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THE NON-GENOMIC EFFECTS OF HIGH DOSES OF ROSIGLITAZONE ON CELL GROWTH AND APOPTOSIS IN CULTURED MONOCYTIC CELLS

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ABSTRACT
Peroxisome Proliferator-Activated Receptor gamma (PPARγ) is a ligand-activated transcription factor which belongs to the nuclear hormone superfamily and has multiple pharmacological ligands called Thiazolidinediones (TZDs). TZDs are a class of drugs used in the treatment of type 2 diabetic patients. Rosiglitazone is one such TZD, and is used clinically to treat type 2 diabetes. In this study, the effect of Rosiglitazone on cell growth and apoptosis in cultured monocytic monomac 6 (MM6) cells was investigated. Over a 14 day period, MM6 cells were cultured in vitro and treated with 1μM and 10μM Rosiglitazone. Cell viability and proliferation were evaluated by Haemocytometer cell count and MTS assay respectively. Turbidity due to cell density was assessed spectrophotometrically. Apoptosis was determined by Caspase-Glo 3/7 assay. Expression of the endoplasmic reticulum (ER) stress-inducible protein sarco-endoplasmic reticulum Ca^{2+} ATPase-2b (SERCA2b) was determined by Western blot. Neither 1μM nor 10μM Rosiglitazone exerted statistically significant inhibitory effects on cell proliferation, turbidity due to cell density, or cell viability ($p > 0.05$ in all cases). In contrast, Rosiglitazone induced increased apoptosis, but a significant difference was only observed in 10μM-treated cells compared with control cells (3.04 ± 0.52 control; $p < 0.05$) while 1μM-treated cells showed a non-significant increase (1.50 ± 0.06 control; $p > 0.05$). Meanwhile the expression of SERCA2b was up-regulated significantly in cells treated for >4hrs (e.g 2.45 ± 0.06 control at 24 hrs; $p < 0.05$) with 10μM Rosiglitazone. It was concluded that high doses (10μM) of Rosiglitazone up-regulate SERCA2b expression and induce apoptosis of MM6 cells by activating an ER stress response via a PPARγ-independent mechanism. The therapeutic relevance of these observations is a matter for further investigations.

Key words: Rosiglitazone, PPARγ, Monocytes, ER Stress, SERCA2b, Apoptosis

INTRODUCTION

Type 2 diabetes mellitus (T2D) is a disease condition characterised by defects in insulin action and secretion resulting from impaired beta cells secretory function. Insulin resistance results in notable decrease in glucose utilisation by the peripheral tissues with an increase in glucose production by the liver. The disease is found in almost every population worldwide. Epidemiological studies have shown that without effective prevention and control programmes, the prevalence of the disease will continue to rise globally (Alberti *et al.,* 2007). No less than 171 million people live with diabetes worldwide in 2000 and this figure likely to increase to about 366 million by the year 2030 (Wild *et al.,*2004).

The thiazolidinediones (TZDs) are a class of oral drugs used for the management of type 2 diabetes mellitus. TZDs are synthetic ligands for the transcription factor Peroxisome Proliferator-Activated Receptor gamma (PPARγ). Binding of TZDs to PPARγ forms a heterodimer with Retinoid X Receptor (RXR) (Olefsky, 2000) that binds to the PPARγ response element (PPRE) within the promoter of PPARγ target genes, whose expression impacts upon different aspects of carbohydrate and lipid metabolism. Rosiglitazone, an example of TZD, is an anti-diabetic agent acting as a potent insulin sensitizer and is used clinically to enhance insulin-stimulated glucose uptake by tissues. Rosiglitazone has been shown to improve glycemic control (Raskin *et al.,* 2001) and reduced glycated haemoglobin levels in patients with type 2 diabetes (Nissen *et al.,* 2007).

