Acute Mitochondrial Actions of Glitazones on the Liver: a Crucial Parameter for their Antidiabetic Properties

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Key Words

Liver • Mitochondria • Glitazones • Direct effects • Oxygen consumption • Oxidative phosphorylation • ROS production • Electron transport chain complexes • PTP

Abstract

Background/aims: Glitazones are synthetic insulin-sensitizing drugs which act as agonists of peroxisome proliferator-activated receptor gamma (PPAR γ). However, TZDs action does not exclude independent PPAR γ -activation effects. Remarkably, direct mitochondrial action of these agents has not been fully studied yet. **Methods:** Oxygen consumption rates (JO_2) were measured using a Clark-type oxygen electrode in intact hepatocytes and isolated liver mitochondria. Mitochondrial reactive oxygen species (ROS) production was quantified by fluorescence assay. Moreover, activities of mitochondrial respiratory chain complex I, II and III were spectrometrically determined. **Results:** Pioglitazone and rosiglitazone inhibited JO_2 in liver cells and mitochondria. This inhibition affected the state 3 of respiration (in the presence of ADP) and the uncoupled state (after addition of dinitrophenol).

Moreover, these agents dramatically reduced mitochondrial ROS production in all situations tested. We also demonstrated that both glitazones specifically inhibited the activities of complex I and complex III, by 50% and 35% respectively. Additionally, they do not modify neither the oxidative phosphorylation yield nor the permeability transition pore opening. **Conclusions:** Pioglitazone and rosiglitazone reduce both respiration intensity and ROS production, acutely and by a probable PPAR γ -independent way, through inhibition of complex I and III activities. This new finding could positively contribute to their anti-diabetic properties.

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Introduction

Glitazones or thiazolidinediones are a unique class of oral glucose-lowering agents that work primarily by activating the nuclear transcription factor peroxisome proliferator-activated receptor gamma (PPAR γ), thereby

turning on and off specific genes for the regulation of glucose and lipid metabolism. There is now considerable evidences to propose that, beyond reducing insulin resistance and providing a durable glycemic control, these agents exert a number of pleiotropic effects that may play an important role in the management of type 2 diabetes mellitus (T2DM) [1].

Type 2 diabetes is a disease associated with mitochondrial dysfunction [2], suggesting that its prevention and/or treatments should focus on the mitochondrial targets [3]. In skeletal muscle, patients with diabetes have a lower mitochondrial mass, thereby implying the decrease of oxidative phosphorylation (OXPHOS) rates [4]. On this matter, investigators have found that lifestyle changes can increase the number of mitochondria and help in control diabetes [5]. Moreover, mitochondrial levels of reactive oxygen species (ROS) are increased in response to hyperglycemia, which could lead to vascular complications such as renal nephropathy [6]. The emergence of oxidative stress has generated much more interest primarily because of its accepted role as a major contributor to the aetiology of normal senescence and severe pathologies such as diabetes, obesity, and atherosclerosis. During the normal metabolism of molecular oxygen in the mitochondrion, ROS are byproducts of reduction-oxidation reactions and are highly reactive molecules that play an important role in cell signaling, acting as a double sword in modulating insulin signaling [7]. On one hand, they are generated in response to insulin and are required for this hormone to exert its complete physiological actions. On the other hand, increasing evidences implicate ROS as negative regulators in the development of insulin resistance and the onset of type 2 diabetes [8, 9]. Increased oxidative stress seems to be the major culprit of diabetes by triggering multiple alterations in chain [10].

PPAR γ is expressed predominantly in adipose tissue and TZDs have been reported to exert receptor-dependent as well as receptor-independent effects. Indeed, several evidences suggest that at least some of the pharmacology of these insulin sensitizers may be independent of PPAR γ [11-13]. Reported receptor-independent effects include suppression of inflammatory gene expression, modification of energy and fuel metabolism, suppression of cell proliferation and induction of cytotoxicity, and perturbation of mitochondrial function. Regarding the acute and PPAR γ -independent effects of TZDs on the mitochondrial function, it has been shown that troglitazone induced a rapid (within minutes) increase in intracellular calcium levels and caspase-3 activation in human HepG2

hepatocarcinoma cells [14]. In astrocytes, TZDs caused a rapid increase in glucose consumption and lactate production (occurring within 1-2 h) followed by a subsequent mitochondrial membrane hyperpolarization [15], and also encouraged ROS production [16]. The Furnsinn's group characterized the inhibitory effects of TZDs on aerobic metabolism and showed that TZDs inhibited complex I activity in liver and skeletal muscle tissue [17]. An inhibitory effect on complex I by ciglitazone was also previously reported using HL-60 cells [18]. In fact, it is not clear whether these effects may be mediated by a recently identified mitochondrial binding site for TZDs, termed as "mitoNEET" [19]. Nonetheless, it is possible that alterations in the mitochondrial running might contribute to the lipid lowering and/or antidiabetic actions of these drugs, mainly in the case of pioglitazone.

