IS THE COMBINATION OF $\beta_3$-AR AGONIST AND PPAR AGONISTS A BETTER TREATMENT OF TYPE II DIABETES AND OBESITY?

R.A. Ngala$^1$, C. Stocker$^2$, J.R.S. Arch$^2$ and M.A. Cawthorne$^2$

$^1$Department of Molecular Medicine, School of Medical Sciences, Kwame Nkrumah University of Science and Technology Kumasi, Ghana
$^2$Clore Laboratory, University of Buckingham, UK MK18 1EG

ABSTRACT

Metabolic syndrome consists of insulin resistance, dyslipidaemia, obesity among other metabolic defects. Treatment of diabetes/obesity goes beyond glycaemic control. Effective management of diabetes involves the improvement of insulin sensitivity, regulation of body weight and improvement of lipid profile. However, there are very few drugs that are able to control more than one or two of these metabolic defects. This work is therefore aimed at studying the effect of a combination of two potential antidiabetic agents in comparison to the effects of the individual agent. Obese mice were treated (p.o) with 10mg/kg GW-800644 for 14 days. Dosing was twice daily at 9.00 and 17.00 hours. After 13 days of dosing, energy expenditure was measured, for three hours and then mice, were dosed with BRL-37344 (1mg/kg, i.p) and measurement continued for another six hours at room temperature (25.5°C). Plasma, glucose, insulin, triglycerides and non-esterified fatty acids were measured after individual treatment and after the combination treatment where possible. Energy expenditure was not significantly increased after treatment with GW80064 alone but was increased by 45% when combined with BRL37344. Insulin sensitivity was improved by 31% with GW80064 alone but by 41% in combination with BRL37344 with a corresponding decrease in plasma glucose. There was a significant improvement in the lipid profile even with the individual agonist. Therefore, the combination of $\beta_3$-AR agonist and PPAR agonists would serve a better treatment of type II diabetes and obesity than the individual agonist.

Keywords: $\beta$-AR, Beta-adrenoceptor PPAR-d Peroxisome Proliferator-Activated delta, HPMC Hydroxypropyl- methylcellulose

INTRODUCTION

There are no drugs that treat all the three features of the metabolic syndrome; insulin resistance, obesity and dyslipidaemia. The PPAR-$\gamma$ agonists actually increase body weight, BAT (brown adipose tissue) is an important site of glucose uptake in response to both $\beta_3$-AR agonists and PPAR-$\alpha$ agonists (Liu et al., 1998; Chou et al., 2002). In rodents, BAT maintains energy balance by allowing oxidation of fat uncoupled to ATP synthesis (thermogenesis) using the mitochondrial protein UCP-1.
(uncoupling protein) (Nedergaard et al., 2001; Arbeeny et al., 1995). Several studies have shown that PPAR-γ activation increases the expression of UCP-1 mRNA in BAT, while at the same time increasing food intake and body weight (Emilsson et al., 2000). This surprising combination of effects suggests that though UCP-1 expression is increased, it is not activated by the sympathetic nervous system, while PPAR-α agonists do not cause significant weight loss in humans.

This has led to many researchers to study the combined effects of PPAR-γ agonists and β3-AR agonists in animal models. In one study it was shown that treatment with the PPAR-γ agonist [2-(4-phenoxy-2-propylphenoxy) ethyl] indole-5-acetic acid] alone induced UCP-1 expression and other genes associated with fat oxidation and thermogenesis in BAT and white adipose tissue (WAT), but did not increase energy expenditure in either lean or obese mice. However, when the mice were treated in combination with CL-316243, the β3-AR agonist, stimulated thermogenesis in lean and ob/ob mice, and the effect was stronger in the PPAR-γ pre-treated mice, which in addition showed lower respiratory quotient, higher oxygen consumption and significant weight and fat loss (Sell et al., 2004). Thurlby et al. (1987) first made a similar report using cigitazone (a PPAR-γ agonist) and BRL-26830A (a β3-AR agonist).

The observation that repeated administration of some β3-AR agonists had a marked influence on body composition and insulin sensitivity in rodents (Arch and Ainsworth, 1983; Cawthorne and Cornish, 1979; Smith et al., 1985), led to intensive research efforts to develop β3-AR agonists for the treatment of obesity and type 2 diabetes in humans. The first generation of β3-AR agonists such as BRL-37344 was also found to increase insulin secretion after a single dose, resulting in lowered fasting blood glucose levels in insulin sensitive animals. Insulin secretion was probably secondary to raised non-esterified fatty acids (NEFA) levels (Arch and Kaumann, 1993). This glucose lowering effect should not be confused with that due to improved insulin sensitivity in animal model of insulin resistance. This latter effect may be due to stimulation of fatty acid oxidation and lowered intracellular levels of fatty acid metabolites such as diacylglycerol (Darimont et al., 2004).

