Rapid non-genomic regulation of Ca^{2+} signals and insulin secretion by PPAR α ligands in mouse pancreatic islets of Langerhans

Ana B Ropero^{1,2}, Pablo Juan-Picó^{1,2}, Alex Rafacho^{1,*}, Esther Fuentes^{1,2}, F Javier Bermúdez-Silva³, Enrique Roche¹, Ivan Quesada^{1,2}, Fernando Rodríguez de Fonseca³ and Angel Nadal^{1,2}

¹Instituto Bioingeniería, Universidad Miguel Hernández de Elche, Elche 03202, Alicante, Spain

²CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Universidad Miguel Hernández de Elche, Elche 03202, Alicante, Spain

³Laboratorio de Medicina Regenerativa and CIBER de Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Hospital Carlos Haya, Fundación IMABIS, Málaga 29010, Spain

(Correspondence should be addressed to A Nadal at Institute of Bioengineering, Miguel Hernández University, 03202 Elche, Alicante, Spain; Email: nadal@umh.es)

*(A Rafacho is now at Department of Physiology and Biophysics, Institute of Biology, State University of Campinas, Campinas, SP, Brazil)

Abstract

PPAR α is a ligand-activated transcription factor belonging to the nuclear receptor superfamily. PPAR α is involved in the regulation of *in vivo* triglyceride levels, presumably through its effects on fatty acid and lipoprotein metabolism. Some nuclear receptors have been involved in rapid effects mediated by non-genomic mechanisms. In this paper, we report the rapid non-genomic effects of PPAR α ligands on the intracellular calcium concentration ($[\text{Ca}^{2+}]_i$), mitochondrial function, reactive oxygen species (ROS) generation, and secretion of insulin in freshly isolated mouse islets of Langerhans. The hypolipidemic fibrate PPAR α agonist WY-14 643 decreased the glucose-induced calcium oscillations in intact islets. This effect was mimicked by the synthetic agonist GW7647 and the endogenous agonist oleylethanolamide. The WY-14 643 action was rapid in

onset (5 min) and was still produced in the presence of protein and mRNA synthesis inhibitors, cycloheximide, and actinomycin-D. This suggests that it is independent of gene transcription. In addition, WY-14 623 impaired mitochondrial function, increased ROS formation and decreased insulin release. PPAR α is present in β -cells, mainly in the cytosol and nucleus, with a small subpopulation localized in the plasma membrane. However, the presence of the PPAR α ligand effects in mice bearing a disrupted *Ppar α* gene raises the possibility that the rapid effects of the agonists in pancreatic β -cells are independent of the receptor. We conclude that PPAR α agonists produce a decrease in glucose-induced $[\text{Ca}^{2+}]_i$ signals and insulin secretion in β -cells through a rapid, non-genomic mechanism.

Journal of Endocrinology (2009) **200**, 127–138

Introduction

Blood glucose is maintained within a very narrow range by the action of two main hormones: insulin and glucagon. Insulin is synthesized and secreted from pancreatic β -cells and glucagon from α -cells. Both cell types are components of the islet of Langerhans, the physiological unit of the endocrine pancreas.

Insulin is the only hormone able to reduce blood glucose levels by decreasing glucose output and increasing the rate of glucose uptake, mainly in the striatal muscle and adipocytes (Pessin & Saltiel 2000). Type II diabetes is characterized by the impaired insulin secretion of pancreatic β -cells in response to glucose and, by insulin resistance.

The stimulus–secretion coupling process in β -cells involves the metabolism of glucose. Oxidative metabolism via the pyruvate dehydrogenase complex and the tricarboxylic acid cycle generates ATP, which triggers glucose-stimulated

insulin secretion. An increase in the (ATP)/(ADP) ratio leads to the closure of the ATP-dependent potassium channels (K_{ATP}), responsible for the resting membrane potential. As a result, the plasma membrane depolarizes, the voltage-gated calcium channels open and thus, $[\text{Ca}^{2+}]_i$ increases (Ashcroft & Rorsman 1989, Rorsman *et al.* 2000). When β -cells are within the islet of Langerhans, their glucose-induced $[\text{Ca}^{2+}]_i$ signal is organized in a synchronous and homogeneous $[\text{Ca}^{2+}]_i$ oscillatory pattern (Santos *et al.* 1991, Valdeolmillos *et al.* 1993, Nadal *et al.* 1999, Fernandez & Valdeolmillos 2000), provoking a pulsatile insulin secretion (Gilon *et al.* 1993, Barbosa *et al.* 1996).

Peroxisome proliferator-activated receptors (PPARs) encompass a three-member subgroup (α , γ , and β/δ) belonging to the nuclear hormone receptor superfamily of ligand activated transcription factors (Robinson-Rechavi *et al.* 2003). PPARs, particularly PPAR α and γ , are regulators of lipid metabolism because they act as lipid sensors.

Therefore, PPAR ligands represent important pharmaceutical agents involved in metabolic disorders that include type II diabetes and dyslipidaemia (Gardner *et al.* 2005a). PPAR ligands act classically through PPAR binding, triggering the transcription of target genes. In addition to this established mechanism of action, growing evidence points to the existence of a non-genomic effect of PPAR ligands via a PPAR-independent pathway. These non-genomic actions include mitochondrial effects, rapid reactive oxygen species (ROS) formation, MAPK activation, and expression of immediate early genes, even in cell types that do not express PPAR (Pauley *et al.* 2002, Gardner *et al.* 2003, Perez-Ortiz *et al.* 2004, 2007, Gardner *et al.* 2005b). These actions are similar to those described for other nuclear receptors (Migliaccio *et al.* 1998, Nadal *et al.* 2001, Losel *et al.* 2003). The physiological dimension of PPAR ligands is reflected by their rapid induction of satiety (Rodriguez *et al.* 2001), insulin-induced glucose uptake by the adipocytes (Gonzalez-Yanes *et al.* 2005), and visceral analgesia (Suardiaz *et al.* 2007).

PPAR α exists in pancreatic β -cells and its expression is down-regulated (60–80% reduction) after being exposed to high glucose for several days (Zhou *et al.* 1998, Roduit *et al.* 2000). Here, we have shown that PPAR α agonists rapidly regulate glucose-induced $[Ca^{2+}]_i$ in a non-genomic manner. In addition, the agonist WY-14 643 acts on mitochondrial function, activates ROS, and decreases insulin release. Moreover, the WY-14 643-induced regulation of $[Ca^{2+}]_i$ signals is still produced in islets from mice with a disrupted *Ppar α* gene, suggesting that the non-genomic action of PPAR α agonists in pancreatic β -cell signaling may be independent of the receptor.