The present study aimed to better define the mechanism underlying TZDs action on hepatocytes respiration and liver mitochondrial function, this latter being evaluated by measurements of JO_2 , oxidative phosphorylation efficiency, mitochondrial ROS formation at the electron transfer chain level, and finally, respiratory complex I, II and III activities. Moreover, due to existence of the permeability transition pore (PTP), a Ca^{2+} -sensitive mitochondrial inner membrane channel involved in several cell death models, we also investigated direct effects of these drugs upon hepatic PTP regulation. This research was conducted with the only two available glitazones in the market, pioglitazone and rosiglitazone, and both products were used in a range of concentrations (μ M) barely superior to therapeutic levels found following an oral drug administration at currently approved dosages [20]. Finally, neither is it intended here to negate reports that rosiglitazone was recently suspended in the European market because of the risk of myocardial infarction associated to its utilization and that pioglitazone is under review due to its probable association with the risk of bladder cancer [21].

Materials and Methods

Chemicals and reagents

Enzymes were acquired by Roche (Mannheim, Germany), all substrates and chemicals by Sigma (Steinheim, Germany), except for sucrose, KCl, KH_2PO_4 , K_2HPO_4 , glucose and inorganic phosphate (Pi) that were obtained in Panreac (Barcelona, Spain). The fluorescent probes, Amplex Red and Calcium Green-5N, were purchased from Biochemika Sigma (Steinheim, Germany) and Invitrogen (Eugene, Oregon, USA), respectively. Pioglitazone and rosiglitazone (both acquired from

Beijing Gaobo Pharm-Chemicals Tech. Co., Ltd, China) were dissolved in a vehicle containing 50% DMSO and 50% ultra-pure water. Importantly, the final concentration of DMSO in all circumstances was not superior to 1% of the total volume.

Isolation of hepatocytes

Liver cells were prepared between 8.00 and 10.00 am from male Wistar rats (200-300 g) fasted 24 h. Animals were anaesthetized with intraperitoneal injection of sodium thiopental (0.2 ml·100 g⁻¹ body weight). Hepatocytes were isolated in the presence of collagenase according to the method of Berry and Friend (1969) [22], modified by Groen et al. [23]. All procedures were performed in accordance with the principles and guidelines established by the European Convention for the Protection of Laboratory Animals.

Isolation of mitochondria

Liver mitochondria from fed male Wistar rats (200-300 g) were prepared according to standard differential centrifugation procedures, in a medium containing 20 mM Tris-HCl (pH 7.2), 250 mM sucrose, 1 mM EGTA [24]. Mitochondrial protein content was determined by the Biuret method [25]. Freshly isolated mitochondria were used immediately for determinations of respiration, oxidative phosphorylation yield, ROS production, or were stored at -80°C until complex activities analysis.

Determination of mitochondrial oxygen consumption rate in intact hepatocytes and isolated mitochondria

Two different approaches were used to study the effect of glitazones on JO_2 . In one case, hepatocytes (final amount of 10 mg dry cells/ml) were incubated in closed vials at 37°C in a shaking water bath with Krebs-bicarbonate calcium buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 1.3 mM $CaCl_2$; pH 7.4), saturated with a mixture of O_2/CO_2 (19:1 by volume) and supplemented with 20 mM dihydroxyacetone (DHA) as substrate and the indicated concentrations of glitazones (25, 50 and 100 μ M). After 30 min of incubation, the cell suspension was saturated again with O_2/CO_2 for 1 minute, then quickly transferred into a stirred oxygraph vessel equipped with a Clark oxygen electrode. JO_2 was measured at 37°C before and after the successive addition of 6 μ g·ml⁻¹ oligomycin and 100 μ M 2,4 dinitrophenol (DNP). On the other hand, isolated mitochondria [1-2 mg of protein ml⁻¹ depending on whether mitochondria were energized with 5 mM succinate plus 0.5 mM malate plus 1.25 μ M rotenone (S/M) or 5 mM glutamate plus 2.5 mM malate (G/M), respectively] were suspended in KCl buffer (125 mM KCl, 1 mM EGTA, 5 mM Pi, 20 mM Tris-HCl, 0.1% FFA-BSA; pH 7.2; 37°C) supplemented or not with either glitazone (pioglitazone or rosiglitazone at doses of 10, 25, 50 and 100 μ M). JO_2 was next measured before and after the successive additions of 1 mM ADP, 1.25 μ g·ml⁻¹ oligomycin, 75 μ M DNP, 0.15 μ g·ml⁻¹ antimycin A and 1mM TMPD (N,N,N',N'-tetramethyl-1,4-phenylenediamine) plus 5 mM ascorbate, in order to respectively assess the phosphorylating state 3, the non-phosphorylating state 4, the uncoupled respiration, and the maximal activity of cytochrome oxidase.