Rosiglitazone has been shown to exert two types of effects: Genomic (PPARγ-dependent) and Non-genomic (PPARγ-independent) modes of actions. Thus, while it exerts anti hyperglycaemic effects by acting as ligands for PPARγ, studies have shown that other effects may occur independent of PPARγ. For example, our research group recently showed that Rosiglitazone induces PPARγ-independent non-genomic effect such as sarco/endoplasmic reticulum calcium ATPase 2b (SERCA2b) inhibition after incubation for periods as brief as 5-30 minutes (Caddy *et al.,* 2008a). However, after extended period of incubation (>24hrs), there was a compensatory up-regulation of SERCA2b mRNA and calcium homeostasis was restored (Caddy *et al.,* 2008a). SERCA2b is an enzyme responsible for translocating calcium from the cytosol to the lumen of the endoplasmic reticulum. Interestingly Zhong *et al.* (2001) reported that SERCA2b inhibition was related to the progression of type 2 diabetes (Zhong *et al.,* 2001). The endoplasmic reticulum (ER) serves several important functions including post-translational modification, folding and assembly of newly synthesised proteins and calcium homeostasis.

Of direct interest here is calcium homeostasis, as it relates to the pathophysiology of type 2 diabetes and its complications (Advani *et al.,* 2004). Thus, this study investigated whether Rosiglitazone exerts adverse effects at a cellular level *in vitro*.

Disturbance of the ER functions (e.g. depletion of calcium from ER lumen) causes a condition collectively termed "ER Stress" (Kaufman *et al.,* 2002). However, to continue to survive in ER stress environment, cells have developed a mechanism to overcome such stress called "ER Stress Response. ER stress responses (also known as unfolded protein responses or UPRs) involve the up-regulation of genes containing ER stress response element (ERSE) and/or unfolded protein response element (UPRE) in their promoters. Examples of such genes are SERCA2b (Caspersen *et al.,* 2000) and BiP (Araki *et al.,* 2003). The effect of ER stress leading to the UPR activation has been linked to the development of insulin resistance and other metabolic diseases (Gregor *et al.,* 2007).

Activation of PPARγ by Rosiglitazone has been shown to inhibit cell growth by inducing apoptosis (Mao *et al.,* 2007). This study focused on ER stress-mediated effects on cell growth, viability and apoptosis in MM6 cells. We show here that exposure of MM6 cells to high dose (10μM) of Rosiglitazone induced an increase in apoptosis. Cell viability was only marginally reduced (compared to control) from day 12 to 14 of MM6 cells incubation in 1μM and 10μM Rosiglitazone. Finally, treatment with Rosiglitazone induced up-regulation of the ER stress-inducible protein, SERCA2b.

MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated. Cell permeabilization reagents and protein assay reagents were obtained from Harlan SERA-LAB Ltd (Loughborough, UK) and Bio-Rad Laboratories (Herts, UK) respectively. Rosiglitazone was obtained from GlaxoSmithKline (Uxbridge, UK). Mouse monoclonal primary anti SERCA2b antibody was purchased from Abcam (Cambridge, UK). HRP-Labelled Donkey anti-mouse IgG secondary antibody was purchased from New England Biolabs (Herts, UK). Cultured MM6 monocytic cells were obtained from the German Collection of Micro-Organisms and Cell Culture (Braunschweig, Germany).

Cell Culture. Monocytic MM6 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) foetal calf serum, 1% (v/v) penicillin (50IU/ml), 1% L-glutamine (2mM), 1% (v/v) non-essential amino acids and 1% (v/v) OPI supplement. The cells were maintained at 37°C with 5% CO_2. The sub-cultured cells were maintained at 0.3 – 1.0 x 10^6 Cells/ml. Cells of passage number < 24 were used in all experiments.

Incubation of Cells with Rosiglitazone. Cells were pooled all together and re-suspended in fresh medium. Two centrifuge tubes were used to spin down the cells using a Harrier Centrifuge 15/18. After spinning, supernatant was discarded and the cells were re-suspended in fresh medium. 0.1% DMSO (v/v), 1μM or 10μM Rosiglitazone (dissolved in DMSO to ≤ 0.1% v/v) was then added to the samples for incubation over the subsequent 1-14 days.