Materials and Methods

Drugs

The PPAR α ligands WY-14 643, oleoylethanolamide, and GW-7647 were purchased from TOCRIS (Biogen, Barcelona, Spain). Lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC) and the remaining reactive and salts were purchased from Sigma Chemicals.

Islet and cell preparation

Swiss albino OF1 male mice (8–10 weeks old) were used and humanely killed by cervical dislocation. An internal animal care and use committee reviewed and approved the method used in strict adherence to the European Community Council Directive 86/609/EEC regulating animal research. Pancreatic islets of Langerhans were isolated by collagenase digestion as previously described (Nadal *et al.* 1998) and loaded with 5 μ M Fura-2 AM for at least 1 h at room temperature. Loaded islets were kept in a medium containing (mM): 115 NaCl, 10 NaHCO₃, 5 KCl, 1.1 MgCl₂, 1.2 NaH₂PO₄, 2.5 CaCl₂, and 25 HEPES; plus 1% albumin and

5 mM D-glucose, continuously gassed with a mixture of 95% O₂ and 5% CO₂ (pH 7.35). Islets were perfused at a rate of 1 ml/min, with a modified Ringer solution containing (mM): 120 NaCl, 5 KCl, 25 NaHCO₃, 1.1 MgCl₂, and 2.5 CaCl₂ (pH 7.35), when gassed with 95% O₂ and 5% CO₂.

Recording intracellular calcium, NADH, and mitochondrial membrane potential ($\Delta\Psi_m$)

Calcium records in whole islets of Langerhans were obtained by imaging intracellular calcium under an inverted epifluorescence microscope (Zeiss, Jena, Germany, Axiovert 200). Images were acquired approximately every 3 s with an extended Hamamatsu Digital Camera C4742-95 (Hamamatsu Photonics, Barcelona, Spain) using a dual filter wheel (Sutter Instrument Co., Nevada, CA, USA) equipped with 340 and 380 nm, 10 nm band-pass filters (Omega Optics, Madrid, Spain). Data were acquired using ORCA software from Hamamatsu (Hamamatsu Photonics). Fluorescence changes are expressed as the ratio of fluorescence at 340 and 380 nm (F_{340}/F_{380}). Results were plotted using commercially available software (SigmaPlot, Jandel Scientific). The amplitude of the $[Ca^{2+}]_i$ oscillations was calculated for a period of 10–15 min before stimuli application (control). It was then measured for 10 min (from 15 to 25 min) while stimuli were being applied. The area under the traces was measured using SigmaPlot for a period of 15 min after the application of stimulatory glucose concentration.

NADH autofluorescence and $\Delta\Psi_m$ were monitored using the same imaging system described in the paragraph above. NADH fluorescence was excited with a 365 nm band-pass filter, while emission was filtered at 445 ± 25 nm (Pertusa *et al.* 2002, Quesada *et al.* 2006). An image was acquired every 60 s. $\Delta\Psi_m$ was measured using rhodamine-123 (Molecular Probes, Eugene, OR, USA). Islets were loaded with 10 μ g/ml rhodamine-123 for 20 min and imaged using conventional fluorescein filters (Pertusa *et al.* 2002, Ravnskjaer *et al.* 2005). An image was acquired every 90 s.

Insulin secretion

After 1 h recovery in the incubator in the isolation medium, groups of five islets were incubated for 1 h in 0.4 ml of a buffer solution containing 120 mM NaCl, 25 mM NaHCO₃, 5 mM KCl, 2.5 mM CaCl₂, and 1.1 mM MgCl₂ (pH 7.35) in the presence of different stimuli. After 1 h, 0.1 ml of the previous buffer solution with 5% BSA was added and gently shaken for homogenization. Then, the medium was immediately collected and insulin was measured in duplicate by RIA using a Coat-a-count (DPC, Los Angeles, CA, USA). Total protein amount was determined by the Bradford method.

ROS measurement

Whole islets of Langerhans were incubated in a modified ringer solution with 4 μ M 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DHCF), di(acetoxymethyl ester),

C2938, Molecular Probes) for 30 min at 37 °C. After this, the islets were transferred to a recording chamber and ROS were imaged using a Zeiss Pascal 5 confocal microscope at 36 °C with 488 nm excitation and 520 nm emission. A concentration of 100 μ M H₂O₂ was used as a control at the end of each experiment. Only those islets that responded to H₂O₂ with a significant increase in fluorescence were taken into consideration for the experiment.

Quantitative real-time PCR

To obtain a measurement of PPAR α expression in several representative tissues from mice, a relative quantification assay of *Ppara* versus *Histone* (housekeeping gene) mRNA was performed. For this purpose, real-time quantitative PCR technology was used. Total RNA isolation from snap frozen tissue samples was obtained using Trizol reagent (Gibco BRL Life Technologies) according to the manufacturer's instructions. All RNA samples showed A260/280 ratios between 1.8 and 2.1. Total RNA from each sample and random hexamers were immediately used to generate first strand cDNA, by using transcriptase reverse transcriptase (Roche Applied Science) according to the manufacturer's instructions. Negative controls included RT reactions omitting reverse transcriptase. The obtained cDNAs were used as the template for real-time quantitative PCR, which was performed using the Opticon Engine System (MJ Research) with the SYBR Green I detection format and using the QuantiTect SYBR Green PCR kit (Qiagen GmbH, Germany). Primers for PCR were designed based on NCBI database sequences of mouse *Ppara* (accession NM_011144.2) and *Histone* (accession NM_175660.1) mRNAs and tested to ensure the amplification of single discrete bands with no primer dimers. Primer sequences were as follows: *Ppara* forward, 5'-TgCTgTCCTCCTTgATgAAC-3'; *Ppara* reverse, 5'-gCTTAAGCACgTgCACAAATC-3' (270 bp product); *Histone* forward, 5'-CTgTgCTggAgTACCTgACg-3'; and *Histone* reverse, 5'-ATTACTTCCCCTTggCCTTg-3'

Figure 1 PPAR α is present in mouse islets of Langerhans. Quantification by real-time PCR of mRNA levels in different tissues from mice. Values are the mean of two independent experiments with 1000 islets each, from 13 mice.