Determination of the oxidative phosphorylation yield (ATP/O ratio)

ATP/O ratio was determined from ATP synthesis rate (J_{ATP} vs JO_2) with an ADP-regenerating system based on hexokinase and glucose [26]. For determination of JO_2 , mitochondria were incubated as described above in the KCl buffer supplemented with 20 mM glucose, 1 mM $MgCl_2$, 125 μ M ATP using S/M plus rotenone (5 mM/0.5 mM/1.25 μ M), G/M (5/2.5 mM) or glutamate/succinate/malate (G/S/M) (5/5/2.5 mM) as respiratory substrates. All the experiments were carried out in the presence of 25 μ M glitazones or their vehicle. The rate of ATP synthesis was followed by glucose 6-phosphate formation, using an ADP regenerating system with increasing concentrations of hexokinase (from 0.17 to 0.7 U/ml).

Determination of mitochondrial ROS production

Generation of ROS was assayed on a Hitachi fluorimeter by monitoring H_2O_2 -induced fluorescence of 1 μ M Amplex Red (excitation: 560 nm; emission: 584 nm) in the presence of horseradish peroxidase (10 U/ml). Isolated mitochondria (0.25 mg/ml) were incubated at room temperature in KCl buffer containing the fluorescent probe and peroxidase as mentioned above, and both glitazones at different concentrations (25, 50 and 100 μ M) or their vehicle. The reaction was started by addition of various substrates: S/M (2.5/0.25 mM), G/M (2.5/1.25 mM) or G/S/M (2.5/2.5/1.25 mM). Mitochondrial H_2O_2 levels were first measured in the basal condition, then 1.25 μ M rotenone and 3 μ M antimycin A were sequentially added. All the values were calculated against a known amount of H_2O_2 .

Respiratory complex activities determination

Measurement of the specific activity of respiratory chain complexes I, II and III was performed spectrophotometrically using liver mitochondrial particles resulting from 3 cycles of freezing-thawing. Enzyme activity was expressed as nanomoles of reduced or oxidized substrate per minute and per milligram of mitochondrial proteins. *Complex I (rotenone-sensitive NADH-ubiquinone oxidoreductase)* was assayed as the rate of NADH oxidation at 340 nm in 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.5) containing 3.75 mg/ml FFA-BSA, 100 μ M decylubiquinone as an electron acceptor and 100 μ M NADH. Addition of 10 μ M rotenone, which allows the calculation of the rotenone-sensitive specific activity, was used to specifically inhibit this enzymatic complex whose activity was deduced from the difference between NADH oxidation without and with rotenone. *Complex II (succinate-ubiquinone reductase)* was quantified by measuring the absorbance decrease due to reduction of 100 μ M 2,6-dichloroindophenol (DCIP) (synthetic acceptor of electrons from respiratory complex II) at 600 nm in 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.5) supplemented with 2.5 mg/ml FFA-BSA, 9.3 μ M antimycin A, 5 μ M rotenone and 30 mM succinate. Enzyme activity was calculated from the difference before and after the addition of 50 μ M decylubiquinone, the electron acceptor from complex II. *Complex III (ubiquinone cytochrome c reductase)* was determined by monitoring the reduction of oxidized cytochrome c at 550 nm. Mitochondria were incubated in 100 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.5) with 1 mg/ml FFA-

BSA, 50 μ M EDTA, 1 mM KCN, 10 μ M oxidized cytochrome c. The reaction was started by the addition of 105.6 μ M decylubiquinol, which was prepared by chemical reduction of decylubiquinone by sodium dithionite. The reduction of cytochrome c was quantified with and without 9.1 μ M antimycin A.

Measurement of Ca^{2+} retention capacity (CRC) in permeabilized hepatocytes and isolated mitochondria

Intact cells (10 mg/ml) were first incubated for 30 min in 2 ml of Krebs-bicarbonate buffer lacking of calcium to avoid any possible interference with the fluorescent probe. Next, 15 mg of cell suspension were harvested, centrifugated (1000 g for 30 s) and resuspended in a standardized buffer (250 mM sucrose, 10 mM MOPS and 1 mM Pi; pH 7.4) containing 40 μ M digitonin for membrane permeabilization. Mitochondrial experiments were performed using 1 mg/ml of isolated organelles. For both conditions, mitochondria fueled with S/M (5/0.5 mM) were incubated in standardized buffer supplemented with 2 μ M Calcium Green-5N (excitation-emission: 506-526 nm). After 1 min of stabilization, the reaction was started by the addition of successive 12.5 μ M Ca^{2+} pulses at 1 min intervals.

Statistics

The results are expressed as means $\pm$ SEM for the indicated number of separate experiments. The statistical significance of differences was calculated using Student's t-test for unpaired samples (Simfit. Version 6.2 software).