Preparation of Total Protein Extracts and Western Blot Analysis. After incubation with DMSO ± Rosiglitazone, cultured cells ($5x10^6$cells/ml) were placed in a sterile centrifuge tube and spun down at 300RCF for 10 minutes. The supernatant was removed and cells were washed in 10ml ice cold PBS buffer. The washing step was repeated twice. Cells were then lysed for 30 minutes on ice in 112μl of RIPA buffer [(100mM NaCl; 10mM Tris HCl; 2mM EDTA; 0.5% (w/v) sodium deoxycholate; 1% (v/v) NonidetP40 (NP40)], 6μl phosphatase inhibitor and 12μl of protease inhibitor cocktail. Sample lysates were sonicated for few seconds on ice to shear DNA to reduce sample viscosity. Protein concentrations of whole cell lysates were then determined using BioRad DC Protein assay according to the manufacturers' instructions (BioRad Laboratories, Herts, UK).

For western blot experiments, Loading buffer was then added to each lysate and then heated for about 5 minutes in a waterbath. Portions (50μg each) of heated protein samples were then loaded into separate wells of the gel. To the first well, MagicMark molecular weight marker was loaded and MultiMark pre-stain molecular marker in the last well. The gel was run at 200 volts for 50 minutes at room temperature. After electrophoresis, samples were transferred on to nitrocellulose membranes. Each membrane was then incubated in primary antibody for 1 hour at room temperature (1:500 dilutions). The membrane was washed 3 times at 5 minutes interval using TBS/Tween20. The membrane was subsequently incubated with HRP-conjugated secondary antibody (1:1000 dilutions) for 3 hours at room temperature before addition of enhanced chemiluminescence-coupled HRP substrate, and visualisation using UVP VisionWorksLs, version 5.54.

Measurement of Cell Viability. Optical density (turbidity) and Trypan blue exclusion methods were used to measure the number of viable cells in the samples. A light scattering technique was used to monitor the concentration of cells in Rosiglitazone treated samples; the amount of light absorbed by the suspension of cells was measured using spectrophotometer at 600nm. A calibration curve was constructed to show that OD_{600} is proportional to cell number. Trypan blue was used to assess the number of viable cells in the sample. Live cells possessed an intact cell membrane which excludes the dye. However, dead cells were stained blue by the dye. Haemocytometry was then used to count the viable cells.

Cell Proliferation Assay. The actively dividing cells in the Rosiglitazone treated samples were assessed using CellTiter 96 Aqueous One Solution cell proliferation assay (MTS assay) according to the manufacturer's instructions.

Apoptosis Assay. Apoptotic cells were determined using Caspase-Glo 3/7 Assay. This was performed in accordance with the manufacturer's instructions.

Statistical Analysis. Data are expressed as the mean of three independent experiments and were compared using Student's *t*-test. $p < 0.05$ was considered statistically significant.

RESULTS

Effect of Rosiglitazone on Cell Proliferation. To examine the effect of Rosiglitazone on cell proliferation, MM6 cell proliferation rate was analyzed by MTS assay. The results show that 1μM and 10μM Rosiglitazone do not exert a statistically significant inhibitory effect on cell proliferation ($p > 0.05$ in both cases) (Figure 1).