PPAR-γ agonists regulate lipid metabolism and alter insulin sensitivity. Two PPAR-γ agonists of the thiazolidinedione class, rosiglitazone and pioglitazone, are used in the treatment of diabetestes. PPAR-α agonists, promote fat oxidation, especially in the liver. PPAR-α agonists, of the fibrate class are used in the treatment of hypertriglyceridaemias. The most widely expressed member of the PPAR family is PPAR-δ. Recently, the PPAR-δ agonist GW-501516 has been shown to regulate fuel metabolism in rat isolated soleus muscle via two pathways. There is a delayed genomic mechanism in which low concentrations of GW-501516 (1×10^{-8} M and 1×10^{-7} M) increased palmitate oxidation and inhibited glucose oxidation, glycogen synthesis and lactate release. Glucose utilization was increased only when palmitate was absent. Secondly, at higher concentrations, greater than 1×10^{-7} M, parallel increases in the oxidation of both glucose and palmitate were observed. The higher concentrations were associated with a direct uncoupling of oxidative phosphorylation and do not appear to be due to PPAR-δ activation (Brunnair et al., 2005).

In this study we investigate the combined metabolic effect of BRL37344 (Fig 2) (a phenylethanolamine), a β3-AR agonist and GW80064 (Fig 1), a PPAR-δ agonist as an alternative for management of diabetes obesity than the individual agonist.

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Fig 1: Chemical structure of GW80064
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MATERIALS AND METHODS

Reagents
GW800644 was obtained from Glaxosmith-kline (GSK), UK. All other reagents were purchased from Sigma-Aldrich, Poole, UK, unless otherwise stated.

Animals
Housing and procedure were conducted in accordance with the UK Government Animal (Scientific procedures) Act 1986 and approved by the University of Buckingham Ethical Review Board. Male C57Bl/6 lean and ob/ob mice aged 6-7 weeks, were obtained from Harlan, UK, and fed ad libitum (free access to water and standard mouse chow) prior to use. The animals were maintained in a constant room temperature (21±2°C), with a fixed 12-hr artificial light/12-hr dark cycle (8.00 am to 8.00 pm).

Treatment
24 C57BL/6 male ob/ob mice, aged 6-7 weeks old, on normal diet, were divided into 2 groups of eight and sixteen mice. Mice in each group were housed in cages of four mice each. Group A (n=8) served as control and was dosed (p.o) with 0.5 % HPMC while group B (n=16) was dosed (p.o) with 10mg/kg GW-800644 for 14 days. Dosing was twice daily at 9.00 and 17.00 hours. After 13 days of dosing, energy expenditure was measured, for three hours and then mice, were dosed with BRL-37344 (1mg/kg, i.p) and measurement continued for another six hours at room temperature (25.5°C).

Analytical procedures
Details of measurement of energy expenditure, blood glucose, triglycerides, NEFA and insulin are discussed in Ngala et al 2009. However, the procedures are briefly described as:

Energy Expenditure
Energy expenditure in mice was measured by open-circuit, indirect calorimetry. This involves estimating heat production from gas exchanges. Mice were dosed as described earlier. Each cage was connected to a rubber tube fitted with a pump to extract expired air from the cage. The flow rate of the extracted air was maintained at a mean 800 ml/min. The air was then passed through a drying system of silica gel. The dried air was analysed by a carbon-oxygen analyser (Sermomex 1440 Gas Analyser). Air was sampled from one chamber at a time, using properly ventilated room air as a reference. The analyser computed the oxygen consumption in each cage every 30 minutes based on the unmodified Weir equation.

Measurement of blood glucose
Mice were injected with 60 mg/kg pentobarbital (sagatol), a general anesthetic, and 20 µl blood samples were taken from a nip in the tail-end of the mouse into disposable micro-pipettes.
and mixed with 0.38 ml of haemolysis reagent (50mg/l digitonin 100mg/l maleimide). Duplicate 20 μl aliquots of the mixture for each individual sample and similarly-treated glucose standards were then analyzed automatically using SpectraMax 250 and SoftMax Pro software (Molecular devices Corporation, 1311 Orleans Drive, Sunnyvale, California, 94089, USA).

