Correction of dysfunctional fatty acid metabolism using peroxisome proliferator activated receptor γ agonists

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INTRODUCTION

Excessive accumulation of fatty acids (FA) or their products, both in the circulation and the tissues, has been implicated in the pathogenesis of several key manifestations of insulin resistance syndrome, including the atherogenic lipoprotein profile, insulin resistance, disturbances in insulin secretion, as well as accelerated apoptosis in pancreatic β-cells and cardiac tissue. In the context of cardiovascular disease and conditions of insulin resistance, these associations provide a reasonable basis for development of strategies, including pharmacological approaches, which correct dysregulation of free fatty acid (FFA) metabolism. Via agonist activation, nuclear receptors (peroxisome proliferator activated receptors [PPARs]) mediate important control over the transcription of many genes involved in FA metabolism. However, there are hardly any detailed *in vivo* investigations of the effects of PPAR agonists on FFA fluxes and metabolic fate. Here, we summarize our own studies of the effects of PPARγ agonists in the obese Zucker rat, a model of insulin resistance and dyslipidaemia. These studies show that PPARγ agonists correct many aspects of dysfunctional FA metabolism in obese Zucker rats. Future clinical studies are required to establish the translation of these beneficial effects in experimental animals to patients with insulin resistance, including type 2 diabetes.

DYSREGULATION OF FA METABOLISM: A METABOLIC DISTURBANCE CENTRAL TO IMPAIRED GLUCOREGULATION AND ARTERIOSCLEROSIS

Dysregulation of FA metabolism, as used here, refers to conditions in which there is an excessive accumulation of FA or their products in plasma and non-adipose tissues. A substantial body of evidence suggests that FA overload is a central causative factor in glucoregulatory disorders and cardiovascular disease in both diabetic and non-diabetic populations. Thirty-eight years ago, Sir Phillip Randle and coworkers[1], in their classic paper, suggested the term 'fatty-acid syndrome' for the situation in which elevated plasma FFA levels cause abnormalities in glucose metabolism. Although the precise biochemical mechanism(s) mediating the *in vivo* interaction remain unclear, there is no doubt that elevated FFA levels decrease insulin-stimulated glucose metabolism in skeletal muscle[2], the major site of insulin-stimulated glucose disposal. In addition, FFA oversupply can reduce the ability of insulin to suppress hepatic glucose production[3] and disturb glucose-stimulated insulin secretion[4]. These glucoregulatory derangements are of major significance in relation to the treatment of patients with type 2 diabetes. Perhaps the most serious consequence of chronically elevated FFA, though, is its probable role in promoting atherogenesis, currently the major cause of early mortality in the industrialized world. Thus, an oversupply of FFA to the liver increases hepatic triglyceride (TG) production, a key event in the generation of the so-called atherogenic lipoprotein profile, characterized by hypertriglyceridaemia, decreased high-density lipoprotein (HDL) cholesterol, and increased numbers of small dense low-density lipoprotein (LDL) particles[5,6]. People with this lipoprotein profile have a greatly increased risk of premature cardiovascular disease.

Elevated plasma FFA levels may also facilitate atherogenesis through modulation of extracellular proteoglycan abundance and composition. Alterations in abundance and composition of the proteoglycans of the vascular intima during atherogenesis and diabetes are well documented[7]. These changes may be a crucial step in atherogenesis by increasing the ability of the extracellular matrix to trap LDL particles. Recently, our laboratory reported, using human arterial smooth muscle cultures, that physiological levels of albumin-bound FFA could induce substantial increases in the expression of the genes for the core proteins of proteoglycans, syndecan, versican and decorin. Most significantly, these changes increased the ability of the cells exposed to the high FFA levels to bind LDL more efficiently[8].

FA overload could theoretically be a consequence of oversupply or underutilization. At the whole-body level, evidence for both of these processes exists, with inability of insulin to adequately suppress FFA levels in patients with familial combined hyperlipidaemia[9], patients with type 2