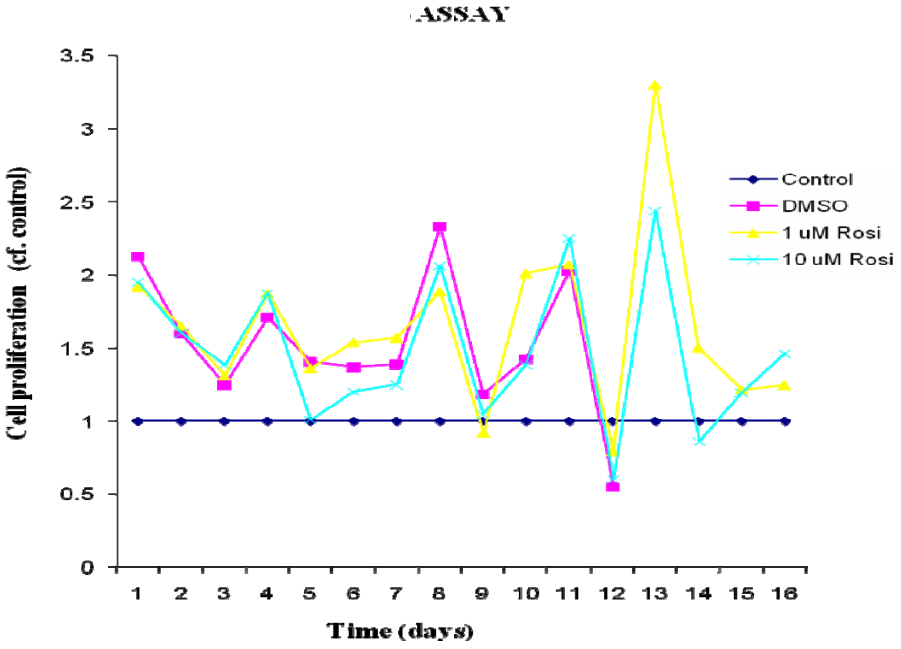

Figure 1: The effect of Rosiglitazone on cell proliferation of MM6 cells treated with 0.1%DMSO, 1µM and 10µM Rosiglitazone.

Effect of Rosiglitazone on Cell Viability

To examine the effect of Rosiglitazone on MM6 cell viability, a direct cell count using haemocytometer was performed. Although cell viability was marginally reduced (compared to control) from day 12 to 14 of the incubation in 1µM or 10µM Rosiglitazone, the effect of both 1µM and 10µM Rosiglitazone did not reach statistical significance (Figure 2).

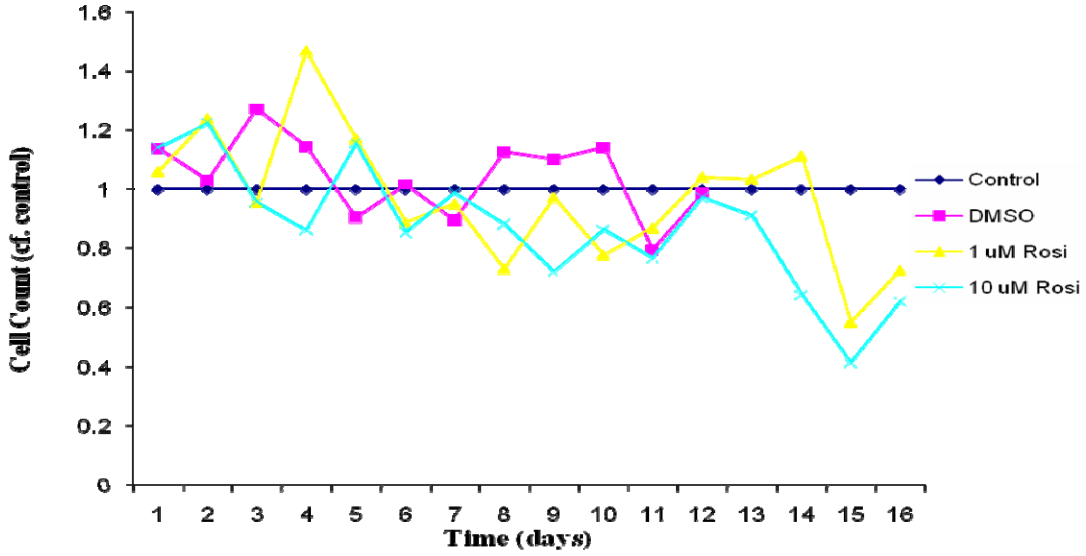

Figure 2: The effect of Rosiglitazone on cellular viability of MM6 cells treated with 0.1% DMSO, 1µM and 10µM Rosiglitazone.

Effect of Rosiglitazone on Turbidity due to Cell Density

Optical density measurements at 600nm were employed to determine the amount of light scattered by the suspension of cells. As the optical density or turbidity of a suspension of cells is directly related to cell number (Fig. 3a), our optical density results allow us to conclude that no significant difference (Fig.3b) in cell density is seen in 1µM and 10µM Rosiglitazone treated samples compared to control ($p > 0.05$ in both cases).