Results

Inhibition of oxygen uptake by pioglitazone and rosiglitazone in rat hepatocytes and liver mitochondria

In a first set of experiments, we assessed the effect of pioglitazone and rosiglitazone on cellular respiration by measuring JO_2 in pre-incubated hepatocytes with or without glitazones at concentrations of 25, 50 or 100 μ M. As shown in Table 1, (only data obtained in the presence of pioglitazone are shown), pioglitazone induced a clear dose-dependent inhibition of the basal JO_2 in comparison with the control situation. Oxygen consumption was reduced about 15%, 19.5% and 30% respectively with 25, 50 and 100 μ M pioglitazone. The subsequent addition of oligomycin, an inhibitor of the ATP synthase- F_0 subunit, diminished cellular respiration irrespective of the presence of the antidiabetic. The JO_2 -inhibition obtained in the presence of oligomycin is not associated with ATP-synthesis process and thus exactly reflects the phenomenon of energy dissipation (protons leak and slippage). Following addition of the uncoupler DNP, pioglitazone (at its highest dose) reduced JO_2 to a degree really closer than above reported. Our data however revealed that respiration intensities under DNP were

	JO_2 (natomsO $\cdot$ min $^{-1}$ ·g of dry hepatocytes $^{-1}$)		
	Basal	Oligomycin	+ DNP
CONTROL	24.51 $\pm$ 0.45	10.34 $\pm$ 0.45	23.83 $\pm$ 1.04
PIO 25 μ M	20.77 $\pm$ 0.62***	10.3 $\pm$ 0.93	19.36 $\pm$ 1.36*
PIO 50 μ M	19.51 $\pm$ 1***	11.99 $\pm$ 0.42	19.51 $\pm$ 0.37*
PIO 100 μ M	17.06 $\pm$ 0.72***	10.89 $\pm$ 0.79	15.57 $\pm$ 0.76***

Table 1. Effects of pioglitazone on oxygen uptake in intact isolated hepatocytes. Hepatocytes (10 mg dry cells/ml) were incubated in closed vials at 37°C in 2.5 ml of Krebs-bicarbonate calcium buffer saturated with a mixture of O $_2$ /CO $_2$ (19:1 by volume) supplemented with 20 mM DHA, and 25 μ M pioglitazone. After 30 min of incubation, the cell suspension was transferred into a stirred oxygraph vessel where JO_2 was measured before and after the successive addition of 6 μ g $\cdot$ ml $^{-1}$ oligomycin and 100 μ M DNP. Data are expressed as means $\pm$ SEM of n=3 different hepatocytes isolation, all tests were performed five independent times for each condition. *p<0.05 and ***p<0.001 as compared to control-vehicle incubated hepatocytes in the same conditions.

	JO_2 (natomsO $\cdot$ min $^{-1}$ ·mg of proteins $^{-1}$)			
	State 3	State 4	+ DNP	+ TMPD-a
G/M	163.65 $\pm$ 6.44	15.47 $\pm$ 0.89	142.48 $\pm$ 9.41	257.89 $\pm$ 13.36
PIO 25 μ M	116.30 $\pm$ 7.50**	20.88 $\pm$ 2.10***	137.54 $\pm$ 12.19	241.63 $\pm$ 15.20
PIO 100 μ M	79.12 $\pm$ 5.74***	29.59 $\pm$ 4.48***	109.04 $\pm$ 9.40	236.66 $\pm$ 13.35
ROSI 25 μ M	129.69 $\pm$ 11.03*	20.39 $\pm$ 1.39***	113.00 $\pm$ 10.83	259.73 $\pm$ 13.27
ROSI 100 μ M	89.63 $\pm$ 7.23***	33.50 $\pm$ 3.14***	90.57 $\pm$ 10.03**	255.32 $\pm$ 19.14
S/M	241.54 $\pm$ 9.60	40.10 $\pm$ 2.38	238.15 $\pm$ 13.50	412.67 $\pm$ 14.36
PIO 25 μ M	224.34 $\pm$ 7.83	57.73 $\pm$ 1.19***	252.69 $\pm$ 15.13	376.51 $\pm$ 16.79
PIO 100 μ M	203.37 $\pm$ 10.36	112.99 $\pm$ 6.14***	244.33 $\pm$ 20.05	342.65 $\pm$ 22.29*
ROSI 25 μ M	232.56 $\pm$ 16.24	54.71 $\pm$ 4.95***	213.41 $\pm$ 18.66	391.96 $\pm$ 29.46
ROSI 100 μ M	227.42 $\pm$ 16.38	227.42 $\pm$ 16.38***	208.61 $\pm$ 16.89	394.08 $\pm$ 30.96

Table 2. Effects of pioglitazone and rosiglitazone on oxidative phosphorylation in rat liver isolated mitochondria. Mitochondria (2 mg/ml with GM and 1 mg/ml with SM) were incubated at 37°C with KCl respiration buffer, pioglitazone or rosiglitazone (25 and 100 μ M), or its vehicle. JO_2 was determined using mitochondria energized with GM (5/2.5 mM) or SM (5/0.5 mM) plus rotenone (1.25 μ M) as energetic substrates. The state 3 respiratory rate was obtained after addition of 1 mM ADP-Tris; the state 4 respiratory rate was obtained by the addition of 1.25 μ g $\cdot$ ml $^{-1}$ oligomycin; and the uncoupled respiratory rate was obtained after the addition of 75 μ M DNP. Finally, the maximal activity of cytochrome oxidase was determined by the addition of 1 mM TMPD and 5 mM ascorbate in the presence of 0.15 μ g/ml antimycin. Data are expressed as means $\pm$ SEM of n=10 different isolated liver mitochondria preparations. All tests were performed for at least five independent times for each condition. *p<0.05; **p<0.01 and ***p<0.001 as compared with control-vehicle incubated mitochondria in the same conditions.