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diabetes[10], and an impaired ability of skeletal muscle to utilize FFA[11]. At the tissue level, FA overload is manifested as an accumulation of metabolic products of FA, ranging from acyl-coenzyme A (acyl-CoA) derivatives to increased acyl-glyceride levels, and has been implicated in several metabolically linked diseases. In skeletal muscles, a number of studies in animals and humans have documented a close correlation between glucose metabolic insulin resistance and the local accumulation of esterified FA (e.g.[12–14]). An oversupply of FFA to the liver might also be involved in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). Patients with NAFLD have a strong tendency to be insulin resistant, with a reduced ability of insulin to suppress plasma FFA[15], possibly combined with an impaired ability to oxidize FFA[16]. Independent of their influence on atherogenesis (see above), an excess of FA or their metabolites has been postulated to play an important role in the pathogenesis of metabolic cardiomyopathies. Cardiac hypertrophy and dysfunction are evident in situations where cardiac FA supply chronically exceeds local oxidation. These include animal models of insulin resistance and diabetes, where circulating FFA and TG are elevated[17]; increased cardiac FFA sequestration by heart-specific overexpression of the enzyme long chain fatty acyl CoA synthetase (ACS)[18]; and conditions of long-chain FA oxidation blockade[19], as well as in patients with genetic defects in the enzymes of FA oxidation[20].

The potential importance of FA dysregulation in the pathophysiology of a number of prevalent metabolic diseases, as outlined above, provides a rational basis for the development of strategies aimed at primary correction of FA overload. In terms of pharmacotherapies, this approach is not new. Nicotinic acid (niacin), which suppresses FFA mobilization from adipose tissue[21], has been used as a lipid-lowering agent since the 1950s[22]. An alternative means of modulating FA availability has emerged more recently with the discovery and elucidation of ligand-activated nuclear transcription factors, the PPARs, which modulate the expression of genes involved in the transport and metabolism of FA. Several recent reviews document the molecular and cellular actions of PPARs (e.g.[23,24]). In brief, PPARs seem to be part of a physiological control system for damping cellular FA levels against fluctuations in FA supply. The natural ligands of PPARs include FA and some of their metabolic products. Physiologically, a high FA supply, through intracellular accumulation of FA or their products, leads to activation of the PPARs. There are three PPAR isotypes: α, β and γ. The PPAR isotype and transcriptional response to activation vary in a tissue-specific manner. Of major relevance to the present work is PPARγ, which in adults is expressed predominantly in adipose tissue. PPARγ, like the other isoforms, is activated by a relatively diverse set of compounds, and it turns out that there are a number of artificial ligands that have a very high potency relative to the natural ligands. Of these, the thiazolidinediones, which are orally available, were the first introduced and are currently the best characterized. Activation of PPARγ induces adipocyte differentiation from adipose precursor cells, increases the expression of adipocyte molecules involved in the uptake and storage of circulating FA, and increases the sensitivity of adipose tissue to the actions of insulin.

EFFECTS OF PPARγ AGONISTS ON *IN VIVO* FA AND TG METABOLISM

Background

Thiazolidinediones were developed and are currently applied clinically for improving blood glucose control in type 2 diabetes. Perhaps for this reason, *in vivo* metabolic research has focused on documenting the effects of these agents on glucose metabolism and its regulation by insulin. Several studies in animals have described *in vivo* effects of PPARγ agonists at the level of gene expression, but little information was available about the functional consequences at the level of FA fluxes and metabolism. This information gap, together with the potential importance of dysregulation of FA metabolism, as outlined above, motivated us to perform detailed *in vivo* studies of the effects of PPARγ agonists on FA metabolism[25].

Animal model used for the studies

The studies described below were conducted in fa/fa Zucker rats. These animals are hyperphagic due to a defect in satiety signalling[26], and progressively develop severe obesity, insulin resistance and dyslipidaemia compared with age-matched lean (FA/?) Zucker rats. Eventually, the obese animals also develop pancreatic failure and severe diabetes. The present studies were performed in animals aged about 12 weeks; at this time, the obese individuals have about four times more fat tissue, and in the fasting state plasma insulin levels that are about 19 times higher and plasma TG levels that are about six times higher than in the lean animals. Gross pancreatic failure at this age is not apparent in the obese animals, as indicated by a relatively mild degree of fasting hyperglycaemia (and the very high insulin levels).

Thiazolidinedione treatment

The results discussed below were based on the effects of two thiazolidinediones, rosiglitazone (Avandia®, 10 μmol/kg/day) and darglitazone (1.3 μmol/kg/day), given to obese Zucker rats for 3 weeks. Both compounds are highly potent and selective PPARγ (versus PPARα) agonists. Rosiglitazone was chosen because it is currently the best characterized of the PPARγ agonists. It is now used clinically to improve glycaemic control in type 2 diabetes patients. Darglitazone

was included to confirm that the observed treatment effects were indeed genuine thiazolidinedione class effects. For much of the work described, effects of both compounds were assessed. While darglitazone exhibited a higher oral potency than rosiglitazone, no qualitative differences were observed between these compounds for any of the many parameters assessed. For this reason, the results are generally discussed in terms of thiazolidinedione effects. At the end of the 3-week treatment period, the thiazolidinediones had virtually normalized fasting glucose and insulin levels, and totally abolished the hypertriglyceridemia in the obese rats.