(234 bp product). They were obtained from ProLigo France SAS (Paris, France). The quantification was carried out based on standard curves run at the same time as samples. *Ppara* and *Histone* standards were generated by PCR amplification from control samples. The PCR product was run in a 1% agarose gel electrophoresis to check the fragment size and the absence of other contaminant fragments, quantified by 260 nm absorbance, and serially diluted to 10⁻⁶ pg/ μ l. Several 10-fold dilutions (10⁻¹–10⁻⁶) were checked for optimal cycling using the Opticon Engine System and 5 were selected to run the standard curve. Each reaction was run in duplicate and contained 3 μ l cDNA template, 5 mM Cl₂Mg, and 0.2–0.4 μ M primers in a final reaction volume of 20 μ l. The cycling parameters were 95 °C for 15 min to activate DNA polymerase, followed by 30–40 cycles of 95 °C for 15 s, annealing temperature for 30 s (*Ppara*: 56 °C, *Histone*: 64 °C) and a final extension step of 72 °C for 30 s in which fluorescence was acquired. Melting curve analysis was performed to ensure that only a single product was amplified. Once the absolute values for both *Ppara* and *Histone* were obtained from each sample, the *Ppara*/*Histone* ratio was calculated.

Immunocytochemistry

Islets isolated as previously described were fixed with Bouin's solution for 5 min and washed with PBS. Then, they were dehydrated with 30, 50, and 70% ethanol, for 3 min each, and washed with PBS. After this, the islets were treated with 0.5% Triton X-100 for 15 min and then washed with PBS. The non-specific staining was blocked with PBS supplemented with 0.1% Triton X-100 and 5% serum from the same host as the secondary antibodies used. After the islets had been incubated for 1 h at room temperature, primary antibodies were added to the blocking solution. These were anti-PPAR α (1:250, PA1-822A, ABR; 1:100, H98, Santa Cruz) and anti-insulin (1:200, Sigma). The islets were incubated overnight with the primary antibodies at 4 °C. Secondary antibodies (Alexa Fluor Molecular Probes) were used at 1:1000 in PBS plus 1% serum from the same host as the secondary antibodies, for 1 h at room temperature. To stain mitochondria, live islets were previously incubated with 500 nM Mitotracker Red CMXRos (Molecular Probes) for 30 min, then washed with PBS and fixed as described. To stain the plasma membrane, 1 μ g/ml wheat germ agglutinin-tetramethylrhodamine was used (WGA, Molecular Probes, W849) and added at the same time as the primary antibody. A confocal Zeiss Pascal 5 microscope and a Zeiss 40 $\times$ objective (numerical aperture = 1.3) were used to obtain images for quantification. The images were analyzed using LSM Zeiss software (Zeiss).

Cell viability assay

Islets were dispersed into single cells with trypsin. Cells were then centrifuged and resuspended in medium containing (mM): 115 NaCl, 10 NaHCO₃, 5 KCl, 1.1 MgCl₂, 1.2

Figure 2 Location of PPAR α in insulin-containing cells. (A) Immunostaining of islets with an antibody against PPAR α (green) and against insulin (red). The right panel shows double staining with both antibodies. (B) Immunostaining with an antibody against PPAR α (green) and wheat germ agglutinin to label membranes (red). The right panel shows colocalization of both signals at the plasma membrane level. (C) Immunostaining with an antibody against PPAR α (green) and Mitotracker (red) to stain mitochondria. The right panel shows that PPAR α does not colocalize with Mitotracker. Calibration bar represents 20 μ m in A and 5 μ m in B and C.

NaH₂PO₄, 2.5 CaCl₂, and 25 HEPES; plus 1% albumin; and 5 mM D-glucose (pH 7.35). They were plated on 24 mm glass covers previously coated with poly-L-lysine. The cells were left to attach for 1 h at 37 °C and the medium was replaced with fresh medium plus the stimuli for 90 min. After this, the cells were washed twice with PBS and incubated with 2 μ M calcein and 4 μ M ethidium homodimer 1 (Live/Dead Viability/Cytotoxicity Kit, Molecular Probes) at room temperature for 45 min whilst being gently shaken. The covers were taken under a fluorescence microscope and live cells were counted as positive for calcein (green cells), while dead cells were positive for ethidium homodimer 1 (red nuclei).

Ppar α gene disrupted mice

Mice homozygous for the Ppar $\alpha^{tm1Gonz}$ targeted mutation were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). They were generated by Lee *et al.* by inserting a 1.14 kb neomycin resistance gene into the exon 8 that encodes the ligand-binding domain in the opposite transcriptional direction of the mouse Ppar α gene. Results showed that

the northern blot analysis did not detect a transcript of wild type size on the RNA derived from the liver of homozygous mice, although a larger abnormal transcript was detected (Lee *et al.* 1995).

Statistical analysis

Data are expressed as mean $\pm$ S.E.M. Comparisons were made using a two-tailed Student's *t*-test, unless stated otherwise. A probability level of <0.05 was considered statistically significant.

Results

PPAR α expression and location within intact islets of Langerhans

Quantitative real-time PCR confirmed that Ppar α is expressed in isolated islets of Langerhans (Fig. 1). Figure 2A–C shows that PPAR α is expressed in insulin containing β -cells and that it is located in the nucleus, as well as in the cytosol

Figure 3 Rapid effect of WY-14 643 on $[Ca^{2+}]_i$ in intact islets of Langerhans. (A) Record of fluorescence versus time from a whole islet of Langerhans. The Ca^{2+} -dependent fluorescence of Fura-2 is expressed as the ratio F_{340}/F_{380} (see Materials and Methods). Stimuli were applied for the period indicated by the horizontal bar. Note that 0.5 μM WY-14 643 decreases the amplitude of $[Ca^{2+}]_i$ oscillations in the presence of 11 mM glucose in 47% of the tested islets (7 out of the 15 islets). (B) 0.5 μM WY-14 643 modified glucose-induced $[Ca^{2+}]_i$ oscillations in 20% of the tested islets (3 out of the 15 islets), in 27% of the islets WY-14 643 had no effect. (C) Mean amplitude ($\Delta F_{340}/F_{380}$) in percentage of $[Ca^{2+}]_i$ oscillations during a period of 5–10 min prior stimuli (control condition in high glucose) and 5–10 min after stimuli application in experiments of the type shown in A. (D) Islets were able to respond to 30 mM K^+ after the application of 5 μM WY-14 643 ($n=3$ islets).

and the plasma membrane (Fig. 2D–I). Similar results were obtained with two different antibodies against PPAR α (see Material and Methods and Supplementary Figure 1, see Supplementary data in the online version of the Journal

of Endocrinology at <http://joe.endocrinology-journals.org/content/vol200/issue2/>).