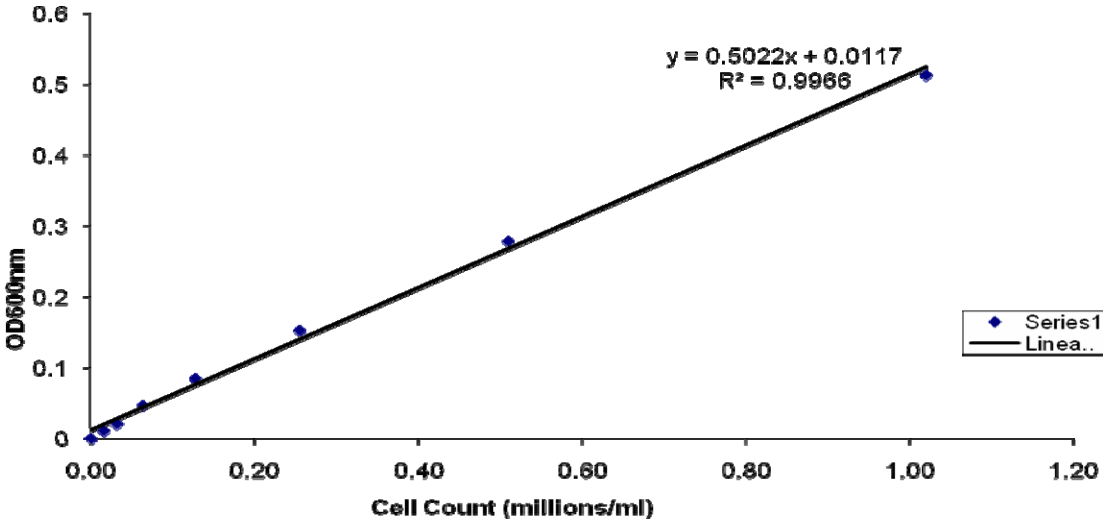

Figure 3a: Optical Density Calibration curve. The calibration curve shows that OD_{600} is proportional to cell number.

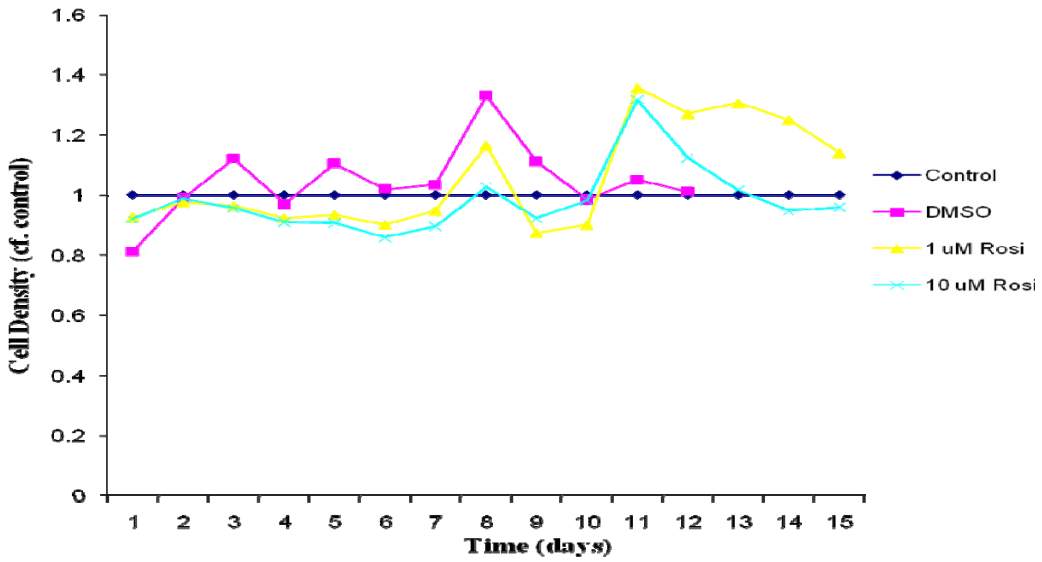

Figure 3b: Effect of Rosiglitazone on Turbidity due to Cell Density for MM6 cells treated with 0.1% DMSO, 1µM and 10µM Rosiglitazone.