similar, even inferior, to basal values. In fact, uncoupling led to a drop in cytosolic ATP/ADP ratio partly preventing

Fig. 1. Relationship between J_{ATP} and JO_2 in liver mitochondria incubated with or without pioglitazone and rosiglitazone. Rat liver mitochondria (2 mg/ml with G/M and 1 mg/ml with S/M or G/S/M) were suspended in KCl buffer supplemented with 20 mM glucose, 1 mM $MgCl_2$, 125 μM ATP, in the presence of 25 μM pioglitazone (open triangles) or rosiglitazone (open circles) compared with control-vehicle (closed squares). The effect of both glitazones was determined by using G/M (panel A), S/M (panel B) and G/S/M (panel C) as energizing substrates. The ATP synthesis flux was modulated by different concentrations of hexokinase (0.17–0.7 U/ml) and monitored by the formation of glucose 6-phosphate. Data are means $\pm$ SEM of $n=7$ different isolated liver mitochondria preparations. All assays were performed at least four independent times for each respiratory substrate.

any further DHA metabolization through glycolysis, and thus its utilization by hepatocytes, so that a slowing down of respiratory flux was found. Results also suggest that pioglitazone, at the cellular level, does not affect neither the proton leak across the inner mitochondrial membrane nor the slipping of the coupling between redox reactions and proton pumping.

Table 2 presents OXPHOS parameters for isolated mitochondria exposed to the presence of pioglitazone and rosiglitazone at doses of 25 and 100 μM , although all determinations were also performed with both agents at the two other ones (10 and 50 μM). As evidenced, in mitochondria energized with the complex I-linked substrate (G/M) or complex II-linked substrate (S/M), both glitazones decreased state 3 respiration (phosphorylating condition in the presence of saturating ADP concentrations) and activated state 4 respiration (non-phosphorylating condition in the presence of oligomycin). Specifically, TZDs increased respiratory state 4 rate in a dose-dependent manner and therefore the largest activation (about 100% or even higher) was obtained with their maximal concentrations. On the other hand, while TZDs-induced inhibition of state 3 was by a dose-dependent way with G/M as a substrate, the decrease did not follow a dose-dependent pattern with S/M, surprisingly obtaining the highest rosiglitazone-induced inhibition rate at its lowest dose of 10 μM (data not shown). Subsequent addition of DNP completely reversed

oligomycin-induced inhibition of respiration and increased JO_2 to similar values of state 3. Nonetheless, DNP did not fully reverse the oligomycin effect in TZDs-exposed mitochondria. Strikingly, this latter effect was significant and dose-dependent with G/M as a substrate (142.48 ± 9.41 , 109.04 ± 9.40 and 90.57 ± 10.03 natoms $O \cdot min^{-1} \cdot mg$ of proteins $^{-1}$ for control and 100 μM pioglitazone and rosiglitazone, respectively), while no significative effect was found in TZDs-exposed mitochondria fueled with S/M. Finally, the lack of a significative action of both glitazones in the presence of TMPD/ascorbate, indicate that glitazones did not affect the complex IV of respiratory chain, despite a slight trend towards the inhibition was observed (412.67 ± 14.36 versus 342.65 ± 22.29 natoms $O \cdot min^{-1} \cdot mg$ of proteins $^{-1}$ in control and 100 μM pioglitazone, respectively; with S/M as a substrate). Taken together, our results suggest that the TZDs-inhibition of JO_2 in liver mitochondria is located at the electron transfer chain, specifically on mitochondrial respiratory complex I.

Neither pioglitazone nor rosiglitazone modify the efficiency of oxidative phosphorylation rate in rat liver mitochondria whatever the substrate employed

As evidenced in Fig. 1, the relationship between J_{ATP} (assessed by glucose-6-phosphate production) and JO_2 , that reflects the yield of oxidative phosphorylation or ATP/

Fig. 2. Effects of pioglitazone and rosiglitazone on mitochondrial H_2O_2 production. Mitochondria (0.25 mg/ml) were incubated with either G/M (panel A), S/M (panel B) or G/S/M (panel C) as respiratory substrates. H_2O_2 formation was assessed by the Amplex Red/ horseradish peroxidase method (see material and methods) before and after the consecutive addition of 1.25 μ M rotenone and 3 μ M antimycin A, in the presence of both agents at concentrations of 10, 25, 50 and 100 μ M, or its vehicle. Data are means $\pm$ SEM of $n=8$ different isolated liver mitochondria preparations. All assays were performed for at least four independent times for each respiratory substrate. * $p<0.05$, ** $p<0.01$ and # $p<0.001$ versus control-vehicle incubated mitochondria in the same conditions.