The hypolipidemic fibrate WY-14 643 regulates $[Ca^{2+}]_i$; signals and insulin secretion in whole islets of Langerhans

To investigate, the effect of the PPAR α agonist WY-14 643 on $[Ca^{2+}]_i$ in β -cells within intact islets of Langerhans, isolated islets were loaded with the fluorescent calcium sensitive dye Fura-2. Changes in the ratio F_{340}/F_{380} were monitored using a conventional imaging system. All the different cell types are present within an islet; however, the average signal of the whole islet corresponds to the β -cell type (Valdeolmillos *et al.* 1993, Nadal *et al.* 1999). The $[Ca^{2+}]_i$ oscillations induced by a stimulatory glucose concentration (11 mM) were inhibited by WY-14 643 in 47% of the islets tested (Fig. 3A and C), while a change in the glucose-induced $[Ca^{2+}]_i$ oscillatory pattern was manifested in 20% of the islets (Fig. 3B). In these cells, the oscillations became wider and less frequent. This inhibitory effect of WY-14 643 was dose-dependent (Supplementary Figures 2A and 3, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol200/issue2/>). The silencing action of WY was not due to a damaging effect of the fibrate, since islets were still able to respond to stimulation with high K^+ concentration (Fig. 3D).

When the islets were pre-incubated for 30 min with 5 μM WY-14 643, as well as during the experiment, the glucose-induced $[Ca^{2+}]_i$ response was partially inhibited to both 7 and 11 mM glucose. This is manifested as a decrease in the global Ca^{2+} amount entering into the islet upon stimulation, measured as the area under the traces (Fig. 4A–C). However, when the islets were exposed to 16 mM glucose, WY was unable to have an effect (Fig. 4C). The basal $[Ca^{2+}]_i$ did not change after the 30 min incubation with WY (data not shown). Additionally, when measured the peak size of the $[Ca^{2+}]_i$ transient in response to 11 mM glucose (peak size, measured as the difference between the calcium level at 3 mM glucose just before the medium was changed and the maximum amplitude of the first calcium transient in response to high glucose concentration), the PPAR α agonist was able to decrease such response in 28% (Fig. 4D). Again, WY had no effect in 16 mM glucose (data not shown).

When the same kind of experiment was performed but stimulating the β -cells with 25 mM K^+ , WY-14 643 affected neither the peak size of the transient or the global calcium entrance (Supplementary Figure 4, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol200/issue2/>). This suggests that the PPAR α agonist WY is not acting through an inhibition of voltage-dependent Ca^{2+} channels.

Insulin secretion is a pulsatile phenomenon that follows the oscillatory $[Ca^{2+}]_i$ pattern (Barbosa *et al.* 1996). The experiment shown in Fig. 4E was performed to evaluate the

Figure 4 Inhibition of the glucose-induced $[Ca^{2+}]_i$ response by WY-14 643. (A) $[Ca^{2+}]_i$ of an islet pre-incubated with vehicle (DMSO) for 30 min prior to the recording; the islet was in 3 mM glucose for a total of 10 min before applying 11 mM glucose. (B) The incubation of islets in the presence of 5 μ M WY-14 643 decreases the $[Ca^{2+}]_i$ signal induced by a stimulatory glucose concentration. (C) Area under the traces measured during a period of 15 min after applying different glucose concentrations; WY decreased the Ca^{2+} entrance to the islet in response to 7 mM and 11 mM glucose. $n=4-7$ islets per condition; # $P<0.01$. (D) WY also decreased the peak size of the Ca^{2+} transient in response to 11 mM glucose. R means the ratio F_{340}/F_{380} . $n=4-7$ islets per condition; * $P<0.05$. (E) Percentage of insulin release in the presence of a non-stimulatory glucose concentration of 3 mM, a stimulatory glucose concentration of 11 and 11 mM glucose in the presence of 5 μ M WY-14 643. The value at 11 mM glucose was set as 100%. $n=9$ mice; * $P<0.05$. Student's *t*-test.

rapid effect of WY-14 643 on insulin secretion. The increase in insulin secretion produced by 11 mM glucose was blocked by the PPAR α agonist.

The action of WY-14 643 on $[Ca^{2+}]_i$ was imitated by the synthetic PPAR α agonist GW7647, which abolished glucose-induced $[Ca^{2+}]_i$ oscillations in 87% of the islets tested (Fig. 5A and B). However, the endogenous agonist oleylethanolamide (OEA; Fu *et al.* 2003) had a double effect, on the one hand it decreased the amplitude of glucose-induced $[Ca^{2+}]_i$ oscillations in 56% (Fig. 5C and D) of the islets while, on the other hand, it increased glucose-induced $[Ca^{2+}]_i$ oscillations in 28% of the islets (Fig. 5E). The latter is presumably due to its action through other receptors, such as GPR119 (Overton *et al.* 2006, Madiraju & Poitout 2007),

since the GPR119 agonists LPC (Fig. 5F) and LPA (data not shown) also increased $[Ca^{2+}]_i$ oscillatory frequency. As well as for WY-14 643, the effect of the two PPAR α agonists was dose-dependent (Supplementary Figures 2 and 3).

Although experiments shown in Figs 3D and 4C, and Supplementary Figure 4 point to the fact that WY-14 643 action is not due to a toxic effect, we sought to perform a viability test to further demonstrate this. We incubated isolated cells from islets for 90 min in the presence of vehicle, 5 μ M WY-14 643 or 1 μ M GW7647. As shown in Supplementary Figure 5, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol200/issue2/>, the percentage of live cells did not vary between the different

Figure 5 WY-14 643 action on $[Ca^{2+}]_i$ is imitated by GW-7647 and OEA. (A) 1 μ M GW-7647 decreased glucose-induced $[Ca^{2+}]_i$ oscillations in 84.5% of the tested islets (16 out of the 19 islets), changed the oscillatory $[Ca^{2+}]_i$ pattern in 10.25% (2 out of the 19 islets) and had no effect in 5.25% (1 out of the 19 islets). (B) Mean amplitude of $[Ca^{2+}]_i$ oscillations in control conditions and after GW-7647 application. (C) 1 μ M OEA decreased the $[Ca^{2+}]_i$ amplitude of glucose induced $[Ca^{2+}]_i$ oscillations in 56% of the tested islets (14 out of the 25). (D) Mean amplitude of $[Ca^{2+}]_i$ oscillations in control conditions and after OEA application. (E) 1 μ M OEA increased the frequency of glucose-induced $[Ca^{2+}]_i$ oscillations in 40% of the tested islets (8 out of the 25 islets). In 4% of the tested islets, OEA had no effect. (F) The GPR119 agonist LPC increased the frequency of glucose-induced $[Ca^{2+}]_i$ oscillations.

conditions. As a positive control to obtain maximal cell death, we used 25% DMSO.