**Measurement of non-esterified fatty acids (NEFA)**

Five microlitres (5μl) of sample and standard were placed in duplicate in a 96-well plate and 100 μl colour reagent A (acyl-Coenzyme A oxidase and peroxidase) was added, mixed well and incubated for 15 min and 200 μl of colour reagent B then added. Absorbance was read at 550 nm, and NEFA was calculated by comparing to the standard value.

**Measurement of Plasma triglycerides**

Plasma was obtained by centrifuging blood at 3000 g for 3 min. Triglycerides were assayed using the Wako Reagent Kit (994-75409) (USA)

Two microlitres of sample and triglycerides standard were pipetted into a 96-well plate in duplicates and 200μl reagent complex added to samples and standard, and incubated in the dark at room temperature for 20 minutes and read at 520 nm. The mean absorbance from each set of duplicates was determined and the triglycerides concentrations read off the standard curve.

**Measurement of plasma insulin**

Plasma insulin concentrations were assayed using a 96-well rat insulin ELISA kit from Crystal Chem Inc. (Chicago, IL, Cat No: INSKR020) according to the manufacturer’s protocol.

Rabbit anti-insulin monoclonal antibodies coated on the microplate bind the insulin from the plasma samples. Addition of guinea pig anti-rat insulin antibodies also bound to the insulin from the plasma samples. This formed an anti-rat insulin monoclonal antibody/mouse plasma insulin/guinea pig anti-rat insulin antibody complex, which was immobilised on the microplate. Repetitive washing removed unbound materials. Anti-guinea pig antibody conjugated with horseradish peroxidase was then bound to the anti-rat insulin monoclonal antibody/mouse plasma insulin/guinea pig anti-rat insulin antibody complex immobilised on the microplate. After the removal of unbound peroxidase conjugated antibody, o-phenylenediamine substrate solution was added to react with the horseradish peroxidase for 30min. The absorbance was read at wavelengths 492 nm and 630 nm. A set of insulin standard supplied by the manufacturer was added and used to plot the standard curve. The mean absorbance from each set of duplicates was determined and the insulin concentrations read off the standard curve (Halaas et al., 1995).

**Data analysis and statistics**

Results were expressed as means ± SEM. Data were analysed by one-way ANOVA followed by the Bonferroni test for multiple comparison using graph Pad Prism version 4 (Graph pad software, San Diego California). Levels of energy expenditure were plotted using Prism. Unpaired Student t-tests were used to assess for significance.

**RESULTS**

*In vivo* treatment of soleus muscles with GW80064 significantly improved *in vitro* soleus muscle glucose-uptake by 1.8 fold, and a 2.2 fold and 3 fold increase in the presence of 10pM BRL37344 and 10nM BRL37344 respectively (P<0.001). The effect of BRL37344 seems to be additive to that of GW80064 (Fig 3).

**DISCUSSION**

A number of studies have shown that PPAR-γ agonist induce UCP1 (Uncoupling protein) mRNA expression in adipose tissue of rodents (Fukui et al., 2000) resulting in increase in lipolysis but without an increase in thermogenesis. At the same time there was an in-
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Fig 3: The effect of BRL37344-stimulated glucose uptake in isolated mouse soleus muscle of ob/ob mouse after in vivo treatment with GW80064 n=6 ** P<0.001 compared to control.

Fig 4: Effect of GW80064 and BRL37344 on energy expenditure in obese mice n=6 ** P<0.01; GW80064-stimulated energy expenditure in ob/ob mice was not significant, however, in the presence of 1µM BRL37344, there was a 45% significant (P<0.01) increase in energy expenditure (Fig 4).
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Fig 5. Effect of GW80064 and BRL37344 on plasma NEFA levels \( n=6 \) **
* \( P<0.001 \) * \( P<0.05 \); compared to control

GW80064 significantly decreased plasma NEFA by 32%. BRL37344 significantly decreased it by 57% (Fig 5)

Fig 6. Effect of GW80064 and BRL37344 on plasma triglycerides in lean C57Bl6 mice. \( n=8 \). * \( P<0.05 \), ** \( P<0.01 \); compared to control

GW80064 significantly increased plasma triglycerides in lean mice by 10% whereas BRL37344 significantly increased it by 47% (Fig 6)
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Fig 7. Effect of GW80064 and BRL 37344 on plasma glucose in lean C57Bl6 mice. 
n=8. 
*P<0.001;  compared to control

GW80064 non-significantly decreased plasma glucose by 13%. The effect of BRL37344 was significantly decreased by 47%. There was also a significant difference between the effect of GW80064 and that of BRL37344 (Fig 7).

