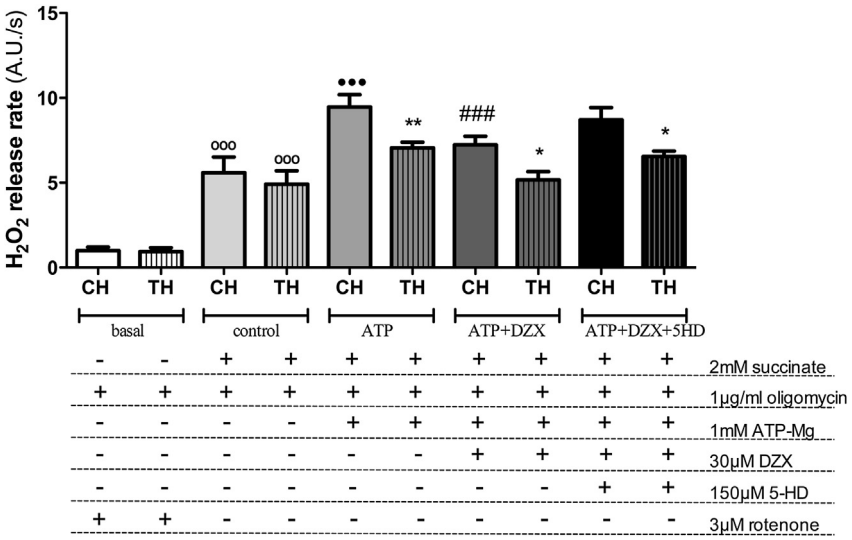

Fig. 5. MitoKATP activity appears to decrease H_2O_2 release in the heart (A). Cardiac mitochondria (100 µg) from control (CH) or TCDD-treated (TH) rats were added to the reaction media (130 mM KCl, 2 mM KH_2PO_4, 2.5 mM $MgCl_2$ and 10 mM HEPES-KOH pH 7.2) in the presence of 5 µM Amplex Red, 1U/L. Depending on the experiment, 2 mM succinate, 1 µg/ml oligomycin, 1 mM ATP, 30 µM DXZ, or 150 µM 5-HD were added to the reaction medium. H_2O_2 release was measured during 30 min at 37 °C. H_2O_2 release rate was determined between 10 and 20 min in K^+ media supplied with the compounds described under the graph (+). Succinate-dependent H_2O_2 release resulted in a rate of 5.6 ± 0.9 A.U. for CH and 4.9 ± 0.8 for TH. Comparisons were performed using one-way ANOVA, followed by Bonferroni multiple comparison, for the following paired observations inside the group, for example for cardiac mitochondria, CH and TH: basal *vs* control (○), control ***vs*** ATP (●), ATP *vs* ATP + DXZ (#), ATP + DZX *vs* ATP + DZX+5HD ($) (one symbol $p < 0.05$; two symbols $p < 0.01$; three symbols $p < 0.001$) and between groups for the same condition: CH basal *vs* TH basal, CH control *vs* TH control, CH ATP *vs* TH ATP, CH ATP + DZX *vs* TH ATP + DZX, CH ATP + DZX+5HD *vs* TH ATP + DZX+5HD ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

Mitochondria are the major source of reactive oxygen in most mammalian cell types, as well as a major target organelle for oxidative damage (Chomyn and Attardi, 2003). The mitoKATP may function in a manner similar to mitochondrial uncoupling proteins (Facundo et al., 2005; Hoerter et al., 2004), which are also activated by oxidative stress, decreasing membrane potential to prevent excessive mitochondrial ROS release (Facundo et al., 2005; Hoerter et al., 2004). Since mitochondrial ROS are involved in regulating pathologic and homeostatic cellular events, it is important to understand the signalling redox pathways involved in the maintenance of proper mitochondrial ROS, including during exposure to environmental chemicals such as TCDD.

By measuring variations in light scattering as means to indirectly follow mitochondrial swelling, an increased activation of mitoKATP in cardiac mitochondria from TCDD-treated rats was detected, the effect being inhibited by mitoKATP antagonists ATP and 5-HD (Figs. 3 and 4). The lack of observable mitochondrial swelling in media without K^+ ions (Fig. 3A and B) provides unequivocal evidence that this effect is related to a selective K^+ transport pathway that promotes mitochondrial swelling and which can be attributable to mitoKATP activity (Facundo et al., 2007). The findings appear to be in accordance with previous results indicating that the mitoKATP is a redox-sensitive channel with increased activity under more oxidative conditions (Facundo et al., 2007; Liu et al., 2002), which is demonstrated here to occur in TCDD-treated rats, as seen by increased carbonyl groups (Fig. 1). Since mitoKATP have been described to be activated when thiol groups are oxidized by exogenous ROS (Zhang et al., 2001), it is logical to predict that TCDD-induced increased ROS generation leads to the oxidation of critical thiol residues and increased open channel probability.