O ratio, was either unaffected or even decreased in TZDs-incubated mitochondria as compared to control. When we focused on complex I by using G/M (panel A) as a substrate, the curve was slightly shifted to the right with both glitazones at 25 μ M, indicating that ATP synthesis was weakly lower in comparison with control situation. Contrary to observations in the presence of G/M, the ATP/O ratio remained linear and parallel regarding to control of either S/M (panel B) or G/S/M (panel C) conditions.

Pioglitazone and rosiglitazone short-term exposure reduce mitochondrial H_2O_2

It is recognized that respiratory complexes I and III are the main sources of mitochondrial ROS production.

Indeed, complex I generate ROS by two distinct mechanisms, (i) in the presence of NADH produce O_2^- through a forward electron flux linked to complex I-substrates (i.e. G/M) and this generation is enhanced by the inhibitor rotenone [27] and (ii) ROS-production linked to complex II substrates (i.e. S/M) through a reverse electron transport, this being abolished by rotenone, thereby evidencing that is due to electrons entering into complex I through the CoQ-binding sites [28]. On the contrary, complex III can be induced to produce O_2^- by its own inhibitor, antimycin A.

In fact, the basal rates of H_2O_2 production by liver mitochondria were absolutely dependent on the substrate provided. Indeed, the amount of basal H_2O_2

Fig. 3. Effects of pioglitazone and rosiglitazone on respiratory complexes I (panel A), II (panel B) and III (panel C) activities. A total of 24 μg mitochondria for complex I and II, or 10 μg mitochondria for complex III were employed. Enzyme activity was expressed as nanomoles of reduced or oxidized substrate per minute and per milligram of mitochondrial proteins. Data are means $\pm$ SEM of $n=3$ different isolated liver mitochondria. All assays were performed at least three independent times for each respiratory complex. *** $p<0.001$ versus control.

produced with complex I-linked substrate (G/M) was the lowest, specifically by 4- and 5-fold decreased as compared with S/M and G/S/M, respectively. As evidenced in Fig. 2, both glitazones at all doses tested (25, 50 and 100 μM) significantly reduced basal H_2O_2 amounts irrespective of the substrate used: G/M (panel A), S/M (panel B) and G/S/M (panel C). This TZDs-mediated inhibition of basal H_2O_2 production was dose-dependent in all cases, with G/M specifically ranged from 51.56 ± 3.15 in control to 28.41 ± 1.70 and 33.76 ± 2.28 $\text{pmol}\cdot\text{min}^{-1}\cdot\text{mg proteins}^{-1}$ in the presence of 100 μM pioglitazone or rosiglitazone, respectively. Interestingly, glitazones-induced inhibition of basal H_2O_2 production was in a much greater extent with succinate as a substrate, by approximately 80% and 70% with S/M and G/S/M, respectively. Rotenone-enhanced ROS production in G/M experiments was unaffected by both glitazones, excluding a slight reduction observed with 100 and 50 μM pioglitazone, while rotenone-inhibited ROS production was greatly diminished by using S/M or G/S/M. On this basis, our results suggest that TZDs-diminished ROS production is mainly due to reverse electron flux from complex II to complex I. As a final point, TZDs-decreased H_2O_2 production remained after the addition of antimycin A, thereby evidencing that the effect of glitazones is also placed at the level of complex III.

Pioglitazone and rosiglitazone rapidly decrease the activities of mitochondrial respiratory complex I and III without modifying complex II

As pioglitazone and rosiglitazone inhibit mitochondrial ROS production and JO_2 , in either intact cells or isolated mitochondria, we postulated that mitochondria, and more particularly the electron transfer chain, could represent one direct target of these pharmacological compounds. To further confirm these questions we determined isolated respiratory complex activities by using disrupted liver mitochondria suspension. By monitoring the complex I and III activities, as shown in Fig. 3 (panels A and C, respectively), we demonstrated that the activities of complex I and III were drastically diminished by both glitazones at all doses tested (10, 25, 50 and 100 μM). However, TZDs-induced respiratory complex inhibition was not dose-dependent, and we noted that the greatest percentages of decrease in most cases were obtained by using either compound at a dose lower than 100 μM . Strikingly, the lowest inhibition of complex III activity by rosiglitazone was found at its maximal concentration, in agreement with our previous results obtained by measuring ROS production in the presence of antimycin A. On the other hand, neither pioglitazone nor rosiglitazone significantly altered complex II activity. Indeed, no difference was observed between the control situation and the majority of glitazones concentrations.

Fig. 4. Effects of pioglitazone and rosiglitazone on the liver calcium retention capacity. Experiment was started by the addition of 15 mg permeabilized hepatocytes (panel A) or 1 mg/ml liver mitochondria (panel B) in a standardized buffer (250 mM sucrose, 10 mM MOPS and 1 mM Pi; pH 7.4). Mitochondria were energized with S/M (5/0.5 mM) in all cases. Extra-mitochondrial Ca²⁺ concentration was measured after successive addition of 12.5 μM Ca²⁺ pulses, in the presence of pioglitazone or rosiglitazone (10, 25 and 50 μM, both cases), and their vehicle. Data are means±SEM of n=3 different hepatocytes isolation; n= 3 different isolated liver mitochondria. All assays were performed at least three independent times for each condition. *p<0.05 versus control mitochondria incubated. ***p<0.001 versus control permeabilized hepatocytes or liver mitochondria.