The action of WY-14 643 on glucose-induced $[Ca^{2+}]_i$ oscillations is through a non-genomic mechanism

When islets were incubated for 4 h in the presence of cycloheximide, a potent inhibitor of protein synthesis, the effect of WY-14 643 on intracellular calcium oscillations was unchanged (Fig. 6A and C). The pretreatment of the islets with actinomycin-D, an inhibitor of RNA synthesis, for at least 3 h, did not prevent the effect of WY-14 643 on $[Ca^{2+}]_i$ (Fig. 6B and C). We obtained the same results when using OEA instead (Supplementary Figure 6, see Supplementary data in the online version of the Journal of Endocrinology at

<http://joe.endocrinology-journals.org/content/vol200/issue2/>). These results, together with the rapid onset of the response, strongly suggest the existence of a non-genomic effect of the PPAR α agonists.

WY-14 643 impairs the mitochondrial function and rapidly activates ROS

The mitochondrial function is essential for an adequate stimulus-secretion coupling in β -cells. It is known that mitochondrial impairment rapidly produces energy depletion and disrupts intracellular Ca^{2+} homeostasis (Freeman *et al.* 2006). Since it has been shown that PPAR ligands can impair mitochondrial oxidative metabolism (Perez-Ortiz *et al.* 2004, Scatena *et al.* 2007), we decided to analyze the mitochondrial

Figure 6 WY-14 643 regulation of Ca^{2+} signals is non-genomic. (A) 5 μ M WY-14 643 abolished $[Ca^{2+}]_i$ oscillations in the presence of cycloheximide. Hundred micromolar cycloheximide was applied at least 3 h before treating the islets with WY-14 643. (B) Effect of WY-14 643 on glucose-induced $[Ca^{2+}]_i$ oscillations after 4 μ M actinomycin-D treatment for at least 4 h. (C) Mean amplitude of $[Ca^{2+}]_i$ oscillations in control conditions and after applying WY-14 643 to islets treated with cycloheximide (CHX) and actinomycin-D (ACT-D).

redox state as well as the electrochemical gradient by monitoring NADH fluorescence and mitochondrial membrane potential ($\Delta\Psi_m$) respectively. Glucose metabolism activates the tricarboxylic acid (TCA) cycle, producing CO_2 and the nucleotide carriers, which reduce the power of NADH, and $FADH_2$, which function as sources of electron transfer in the oxidative phosphorylation reactions that mediate ATP production (Quesada *et al.* 2006, Newsholme *et al.* 2007). As we observe in Fig. 7A, the elevation of glucose to 8 mM induced an increase in NADH autofluorescence. Application of WY-14 643 further enhanced this signal. This effect was also reproduced by GW7647 (Fig. 7B). It has been reported that PPAR ligands can inhibit electron transport,

particularly at the level of NADH oxidation (Scatena *et al.* 2004). Our observations also point to a similar action of WY-14 643. Blockade of the electron transport can decrease the re-oxidation of the NADH molecules generated by the TCA cycle, producing an accumulation of the reduced forms and thus, an increase in NADH fluorescence (Quesada *et al.* 2006). This is the case of the inhibitor cyanide (CN; Fig. 7A and B). Given that mitochondrial ATP synthesis is largely dependent on $\Delta\Psi_m$ (Newsholme *et al.* 2007), we also explored this parameter. The change in glucose concentrations from 3 to 8 mM produced the characteristic hyperpolarization of $\Delta\Psi_m$ (Pertusa *et al.* 2002), which corresponds to a decrease in rhodamine-123 fluorescence (Fig. 7C). Interestingly, the application of WY-14 643 induced a progressive loss of $\Delta\Psi_m$, thus indicating an uncoupling effect on mitochondrial function. These findings are in agreement with previous reports showing similar effects with PPAR ligands (Perez-Ortiz *et al.* 2004). Finally, because mitochondrial inhibitors can elevate the steady-state levels of ROS (Perez-Ortiz *et al.* 2004), we also examined ROS production in the presence of WY-14 643. The dye DDCF was used to measure ROS production in whole islets (Fig. 7D). When the islets were exposed to 8 mM glucose, ROS production did not vary. However, the addition of WY-14 643 produced a dramatic rise in ROS production in 50% of the tested islets (Fig. 7E and F). Similar results have been found in other cellular systems (Rose *et al.* 1999, Perez-Ortiz *et al.* 2004). Therefore, the analysis of the redox state, $\Delta\Psi_m$ and ROS production indicate that WY-14 643 affects mitochondrial function, in agreement with previous studies about the effect of PPAR α agonists (Rose *et al.* 1999, Perez-Ortiz *et al.* 2004, Scatena *et al.* 2004).

Rapid WY-14 643 effect on $[Ca^{2+}]_i$ in *Ppar α* gene disrupted mice

In order to demonstrate whether PPAR α mediated the rapid action of WY-14 643, we sought to perform Ca^{2+} imaging experiments in islets of Langerhans from wild type (WT) and *Ppar α* gene disrupted mice. In islets obtained from WT mice, WY-14 643 completely abolished glucose-induced $[Ca^{2+}]_i$ oscillations (Fig. 8A and B). Remarkably, when islets from *Ppar α* gene disrupted mice were used, the same inhibition of $[Ca^{2+}]_i$ signals was obtained by applying WY-14 643 (Fig. 8A and B). This result indicates that the rapid, non-genomic action of the PPAR α agonist did not require the full-length *Ppar α* .