Expression of an ER Stress-Inducible protein (SERCA2b) in Rosiglitazone Treated MM6 cells

To investigate the effect of 10µM Rosiglitazone on the expression of SERCA2b protein, a Western Blot analysis was performed. SERCA2b was detected by the Western Blot as approximately 105 KDa band (Fig. 4a) in the total protein lysate of the MM6 Rosiglitazone treated cells. The result (Fig. 4b) showed an increase (approx. x2.5 fold) in expression of this protein in the MM6 cells treated for >4hrs with 10µM Rosiglitazone. Thus, it can be concluded that Rosiglitazone induces an ER stress response in these cells.

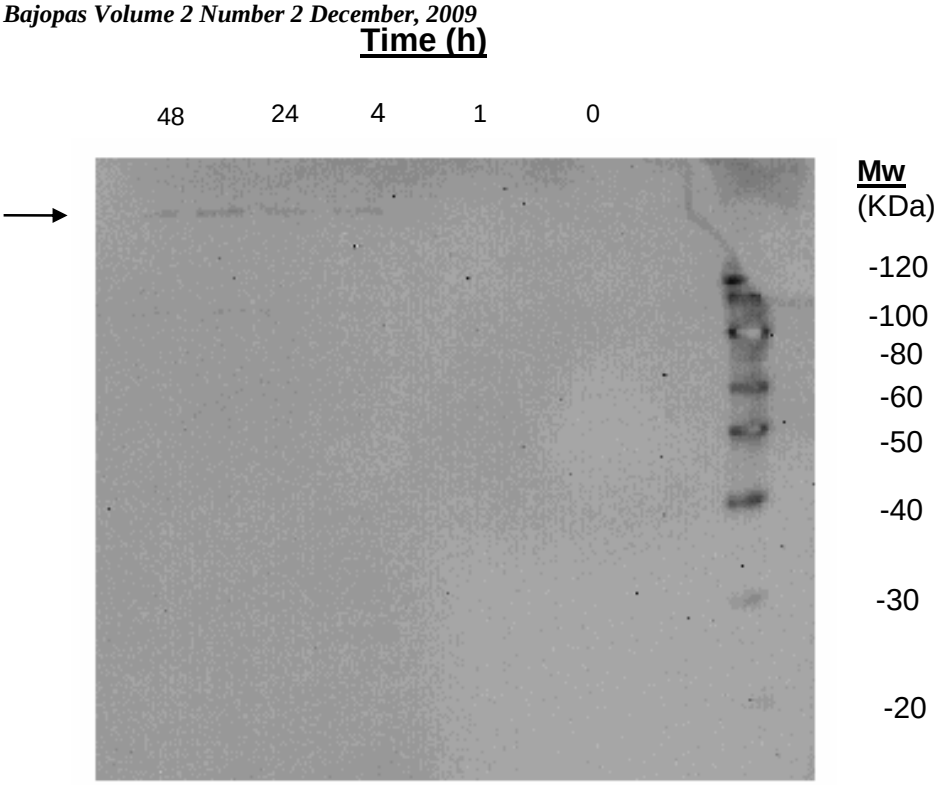

Figure 4a: Western Blot analysis of SERCA2b in MM6 cells total protein lysate. SERCA2b was detected using anti-SERCA2b primary antibodies and HRP-labelled secondary antibodies. Arrow indicates 105KDa molecular weight bands, which correspond to SERCA2b. Image is representative of three separate experiments