Methods for assessing FA loading at the whole-body and individual tissue levels

In these experiments, a FFA tracer (^{3}H-palmitate) was used to assess comprehensively FFA fluxes and metabolic fate *in vivo*. The data obtained provide information about the magnitude and kinetic mechanisms of FA loading at both the whole-body and individual tissue levels. At the whole-body level, plasma FFA concentration represents an ongoing balance between the rate of entry of FFA into plasma and the efficiency with which the tissues of the body extract FFA from the plasma. In kinetic terminology, rate of appearance (R_a) defines the former process and metabolic clearance rate (K_p) indexes the latter process. An increased plasma FFA could be a consequence of either increased R_a (due to increased release of FA from adipose tissue), decreased K_p, or a combination of these processes. FFA oxidation is the only means of irreversible loss of FFA from the body and, as discussed above, defective FFA oxidation may be an important determinant of FA overload. Whole-body FA oxidation rate (R_{ox}) was calculated based on the production of ^3H_2O from ^{3}H-palmitate. In addition to providing data at the whole-body level, the tracer protocol for these studies was designed to yield information about non-oxidative FFA disposal into individual tissues. Quantification of this pathway was especially interesting because of the consequences of overaccumulation of esterified FA and their metabolic products in non-adipose tissues, including the heart and liver, as described above. The final point to be made about the methodology concerns the conditions under which the animals were studied. To examine the effect of a physiologically relevant range of insulin levels, studies were conducted in both the basal 7-h fasting state and under conditions of a euglycaemic-hyperinsulinaemic clamp, at plateau insulin levels corresponding to those observed in freely fed postprandial animals.

Methods for assessing plasma TG kinetics

In order to reveal the kinetic mechanisms of plasma TG lowering by thiazolidinediones, we employed a method based on the use of Triton WR 1339. This is a non-ionic detergent that blocks the clearance of plasma TG[27]. When applied in the postabsorptive state, TG continues to be constantly secreted by the liver for several hours, as indicated by a remarkable, highly linear accumulation of TG in plasma. The rate of hepatic TG production (HTGO) was calculated from the rate of TG accumulation. Plasma TG clearance (K_{TG}), the other kinetic parameter that governs TG levels, is an index of the combined ability of the tissues to remove TG from the circulation. K_{TG} was calculated from basal (pre-Triton WR 1339) plasma TG and HTGO as the ratio $HTGO/C_{TG}$.

PPARγ agonist effects on systemic FFA load

In initial experiments, we were puzzled by the finding of variable FFA levels in short-term-fasted, thiazolidinedione-treated animals. This prompted us to scrutinize thoroughly sample handling and analytical procedures involved in the analysis of this notoriously difficult-to-measure variable. We had expected that treatment would generally limit systemic FA mobilization, but it turned out that the treatment effects were very dependent upon the prevailing insulin levels. At high physiological levels of insulin, plasma levels of FFA were indeed lowered dramatically by thiazolidinedione treatment due to enhanced suppression of systemic FFA mobilization (and increased plasma clearance of FFA). Thus, under euglycaemic clamp conditions, hyperinsulinaemia (approximating levels in postprandial freely fed animals) induced only a partial suppression of FFA and basal R_a (of 44%) in the insulin-resistant, untreated animals. By contrast, in the treated animals, a much lower absolute level of hyperinsulinaemia induced an almost total suppression of R_a (by 92%) to a much lower level than in the untreated animals. Since the treated animals received only half the exogenous insulin infusion, these results demonstrated a remarkable enhancement of insulin sensitivity in the adipose tissue of these animals. A big surprise came when we looked at the situation at relatively low insulin levels achieved in animals following a fast of moderate duration (7 h). In this situation, R_a in the treated animals was not decreased but actually greatly elevated. We first observed this treatment effect in anaesthetized, acutely catheterized rats and assumed that it might have been some sort of artefact. Propranalol administration was unable to abolish this elevation in R_a, excluding a possible major role of elevated β-adrenergic activation in the treated animals (unpublished observations). Tracer studies were then repeated in conscious, chronically catheterized animals. The results from these studies confirmed the original findings that in the basal state, R_a was substantially elevated in the thiazolidinedione-treated animals. On its own, an elevation in R_a should produce an increase in plasma FFA concentration. But despite all the extra FFA flooding in, the

plasma FFA level in treated animals was similar to the level in the untreated animals. This was due to a substantial treatment-induced increase in K_p, largely a result of a greatly enhanced ability of adipose tissue to take up and store FFA, as discussed below.