Discussion

This report demonstrates that the PPAR α agonist WY-14 643 rapidly regulates glucose-induced $[Ca^{2+}]_i$ oscillations and insulin secretion. The rapid onset of the response indicates that it is a non-genomic action. This is further confirmed by the absence of effect of the mRNA synthesis inhibitor

Figure 7 WY-14 643 affects mitochondrial function and ROS production. (A) Temporal pattern of NADH autofluorescence in response to glucose and WY-14 643 ($n=3$). Activation of the tricarboxylic acid cycle by glucose increases NADH production. The effect of 2 mM cyanide (CN) is also shown. Arbitrary units (a.u.). (B) Effect of GW-7647 on NADH autofluorescence ($n=3$). (C) Temporal changes in $\Delta\Psi_m$ by measurement of rhodamine-123 (Rhod 123) fluorescence ($n=4$). The experiment illustrates the opposite actions of glucose and WY-14 643 on $\Delta\Psi_m$. The uncoupling effect of the protonophore carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP; 2 μ M) is also shown. (D–F) ROS measurement using the fluorescent dye DHCF in whole islets of Langerhans. (D) Representative images of DHCF fluorescence in points 'a' and 'b' of the graph in E. The upper panel shows the picture of the control islet in the presence of 8 mM glucose. The lower panel shows the picture of a WY-14 643 responsive islet when the PPAR α agonist begins to be applied ('a') and after 20 min of applying WY-14 643 ('b'). (E) Fluorescence of DHCF versus time of islets in D. (F) Data of the mean $\pm$ S.E.M. of nine islets responsive to WY-14 643. In 9 out of the 18 tested islets, WY-14 643 had no effect.

actinomycin-D, and the protein synthesis inhibitor cycloheximide. $[Ca^{2+}]_i$ signals were affected in this manner not exclusively by WY-14 643, since other synthetic and endogenous PPAR α agonists, such as GW7647 and OEA, produced the same action. Regarding the mechanism for the

action of the PPAR α ligands, we show here that they also affect the intracellular NADH concentration, the mitochondrial membrane potential, and ROS production.

Reports have shown that all members of the PPAR family are expressed in pancreatic β -cells (Zhou *et al.* 1998).

Figure 8 WY-14 643 rapidly blocks $[\text{Ca}^{2+}]_i$ signals in islets from *Ppar α* gene disrupted mice. (A) Application of $0.5 \mu\text{M}$ WY-14 643 blocks glucose-induced $[\text{Ca}^{2+}]_i$ oscillations in islets from *Ppar α* gene disrupted mice. (B) Mean amplitude in percentage of $[\text{Ca}^{2+}]_i$ during a period of 5–10 min prior stimuli (control condition in high glucose) and 10 min after WY-14 643 application in high glucose. $n=6$ islets $P<0.05$. Student's *t*-test.

However, the role of PPAR α in regulating GSIS is still unclear. Some studies point to a significant reduction in GSIS. Both the ectopic PPAR α expression as well as the application of the PPAR α agonist clofibrate led to the conclusion that the PPAR α activity could cause β -cell dysfunction, possibly through an induction of UCP2 (Tordjman *et al.* 2002). Other authors showed that an acute over-expression of PPAR α and RXR potentiates GSIS in INS1E cells and rat islets, although the PPAR α agonist WY-14 643 diminishes insulin release in these islets (Ravnskjaer *et al.* 2005). We have demonstrated here that GSIS is diminished by PPAR α agonists. However, WY-14 643 was unable to affect intracellular calcium responses at 16 mM glucose or higher levels. At these concentrations, β -cells are stimulated close to the maximal response, and thus, WY-14 643 probably may not counteract the glucose effect.

Rapid non-genomic actions of PPAR α ligands have been described in several cell systems. For instance, it has been reported that PPAR α agonists rapidly induce MAP kinases activation (Rokos & Ledwith 1997, Gardner *et al.* 2005b) independently of PPAR α (Gardner *et al.* 2003). In addition, PPAR α ligands have been found to induce expression of immediate early genes in cell lines that do not express this receptor (Pauley *et al.* 2002). We have shown here that the PPAR α agonist WY-14 643 modifies $[\text{Ca}^{2+}]_i$ signals in islets

from wild type as well as from *Ppar α* gene disrupted mice. These mice were designed with a 1 kb fragment inserted in the last exon of the gene (exon 8), the one encoding the domain that enables the protein to act as a transcription factor. Therefore, the mRNA might still be present, although 1 kb longer, and therefore a disrupted protein would be synthesized. This protein, although unable to act as a transcription factor, may still be able to have other functions, as it may be to inhibit insulin secretion in isolated islets of Langerhans through a non-genomic mechanism. The inhibitory effect of the PPAR α agonist in mice with the *Ppar α* gene disrupted together with the rapid onset of the effect after applying WY-14 643 and the absence of inhibition by actinomycin D and cycloheximide, strongly suggests that the PPAR α transcriptional activity in the regulation of $[\text{Ca}^{2+}]_i$ signals is not necessary. Therefore, these data provide further evidence supporting the existence of rapid non-genomic physiological effects of PPAR α ligands.

Recent studies provide further support to the physiologically relevant rapid actions of these compounds. Thus, OEA modulates glucose transport in adipocytes through a non-genomic, extracellular-regulated kinases-mediated mechanism (Gonzalez-Yanes *et al.* 2005). Both OEA and WY-14 643 induce rapid visceral analgesia that is also present in *Ppar α* gene disrupted mice (Suardiaz *et al.* 2007). However, in other models, such as satiety, the PPAR α receptor independence was not fully demonstrated (Fu *et al.* 2003), indicating a potential contribution of the PPAR α receptor to the rapid non-genomic satiety induced by PPAR α receptor ligands. Further research is needed to identify the mechanism of action of these ligands. Dual genomic/non-genomic effects have been observed in several ligand-activated transcription factors (Nadal *et al.* 2001, Naranjo & Mellstrom 2007). Whether PPAR α receptors display this dual profile or not, remains to be determined.

The stimulus–secretion coupling in β -cells depends on mitochondrial activation (Detimary *et al.* 1994, Quesada *et al.* 2006). PPAR α agonists have been shown to rapidly alter mitochondrial metabolism and to increase ROS (Rose *et al.* 1999, Perez-Ortiz *et al.* 2004, Scatena *et al.* 2004, Gardner *et al.* 2005a). In agreement with these studies, we show here that WY-14 643 can induce alterations in the mitochondrial function at multiple levels: redox state, $\Delta\Psi_m$, and ROS concentrations. The changes observed strongly indicate that WY-14 643 impairs the mitochondrial function. Since multiple mitochondrial processes such as ATP synthesis and Ca^{2+} uptake depend on mitochondrial electrochemical gradients, the loss of $\Delta\Psi_m$ affect these functions (Newsholme *et al.* 2007, Quesada *et al.* 2008). Moreover, it has been demonstrated in β -cells that ROS can decrease ATP synthesis through activation of UCP2 (Krauss *et al.* 2003, Newsholme *et al.* 2007). Interestingly, PPAR α activation can induce UCP2 expression and decrease insulin secretion in a β -cell line (Tordjman *et al.* 2002). In addition to mitochondrial effects, ROS can also activate stress-sensitive intracellular signaling pathways, as well as several kinases, that can affect

glucose-induced signaling and insulin secretion (Newsholme *et al.* 2007). Therefore, multiple processes derived from the altered mitochondrial function observed here with PPAR α agonists could contribute to the decrease in glucose-induced $[Ca^{2+}]_i$ oscillations, as well as in glucose-stimulated insulin secretion (GSIS; Freeman *et al.* 2006).