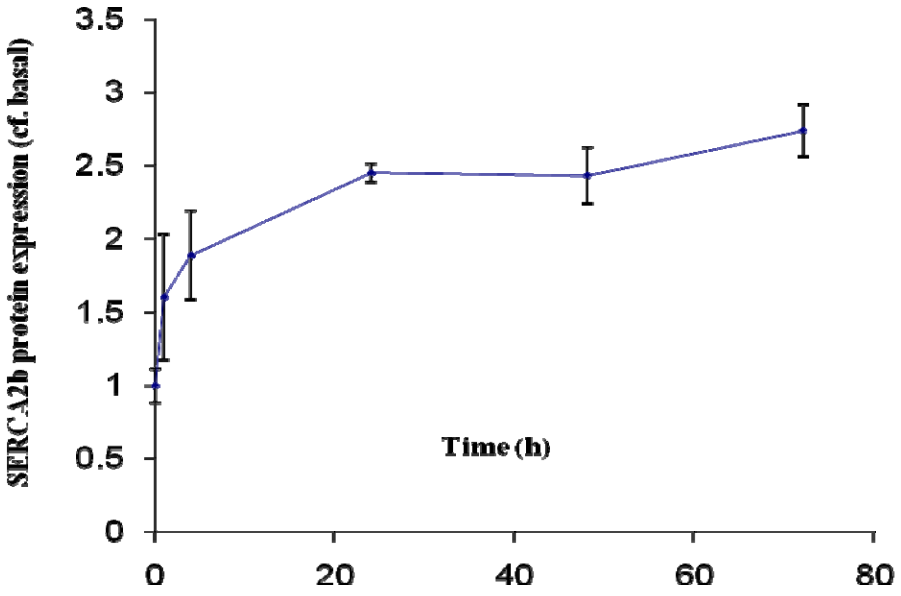

Figure 4b: Densitometric analysis of SERCA2b protein expression in MM6 cells treated with 10µM Rosiglitazone. >4hrs incubation with 10µM Rosiglitazone showed an increase (approx. x2.5 fold) in expression of SERCA2b. Data are from 3 separate experiments.

Induction of MM6 Apoptosis by Rosiglitazone

As ER stress responses can lead to either restoration of normal cell physiology or to apoptosis, MM6 cells were treated with 1µM and 10µM Rosiglitazone for 14 days and subsequent apoptosis was assessed by Caspase-Glo 3/7 Assay. Although 14 days exposure to Rosiglitazone induced increases in apoptosis in MM6 cells, the results (Fig. 5) showed a significant difference only in 10µM treated cells compared with control cells (3.04 ± 0.52 control; $p < 0.05$) while 1µM Rosiglitazone treated cells showed only a non-significant increase (1.50 ± 0.06 control; $p > 0.05$).

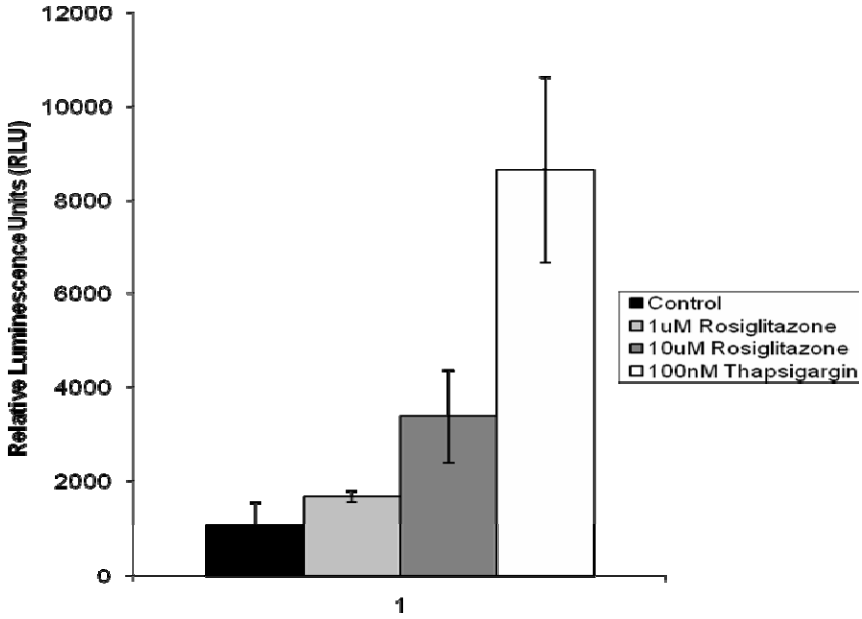

Figure 5: Effect of Rosiglitazone on Apoptosis of MM6 cells by Caspase-Glo 3/7 assay. Data are expressed as the mean of three independent experiments (NB: Cells were treated with thapsigargin (Tg) for only four days, as longer duration of Tg treatment proved cytotoxic).