Cellular remodelling of adipose tissue

Most of the FFA entering plasma in the postabsorbative phase is released from adipose tissue, and it is likely that metabolic heterogeneity of adipocytes played an important role in determining the systemic FFA mobilization rates described above. Very large insulin-resistant cells populate the adipose tissue of untreated obese Zucker rats (Figure 1). The lack of suppressibility of R_a in these animals even at very high insulin levels implies that these large cells continuously leak FA. Following the commencement of treatment, our data suggest not only that this process continues but also that it is actually accelerated, perhaps due to the anti-hyperinsulinaemic effect of treatment. This would explain the very high R_a observed in the obese treated animals in the basal fasting condition. While the original large cells leak FFA, the newly differentiated adipocytes (induced by PPARγ activation) probably have an increased ability to soak up FFA (accounting for the substantial overall enhancement in adipose tissue FFA uptake described below). A dynamic situation results, with the original large cells shrinking and the newly differentiated cells expanding. To enable this exchange, a net FFA flux of sufficient magnitude must exist from the large original adipocytes to plasma, and then to the small, newly differentiated adipocytes. Indeed, the potential for this does exist, as revealed by a quantitative analysis of FFA fluxes, showing that in the treated obese animals, at least 40% of FFA released from adipose tissue is subsequently taken up and stored in adipose tissue. As the original large cells shrink, their insulin sensitivity increases, perhaps as a consequence of lipid unloading. Shrinkage probably proceeds until the new low levels of insulin in the treated animals are sufficient to shut off their FFA leak. This situation was apparently being approached in our studies (following 3 weeks of treatment), as postprandial insulin levels were able to suppress R_a very effectively in the treated animals. Following this length of treatment, the adipose tissue has undergone substantial remodelling (*see* Figure 1). The original, large adipocytes have effectively disappeared, presumably due to their shrinkage, and bunches of small adipocytes have appeared—probably the newly differentiated cells that have filled rapidly with TG.

PPARγ agonists alter the tissue distribution of FFA

PPARγ agonist treatment greatly enhanced (more than doubled) the ability of adipose tissue to take up and store FFA. This effect, allied with the large adipose tissue mass in the obese animals, was responsible for the increase in whole-body FFA clearance. By combining flux and body composition measurements, we were able to calculate the contribution of adipose tissue FFA uptake and storage to whole-body FFA disposal. Thiazolidinedione treatment of obese rats increased this fraction from approximately 25% to 40% (irrespective of insulin level). So, treatment had a major quantitative impact on the tissue distribution of FFA, trafficking it into adipose tissue and away from non-oxidative disposal in other tissues, including skeletal muscles and liver. This secondary effect in non-adipose tissues is very significant because, as we summarized above, accumulation of FA and their products in non-adipose tissues can have serious consequences. What about the molecular mechanisms responsible for the enhanced capacity of adipose tissue to take up FFA? There have been several reports that PPARγ agonists increase the gene expression of a number of molecules involved in the transport and metabolism of fatty acids (e.g.[28,29]). Our studies provided evidence that these changes in gene expression actually translated into functional changes in *in vivo* FFA metabolism.

PPARγ agonists ameliorate metabolic inflexibility

One important action emerged out of our studies that could not be explained in terms of direct effects in

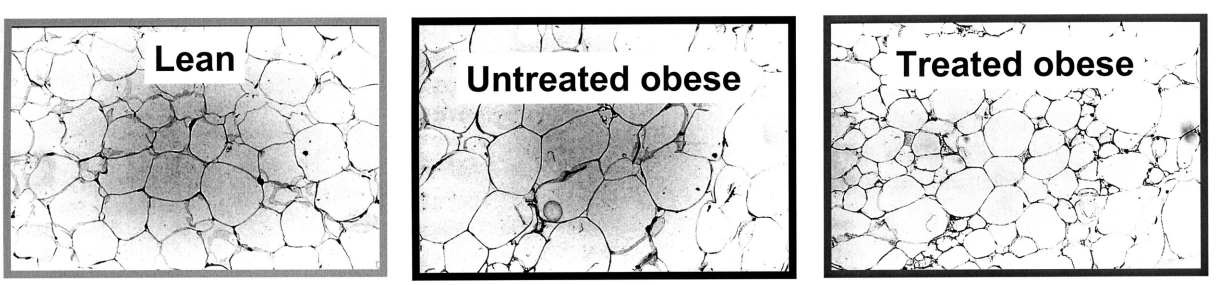

Figure 1 **Epididymal white adipose tissue in untreated lean, untreated obese, and obese Zucker rats treated with darglitazone (1.3 μmol/kg/day) for 3 weeks (Actual field width=1.2 mm)**