Taken altogether, and considering the physiological relevance of OEA as a modulator of energy and metabolism, the present results support the existence of rapid modulatory effects of PPAR α receptor ligands on glucose-stimulated insulin secretion, through the modulation of glucose-induced $[Ca^{2+}]_i$ oscillations.

Declaration of interest

Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by the Spanish Ministry of Science and Innovation, grant BFU2005-01052, BFU2008-01492, BFU2007-67607, MEC 2004/07762, and Instituto de Salud Carlos III, grants RCMN (C03/08), 03/0178, 07/0880, 07/1226; ISCIII-RETIC RD06/001, and PND 06/142. CIBERDEM and CIDEROBENU are an initiative of Instituto de Salud Carlos III.

Acknowledgements

We thank Ms Francisca Almagro, M^a Luisa Navarro and Ana B. Rufete for their excellent technical assistance.

References

Ashcroft FM & Rorsman P 1989 Electrophysiology of the pancreatic beta-cell. *Progress in Biophysics and Molecular Biology* **54** 87–143.

Barbosa RM, Silva AM, Tome AR, Stamford JA, Santos RM & Rosario LM 1996 Real time electrochemical detection of 5-HT/insulin secretion from single pancreatic islets: effect of glucose and K⁺ depolarization. *Biochemical and Biophysical Research Communications* **228** 100–104.

Detimary P, Gilon P, Nenquin M & Henquin JC 1994 Two sites of glucose control of insulin release with distinct dependence on the energy state in pancreatic B-cells. *Biochemical Journal* **297** 455–461.

Fernandez J & Valdeolmillos M 2000 Synchronous glucose-dependent $[Ca^{2+}]_i$ oscillations in mouse pancreatic islets of Langerhans recorded *in vivo*. *FEBS Letters* **477** 33–36.

Freeman H, Shimonura K, Horner E, Cox RD & Ashcroft FM 2006 Nicotinamide nucleotide transhydrogenase: a key role in insulin secretion. *Cell Metabolism* **3** 35–45.

Fu J, Gaetani S, Oveisi F, Lo VJ, Serrano A, Rodriguez DF, Rosengarth A, Luecke H, DiGiacomo B, Tarzia G *et al.* 2003 Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR α . *Nature* **425** 90–93.

Gardner OS, Dewar BJ, Earp HS, Samet JM & Graves LM 2003 Dependence of peroxisome proliferator-activated receptor ligand-induced mitogen-activated protein kinase signaling on epidermal growth factor receptor transactivation. *Journal of Biological Chemistry* **278** 46261–46269.

Gardner OS, Dewar BJ & Graves LM 2005a Activation of mitogen-activated protein kinases by peroxisome proliferator-activated receptor ligands: an example of nongenomic signaling. *Molecular Pharmacology* **68** 933–941.

Gardner OS, Shiau CW, Chen CS & Graves LM 2005b Peroxisome proliferator-activated receptor γ -independent activation of p38 MAPK by thiazolidinediones involves calcium/calmodulin-dependent protein kinase II and protein kinase R: correlation with endoplasmic reticulum stress. *Journal of Biological Chemistry* **280** 10109–10118.

Gilon P, Shepherd RM & Henquin JC 1993 Oscillations of secretion driven by oscillations of cytoplasmic Ca^{2+} as evidences in single pancreatic islets. *Journal of Biological Chemistry* **268** 22265–22268.

Gonzalez-Yanes C, Serrano A, Bermudez-Silva FJ, Hernandez-Dominguez M, Paez-Ochoa MA, Rodriguez DF & Sanchez-Margalet V 2005 Oleylethanolamide impairs glucose tolerance and inhibits insulin-stimulated glucose uptake in rat adipocytes through p38 and JNK MAPK pathways. *American Journal of Physiology: Endocrinology and Metabolism* **289** E923–E929.

Krauss S, Zhang CY, Scorrano L, Dalggaard LT, St-Pierre J, Grey ST & Lowell BB 2003 Superoxide-mediated activation of uncoupling protein 2 causes pancreatic beta cell dysfunction. *Journal of Clinical Investigation* **112** 1831–1842.

Lee SS, Pineau T, Drago J, Lee EJ, Owens JW, Kroetz DL, Fernandez-Salguero PM, Westphal H & Gonzalez FJ 1995 Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Molecular and Cellular Biology* **15** 3012–3022.

Losel RM, Falkenstein E, Feuring M, Schultz A, Tillmann HC, Rossol-Haseroth K & Wehling M 2003 Nongenomic steroid action: controversies, questions, and answers. *Physiological Reviews* **83** 965–1016.

Madiraju SR & Poitout V 2007 G protein-coupled receptors and insulin secretion: 119 and counting. *Endocrinology* **148** 2598–2600.

Migliaccio S, Bernardini S, Wetsel WC, Korach KS, Faraggiana T & Teti A 1998 Protein kinase C modulates estrogen receptors in differentiated osteoblastic cells *in vitro*. *Steroids* **63** 352–354.

Nadal A, Rovira JM, Laribi O, Leon-quinto T, Andreu E, Ripoll C & Soria B 1998 Rapid insulinotropic effect of 17 β -estradiol via a plasma membrane receptor. *FASEB Journal* **12** 1341–1348.

Nadal A, Quesada I & Soria B 1999 Homologous and heterologous asynchronicity between identified alpha-, beta- and delta-cells within intact islets of Langerhans in the mouse. *Journal of Physiology* **517** 85–93.

Nadal A, Diaz M & Valverde MA 2001 The estrogen trinity: membrane, cytosolic, and nuclear effects. *News in Physiological Sciences* **16** 251–255.