Using cardiac isolated mitochondria (Fig. 5), the results appear to suggest that mitochondrial ROS generation decreases when mitoKATP is in the open state. MitoKATP-modulated oxygen free radical production also exhibited the expected pharmacological response, with the addition of ATP resulting in an increase of ROS release, DZX reversing ATP inhibition and DZX effects being by its turn reversed by the addition of the mitoKATP inhibitor 5-HD. Mitochondria incubated in K^+-free, Na^+-containing media presented higher H_2O_2 release rates. The data suggests that the mitoKATP may be acting as a reactive oxygen species sensor that acts to decrease mitochondrial free radical generation in response to enhanced local levels of oxidants, as previously described (Facundo et al., 2007). It has been previously suggested that the effects of DZX on oxidative stress modulated by channel activity results from succinate dehydrogenase inhibition (Facundo et al., 2007); in the present study, a concentration of 30 µM of DZX was used, which is three-fold lower than concentrations already described to inhibit complex II (Dzeja et al., 2003; Kowaltowski et al., 2001). The physiological mitoKATP agonist GTP decreases mitochondrial ROS release only when ATP inhibits the channel and K^+ is available for transport, confirming that mitoKATP activity prevents mitochondrial ROS release (Facundo et al., 2007). The protective activity of mitoKATP is also supported by the fact that mitochondrial uncoupling resulting from expressing mitochondrial uncoupling proteins (UCP) or from mild treatment with protonophores are also strongly cardioprotective, decreasing oxidative stress (Hoerter et al., 2004). Besides the possible mild mitochondrial uncoupling, activation of mitoKATP can also protect mitochondria during stressful events due to the promotion of mild mitochondrial swelling, which decreases the spatial distance between the inner and outer mitochondrial membranes, thus contributing to improve oxidative metabolism and decreasing ROS production by the mitochondrial respiratory chain (Facundo et al., 2006).

Contrary to our findings, some groups have proposed that mitoKATP activity contributes to an increase in ROS release (Andrukhiv et al., 2006). However, the increase is unrelated to the mitoKATP and attributable to interferences in ROS measurements using dichlorofluorescein in the presence of DZX (Facundo et al., 2007). In our experiments, we used the specific, high sensitivity and low noise probe Amplex Red, which is oxidized specifically by HRP-bound H_2O_2 to form the fluorescent product resorufin (Facundo et al., 2007; Zhou et al., 1997).

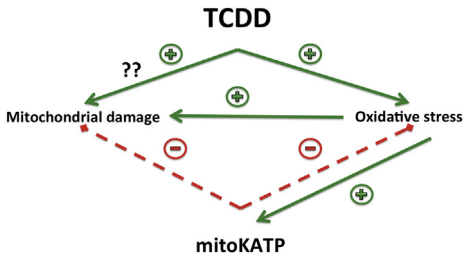

Fig. 6. Proposed interplay between cardiac mitoKATP and TCDD. From the data reported in the present work, we propose that TCDD causes mitochondrial oxidative stress, which can contribute to induce degeneration of mitochondrial function. It is also likely that TCDD causes mitochondrial dysfunction by ROS-independent factors, e.g. by altering mitochondrial membrane order. TCDD-induced oxidative stress signals through the mitoKATP, increasing their open state probability. The mitoKATP will act downstream to decrease ROS production by the respiratory chain and contribute to decrease the overall oxidative mitochondrial environment, in an attempt to adapt to TCDD increased oxidative stress. Circles with a "+" sign (full lines in green) denote a positive effect, while circles with a "−" (dashed lines in red) denote a negative effect. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Interestingly, the fact that mitoKATP were more active in hearts from TCDD-treated rats can contribute to the decrease in mitochondrial transmembrane potential in the hearts of treated rats, although the results were only significant when succinate was used as substrate. This is hardly surprising as it has been described that the effects of mitoKATP on mitochondrial membrane potential are not easily detected with conventional methods (Facundo et al., 2006).

Despite the obvious advances in the understanding of mitoKATP as a redox sensor during dioxin-induced mitochondrial toxicity, a few limitations exist in the present work. One includes the mechanisms of TCDD-induced oxidative stress on cardiac mitochondria. It has been reported that TCDD causes oxidative stress on liver mitochondria although the authors could not exactly pinpoint the exact mechanism of increased ROS generation (Senft et al., 2002). The second limitation is to identify the links between TCDD-induced oxidative stress, increased mitoKATP activation and consequent response by decreasing ROS production by mitochondria, which to be fair is not a solo limitation of the present work, since the literature is far from an agreement (Andrukhiv et al., 2006; Facundo et al., 2007).

In conclusion, this study indicates that acute TCDD toxicity causes negative alterations on heart mitochondria and enhanced carbonylation of proteins. TCDD also caused an alteration of mitoKATP modulation, which appears to result in a decreased production of ROS by the mitochondrial respiratory chain in the treated animals. Independent of the controversy regarding the activity of mitoKATP, our results appear to suggest that the activity of these channels may decrease mitochondrial ROS release. Our data and that of others suggest that mitoKATP regulate ROS generation, thus minimizing ROS-induced oxidative damage caused by TCDD to mitochondrial structures, which is already apparent from increased protein carbonylation (Fig. 6). On the other hand, the present work highlights the need to explore alternative targets of known xenobiotics, that are known to cause oxidative stress in mitochondria, and which may alter the regulation of the mitoKATP and thus cause physiological alterations which may positively or negatively impact the outcome of toxicant interactions.

Author disclosure statement

None of the authors have any disclosures as no competing financial interests exist.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2013.05.049.

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