Fig 8 Effect of GW80064 and BRL 37344 on insulin secretion  in ob/ob C57Bl6 mice 
n=8  * P<0.05;  * *P<0.01 compared to control

GW80064 significantly decreased plasma insulin in obese mice by 31% whiles in combination with BRL37344 significantly decreased it by 41% (Fig 8)

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crease in food intake and body weight suggesting a lack of coupling between raised UCP1 expression and thermogenic activity, possibly due to reduced sympathetic derive (Wang et al., 1997). However, dual treatment with PPAR-γ and β3-AR agonists led to increase in thermogenesis and weight loss in lean and ob/ob mice relative to treatment with each agonist alone (Sell et al., 2004). The effect of GW80064, a PPAR-ö in this study was very similar to the effect of the PPAR-γ agonist [2-(2[4-phenoxy-2-proplyphenoxy]ethyl) indole-3-acetic acid] activation. Activation by the GW80064 alone significantly increased lypolysis as shown by the increase in plasma triglycerides (Fig 6). However, this was not accompanied by a concomitant increase in energy expenditure (Fig 3). Energy expenditure was only significantly increased after the subsequent administration of BRL37344, a β3-AR agonist. This suggests the lack of coupling between increased lypolysis and energy expenditure. The sympathetic effect was increased when BRL37344 was administered that led to the significant increase in energy expenditure. The metabolic effects of the activation of PPAR-ö on fuel metabolism are attributed to the expression of genes involved in lipid metabolism, uncoupling, and mitochondrial biogenesis, increasing fat and energy expenditure (Brunmair et al., 2005). The rate of weight gain and food intake in the GW80064 treated compared to the control in the obese mice after the two weeks treatment was significantly reduced (data not shown).

Type 2 diabetes and obesity are associated with pancreatic β-cell dysfunction as a result of increased plasma levels of non-esterified fatty acids and triglycerides. High levels of plasma lipids accumulation leads to cellular dysfunction and death in non-adipose tissues including the heart, pancreas and liver and therefore inhibiting β-cell insulin secretion and reduction in glucose uptake, referred to as ‘lipotoxicity’ (Unger, 1995). PPAR-γ activation through the binding of the synthetic glitazone results in marked improvement in type 2 diabetic patients of insulin and glucose metabolism hence an improvement of whole body insulin sensitivity. GW80064 similarly improved insulin sensitivity in ob/ob mice significantly (Fig 8). The effect was even much better when administered together with BRL37344. The increased insulin sensitivity seems to correspond with an increased oxidation of lipids as depicted by the decrease in NEFA (Fig 5). High plasma lipids increase insulin resistance in obese mice and High fatty acid oxidation improves insulin sensitivity a typical effect of thiazolidinediones (Yamauchi et al., 2001). Indeed, the activation of PPAR-ö can induce reverse cholesterol transport, correct lipoprotein profiles and triglyceride levels in obese rhesus monkeys (Oliver et al., 2001).

Accumulation of different fat species in the skeletal muscle in type 2 diabetes may occur due to an impairment of oxidation of FFAs by the mitochondria resulting in insulin resistance (Petersen et al., 2005). The improved insulin sensitivity by the individual activation of GW80064 and BRL37344 was in agreement with the increased glucose utilization (Fig 7), probably due to the increased oxidation of free fatty acids as shown by the reduction of non-esterified fatty acids (Fig 5). Therefore, the pretreatment (in vivo) with GW80064 and subsequent in vitro treatment with BRL37344 significantly improved glucose uptake in the isolated soleus muscle up to between 2.2 fold and 3 fold increases in glucose uptake (Fig 3) compared to the glucose utilization.

CONCLUSION

Energy expenditure was not significantly increased after treatment with GW80064 alone but was 45% increased when combined with BRL37344. Insulin sensitivity was improved by 31% with GW80064 alone but by 41% in combination with BRL37344 with a corresponding decrease in plasma glucose.

Since the activation of PPAR-ö by specific agonists ameliorates hyperglycaemia, insulin resistance and dyslipidaemia in animal models of type 2 diabetes, PPAR-ö can be regarded as a new promising target in the treatment of meta-
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bolic disorders and when in combination with the beta-adrenoceptor BRL37344, energy expenditure is increased which could lead to weight loss. Management of diabetes/obesity would therefore, be more effective with a combined therapy of PPAR-ò, GW80064 and the beta-adrenoceptor BRL37344.

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