DISCUSSION

The discovery of TZDs as potent insulin sensitizers in diabetes patients has generated substantial interest into their pharmacological actions. The molecular mechanisms by which TZDs exert their actions are quite complex and studies have shown that they may have differential effects in some tissues (Camp *et al.,* 2000). However, TZDs have been shown to exert two types of effects: PPARγ-dependent and PPARγ-independent modes of action. Thus, although Rosiglitazone exerts anti-hyperglycaemic effects by acting as a ligand for the nuclear receptor PPARγ, studies have shown that effects such as those observed in the present study may occur independent of PPARγ (so called non-genomic effects). It should be noted that disruptions in calcium homeostasis, which have been associated with the pathophysiology of type 2 diabetes and its complications (Zhong *et al.,* 2001), have been linked to the non-genomic effects of PPARγ ligands in this and previous studies. For example, a recent study in our laboratory has shown that short-term (< 30 mins) incubation with Rosiglitazone inhibits SERCA2b via a PPARγ-independent (non-genomic) manner (Caddy *et al.,* 2008a). However, after extended period of incubation (>24hrs), there was a compensatory up-regulation of SERCA2b and restoration of calcium homeostasis in monocytic cells (Caddy *et al.,* 2008a). As mentioned earlier, the endoplasmic reticulum (ER) serves several important functions including calcium homeostasis. Recently, Rosiglitazone has been linked to disruption of ER (Caddy *et al.,* 2008b), which may be suggested to lead to induction of an ER stress response. ER stress responses cause up-regulation of ER stress-inducible genes such as SERCA2b (Caspersen *et al.,* 2000). In this study, SERCA2b was detected by western blot as approximately 105 KDa band in the total protein lysate of Rosiglitazone-treated MM6 cells.

The results showed an increase (approx. x2.5 fold) in expression of the 105KDa (Fig. 4b) protein in MM6 cells treated for >4hrs with 10μM Rosiglitazone. Rosiglitazone has also been shown to inhibit cell growth by inducing apoptosis (Mao *et al.,* 2007), and ER stress responses can lead to apoptosis (Di Sano *et al.,* 2006; Szegedi *et al.,* 2006); therefore this study focused on ER stress-mediated inhibition of cell growth and viability in MM6 cells. Although cell viability was marginally reduced (compared to control) from day 12 to 14 of the incubation in 1μM and 10μM Rosiglitazone, the effect in both cases did not reach a statistical significance ($p > 0.05$). The results suggest that up-regulation of SERCA2b helps to largely restore normal intracellular calcium homeostasis. While incubation of MM6 cells with 10μM Rosiglitazone leads to approximately 3 fold increases in apoptosis, this effect is much less marked than that seen with the classical SERCA2b inhibitor Thapsigargin (Tg). Moreover, 1uM Rosiglitazone (which approximated to the concentration seen in T2D patients *in vivo* (Niemi *et al.,* 2003)) does not have a significant effect in this regard.

In conclusion, results from this study show an increase (approx. x2.5 fold) in expression of SERCA2b in MM6 cells treated with Rosiglitazone. This up-regulation of SERCA2b may be important in maintaining calcium homeostasis in the cells of patients undergoing Rosiglitazone therapy. Since TZDs have been shown in several studies to be associated with unfavourable side effects (Soroceanu *et al.,* 2004; Nissen *et al.,* 2007; Singh *et al.,* 2007; Sarafidis 2008), there is a need to identify natural and/or synthetic PPARγ ligands that have the ability to

exert PPARγ-dependent anti-hyperglycemic effects, without also inducing deleterious side-effects. There is also the need to investigate whether the mechanism via which Rosiglitazone exerts its effect in monocytes also occur in other cell types, like skeletal muscle and cardiomyocytes. While pharmacological doses of Rosiglitazone (Rosiglitazone has been shown to exist in the plasma at concentration approximately 1μM, (Niemi *et al.,* 2003)) did not significantly induce apoptosis, high doses of Rosiglitazone (10μM) could inhibit cell growth by inducing apoptosis via the ER stress response pathway. Since ER stress responses have been linked to the development of insulin resistance and other metabolic diseases (Gregor *et al.,* 2007), discoveries in this aspect indicates the importance of ER stress responses in type 2 diabetes, and suggest that they could be a target for the management of the disease.

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