Naranjo JR & Mellstrom B 2007 Split personality of transcription factors inside and outside the nuclear border. *Science's STKE* **2007** e5.

Newsholme P, Haber EP, Hirabara SM, Rebelato EL, Procopio J, Morgan D, Oliveira-Emilio HC, Carpinelli AR & Curi R 2007 Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity. *Journal of Physiology* **583** 9–24.

Overton HA, Babbs AJ, Doel SM, Fyfe MC, Gardner LS, Griffin G, Jackson HC, Procter MJ, Rasamison CM, Tang-Christensen M *et al.* 2006 Deorphanization of a G protein-coupled receptor for oleylethanolamide and its use in the discovery of small-molecule hypophagic agents. *Cell Metabolism* **3** 167–175.

Pauley CJ, Ledwith BJ & Kaplanski C 2002 Peroxisome proliferators activate growth regulatory pathways largely via peroxisome proliferator-activated receptor alpha-independent mechanisms. *Cell Signalling* **14** 351–358.

Perez-Ortiz JM, Tranque P, Vaquero CF, Domingo B, Molina F, Calvo S, Jordan J, Cena V & Llopis J 2004 Glitazones differentially regulate primary astrocyte and glioma cell survival. Involvement of reactive oxygen species and peroxisome proliferator-activated receptor-gamma. *Journal of Biological Chemistry* **279** 8976–8985.

Perez-Ortiz JM, Tranque P, Burgos M, Vaquero CF & Llopis J 2007 Glitazones induce astrogloma cell death by releasing reactive oxygen species from mitochondria: modulation of cytotoxicity by nitric oxide. *Molecular Pharmacology* **72** 407–417.

Pertusa JA, Neshier R, Kaiser N, Cerasi E, Henquin JC & Jonas JC 2002 Increased glucose sensitivity of stimulus-secretion coupling in islets from Psammomys obesus after diet induction of diabetes. *Diabetes* **51** 2552–2560.

- Pessin JE & Saltiel AR 2000 Signaling pathways in insulin action: molecular targets of insulin resistance. *Journal of Clinical Investigation* **106** 165–169.
- Quesada I, Todorova MG & Soria B 2006 Different metabolic responses in alpha-, beta-, and delta-cells of the islet of Langerhans monitored by redox confocal microscopy. *Biophysical Journal* **90** 2641–2650.
- Quesada I, Villalobos C, Núñez L, Chamero P, Alonso MT, Nadal A & García-Sancho J 2008 Glucose induces synchronous mitochondrial calcium oscillations in intact pancreatic islets. *Cell Calcium* **43** 39–47.
- Ravnskjaer K, Boergesen M, Rubi B, Larsen JK, Nielsen T, Fridriksson J, Maechler P & Mandrup S 2005 Peroxisome proliferator-activated receptor alpha (PPARalpha) potentiates, whereas PPARgamma attenuates, glucose-stimulated insulin secretion in pancreatic beta-cells. *Endocrinology* **146** 3266–3276.
- Robinson-Rechavi M, Escriva GH & Laudet V 2003 The nuclear receptor superfamily. *Journal of Cell Science* **116** 585–586.
- Rodriguez DE, Navarro M, Gomez R, Escuredo L, Nava F, Fu J, Murillo-Rodriguez E, Giuffrida A, LoVerme J, Gaetani S *et al.* 2001 An anorexic lipid mediator regulated by feeding. *Nature* **414** 209–212.
- Roduit R, Morin J, Masse F, Segall L, Roche E, Newgard CB, Assimakopoulos-Jeannet F & Prentki M 2000 Glucose down-regulates the expression of the peroxisome proliferator-activated receptor-alpha gene in the pancreatic beta-cell. *Journal of Biological Chemistry* **275** 35799–35806.
- Rokos CL & Ledwith BJ 1997 Peroxisome proliferators activate extracellular signal-regulated kinases in immortalized mouse liver cells. *Journal of Biological Chemistry* **272** 13452–13457.
- Rorsman P, Eliasson L, Renstrom E, Gromada J, Barg S & Gopel S 2000 The cell physiology of biphasic insulin secretion. *News in Physiological Sciences* **15** 72–77.
- Rose ML, Rivera CA, Bradford BU, Graves LM, Cattley RC, Schoonhoven R, Swenberg JA & Thurman RG 1999 Kupffer cell oxidant production is central to the mechanism of peroxisome proliferators. *Carcinogenesis* **20** 27–33.
- Santos RM, Rosario LM, Nadal A, Garcia-Sancho J, Soria B & Valdeolmillos M 1991 Widespread synchronous [Ca²⁺]_i oscillations due to bursting electrical activity in single pancreatic islets. *Pflügers Archiv: European Journal of Physiology* **418** 417–422.
- Scatena R, Bottoni P, Martorana GE, Ferrari F, De Sole P, Rossi C & Giardina B 2004 Mitochondrial respiratory chain dysfunction, a non-receptor-mediated effect of synthetic PPAR-ligands: biochemical and pharmacological implications. *Biochemical and Biophysical Research Communications* **319** 967–973.
- Scatena R, Bottoni P, Martorana GE, Vincenzoni F, Botta G, Pastore P & Giardina B 2007 Mitochondria, ciglitazone and liver: a neglected interaction in biochemical pharmacology. *European Journal of Pharmacology* **567** 50–58.
- Suardiaz M, Estivill-Torrus G, Goicoechea C, Bilbao A & Rodriguez DF 2007 Analgesic properties of oleoylethanolamide (OEA) in visceral and inflammatory pain. *Pain* **133** 99–110.
- Tordjman K, Standley KN, Bernal-Mizrachi C, Leone TC, Coleman T, Kelly DP & Semenkovich CF 2002 PPARalpha suppresses insulin secretion and induces UCP2 in insulinoma cells. *Journal of Lipid Research* **43** 936–943.
- Valdeolmillos M, Nadal A, Soria B & Garcia-Sancho J 1993 Fluorescence digital image analysis of glucose-induced [Ca²⁺]_i oscillations in mouse pancreatic islets of Langerhans. *Diabetes* **42** 1210–1214.
- Zhou YT, Shimabukuro M, Wang MY, Lee Y, Higa M, Milburn JL, Newgard CB & Unger RH 1998 Role of peroxisome proliferator-activated receptor alpha in disease of pancreatic beta cells. *PNAS* **95** 8898–8903.

Received in final form 6 November 2008

Accepted 11 November 2008

Made available online as an Accepted Preprint
17 November 2008