Glitazones do not significantly affect PTP opening in permeabilized hepatocytes and liver mitochondria

PTP opening plays a key role in the release of mitochondrial pro-apoptotic factors such as cytochrome c into the cytoplasm, and it also participates in the distribution of nucleotides and the mitochondrial depolarization [29]. We therefore decided to study whether pioglitazone or rosiglitazone could exert an acute effect on PTP regulation. By using permeabilized hepatocytes (Fig. 4A) or isolated liver mitochondria (Fig. 4B), both models being energized with S/M, it seemed that glitazones tended to diminish the Ca²⁺ load required to induce PTP opening, but this response was only significative with 50 μM pioglitazone on isolated mitochondria. Expectedly, the Ca²⁺ load required to open the pore was increased by 1 μM cyclosporin A (CsA), the PTP reference blocker.

Discussion

Unpublished data from our laboratory, focusing on the direct metabolic effects of pioglitazone and rosiglitazone in perfused rat hepatocytes, suggested that cellular respiration could also been affected because of the rapid activation of glycolysis and the fall in both cytosolic and mitochondrial ATP/ADP ratio. So, our next objective was to investigate whether both glitazones exert direct actions on liver cell respiration that could contribute

to their antidiabetic properties or amplify them. The cell model of hepatocytes was chosen because the liver plays a crucial role in maintaining glucose homeostasis during both fasting and feeding. Indeed, increased hepatic glucose production is a major cause of hyperglycemia in patients with T2DM, leading to an exacerbated ROS production as a result of elevated mitochondrial membrane potential ($\Delta\psi_m$) values [30]. Hence, we report in the present study that both glitazones immediately (within seconds) diminished the oxygen uptake in intact hepatocytes as well as in liver mitochondria, through a direct inhibition of complex I and III activities. The TZDs-induced inhibition of complex I and III activities is related to the remarkable and acute decrease in mitochondrial H₂O₂ production that we have also demonstrated. However, it remains to be elucidated whether this event contributes to the long-term antidiabetic potential of glitazones *in vivo*. In favor of this, it should be consider that some mitochondrial effects associated with glitazones chronically used, have been documented in various cell types, through an induction of mitochondrial biogenesis and an improvement of oxidative stress related to the diabetes disease [31, 32]. Most importantly, the concentrations of glitazones here tested were almost similar to those used in other *in vitro* studies [17, 33], while maximum plasma concentrations of glitazones in human is of 1865±605.3 ng/ml and 603 ng/ml after a single oral dose of 45 mg pioglitazone and 8 mg rosiglitazone, respectively [34, 35]. It must be also taken into account that both glitazones are metabolized in the liver, leading to an intrahepatic concentration largely

superior to 25 μM . In the same line, as glitazones were targeting mitochondria, accumulation could lead to even higher cytosolic levels.

There are few relevant studies in the recent literature regarding acute actions of glitazones on cell respiration and mitochondrial function in the liver. It should be also noticed that many other short-term effects of glitazones pointed towards an intracellular damage; i.e. in human and rat liver cells, TZDs caused a mitochondrial dysfunctioning that was followed by an augmented membrane permeability in a range of potency troglitazone > pioglitazone > rosiglitazone [36]; troglitazone (25–100 μM) activated the caspases pathway in human hepatocarcinoma cells [14]; in astrocytes and astrocytoma cells, rapid effects increasing ROS formation were observed in the presence of ciglitazone, pioglitazone and rosiglitazone [16]. This TZDs-induced ROS stimulation, contrary to what we herein described, could lead to the progressive damage of cancer cells, thus favouring the hypothesis that postulates glitazones as a possible anti-cancer therapy [37]. Notably, the recognized harmful effect of troglitazone upon mitochondrial function was not evidenced in our case, when using pioglitazone and rosiglitazone. Because of its idiosyncratic hepatotoxicity, troglitazone was withdrawn from the market in 2000. Many factors have been proposed to explain this severe liver failure, and an activation of PTP opening has been suggested [38, 39]. Of interest is that no relevant changes in this phenomenon were seen in our work with pioglitazone and rosiglitazone whatever the model studied (permeabilized cells or mitochondria). In addition to this, the degradation of hepatic PGC-1 α that selectively provokes troglitazone, but not rosiglitazone or pioglitazone, leads to a declined mitochondrial mass and reduced expression of superoxide dismutase, both events being involved in the cytotoxicity of troglitazone [40].

The novelty of the results presented is based on the finding that TZDs-induced JO_2 diminution in intact cells, surprisingly not investigated until now, was reproduced when working with isolated mitochondria. This latter is in agreement with previous reports in which state 3 respiration was inhibited [17, 33]. Contrary to our results, glitazones unsuccessfully reduced state 3 when mitochondria were energized with complex II-linked substrates (succinate), and an activation of state 4 was only found at the highest concentration of rosiglitazone. Furthermore, our data were obtained in hepatocytes as well as in mitochondria using more experimental conditions than before, including DNP-induced respiration, in both cases, and the use of TMPD-ascorbate, exclusively in

the case of isolated mitochondria. Although the activation of state 4 could probably lead to a situation of partial uncoupling, the subsequent inhibitions of phosphorylating and uncoupled states counteracted the initial effect observed. Taken together, our results indicate that the effect of both glitazones would be mainly located at the level of complex I and III of the respiratory chain. Furthermore, the fact that the oxidative phosphorylation yield was only affected in the presence of complex I-linked substrate (G/M) (see Fig. 1A) could be the consequence of a slipping at the level of this complex. This slipping phenomenon is also involved in the decrease of ROS production due to the reverse electron transfer process at complex I.

As earlier mentioned in the text, mitochondrial dysfunction is intrinsically related to the onset and progression of type 2 diabetes. Traditional hyperglycemia together with the stimulation of respiratory rates lead to enlarge mitochondrial ROS production that hampers the insulin action and participates to the development of insulin resistance [30, 41, 42]. Our results demonstrate, for the first time, a rapid and drastic decrease in mitochondrial H_2O_2 levels in the liver by direct actions of glitazones. This effect is in accordance with previous reports showing that an improvement in inflammatory markers and oxidative stress was due to diminished ROS levels in endothelial cells [43, 44]. Moreover, it is worth keeping in mind that most of TZDs-induced enhancement of oxidative stress in endothelial cells are independent of PPAR γ -activation [45]. Hence, a moderate inhibition of electron flux through the respiratory chain complex I has been demonstrated to protect cells against oxidative stress-induced cell death [46]. In our case, the great diminution of respiratory complex I and III activities is related to the marked inhibition of ROS production. Surprisingly, rosiglitazone at its highest dose of 100 μM turned out the less active form or even toxic. This detrimental effect could contribute to the elucidation of minor toxicity associated to the use of rosiglitazone, that caused its temporary suspension from the European market.

All together, our data are consistent with several data previously reported [17, 33], and clearly suggest a predominant implication of respiratory complex I and III in the mitochondrial action of glitazones, without a significant involvement of complex II. Nonetheless, our results should be carefully interpreted without assuming that glitazones act on mitochondrial respiration exclusively by decreasing complex I and III activities. The mechanism by which glitazones reduce the activities of complex I

and III while impacting on ROS formation remains to be fully elucidated. This TZDs-induced inhibition of OXPHOS would eventually brake the fatty acids β -oxidation, causing an accumulation of lipids in the liver of diabetic patients. However, it is important to recall that our findings arise from *in vitro* studies and very brief effects, i.e. a setting for which the extent of obtained results to manifestations over many days of treatment is uneasy. On the other hand, it has been well demonstrated in man that a chronic treatment with pioglitazone induced mitochondrial biogenesis in adipose tissue as well as expression of PGC-1 α and genes of the fatty acid β -oxydation path [47], and reduced the fat deposition in the liver through a mechanism involving adiponectin [48, 49].

On this basis, TZDs behave similarly as metformin, the most widely prescribed oral antidiabetic drug [50]. This parallelism suggests that the improvement of mitochondrial function might contribute to the antidiabetic properties of these agents. However, it is necessary to remind that metformin requires total cell integrity to exert its mitochondrial action, since this molecule failed to affect JO_2 in isolated mitochondria [51]. By contrast, glitazones are able to accumulate in the vicinity of the outer mitochondrial membrane due to their highly lipophilic nature. The late evidence that a specific and saturable binding site for pioglitazone (the protein named mitoNEET) has been solubilized from the mitochondrial fraction of rodent brain, liver and skeletal muscle, could partially explain the direct actions of glitazones at this intra-cellular level [19], without any involvement of PPAR γ -mediated activation. TZDs were found to activate, again within minutes, AMP-activated protein kinase *in vivo* in selected mammalian tissues [52]. This result together with our own are clearly associated with a change in cellular energy status, and all these events

occur too briefly to be primarily attributable to transcription. It is obvious that TZDs exert a portion of their activity via PPAR γ -mediated transcriptional events but that non-genomic mechanisms, perhaps directly impacting on mitochondrial function, are significant and potentially relevant to their clinical effects.

Abbreviations

ATP/O (oxidative phosphorylation yield); CRC (Ca²⁺ retention capacity); JO_2 (oxygen consumption rate); OXPHOS (oxidative phosphorylation); Pi (inorganic phosphate); PPAR γ (peroxisome proliferator-activated receptor gamma); T2DM (type 2 Diabetes Mellitus); TMPD-a (N,N,N',N'-tetramethyl-1,4-phenylene-diamine/ascorbate); TZDs (thiazolidinediones or glitazones); $\Delta\psi_m$ (mitochondrial membrane potential).

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