adipose tissue. Thiazolidinedione treatment did augment metabolic flexibility in the obese animals. As described above, metabolic flexibility implies relatively high rates of lipid oxidation in the basal fasting state and an efficient ability to suppress lipid oxidation under insulin-stimulated conditions. When metabolically inflexible obese Zucker rats were treated with thiazolidinediones, basal FFA oxidation was increased substantially (by approximately 50%), while insulin-mediated suppression of FFA oxidation was greatly enhanced. Higher plasma FFA levels did not drive the higher rates of basal FFA oxidation. As mentioned already, basal FFA levels were similar in the treated and untreated animals. Increased skeletal-muscle FFA oxidation was most likely responsible for the elevated whole-body FFA oxidation in the basal state. First, skeletal muscle is a quantitatively important site of oxidative FA metabolism in the fasting state. Second, there is evidence, based upon *in vitro* studies, that thiazolidinediones can substantially enhance muscle FA oxidation[30,31]. In untreated obese Zucker rats, the ability of a physiological elevation of insulin to stimulate glucose utilization and suppress FFA utilization is limited. Thiazolidinedione treatment reverses this metabolic rigidity, enhancing insulin-mediated stimulation of glucose metabolism and the suppression of FFA oxidation. Two mechanisms were involved in this latter effect: greater suppression of systemic FFA mobilization and a decrease in the fraction of FFA undergoing oxidative metabolism. Our data indicated that the tissue locus of the second of these mechanisms was in skeletal muscle, as evidenced by a corresponding increase in muscle non-oxidative FFA disposal.

PPARγ agonists reverse FA overload in non-adipose tissues

Untreated obese Zucker rats exhibit a widespread excessive accumulation of FA products, including TG, among non-adipose tissues, notably skeletal muscles, liver, pancreas and the heart[32]. As described above, two thiazolidinedione effects on adipose tissue decreased FFA supply to non-adipose tissues: increased insulin suppressibility of FFA mobilization and enhanced trafficking of FFA into adipose tissue. In addition, darglitazone also enhanced basal FFA oxidation. The combination of decreased FFA supply and increased oxidation resulted in substantial reversal of the non-adipose tissue FA overload, i.e. PPARγ activation by darglitazone reduced TG content in liver and heart by 72% and 69%, respectively.

Effects of PPARγ agonists on TG kinetics

Hypertriglyceridemia in the untreated obese Zucker rat is attributable largely to a greatly increased HTGO, but it is also due to impaired K_{TG}. The result of these disturbances is a tremendous elevation of very-low-density lipoprotein (VLDL) in the plasma, resulting in the elevation of total plasma TG. Thiazolidinedione treatment completely eliminated the extreme hypertriglyceridaemia in the obese animals. A substantial component of this TG lowering was effected by a reduction (of about 50%) in HTGO. The major mechanism of the anti-hypertriglyceridaemic action was, however, enhanced K_{TG}, reflecting an accelerated conversion of VLDL to TG-poor lipoprotein remnants, probably by enhanced lipoprotein lipase activity in adipose tissue. In this context, it should be noted that the total plasma content of TG is a minute fraction of the whole-body TG pool, and thus accelerated removal of TG from plasma to adipose tissue does not contribute to obesity *per se*.

CONCLUSIONS

There is now a mass of evidence that dysregulation of FFA metabolism plays a central role in several major diseases afflicting the industrialized world, including impaired glucose control in type 2 diabetes. Design of therapies aimed at primary correction of FFA metabolism is therefore worthy of serious effort. Our own studies demonstrate that PPARγ agonists have a remarkable capacity to correct the extreme dysregulation of FFA and TG metabolism in the obese Zucker rat, as studied across a physiological range of insulin levels corresponding to the fed and fasted states. If translatable to the clinical setting, then these results suggest that the principle of PPARγ agonism could be applied very usefully for indications far beyond blood glucose control, including atherosclerosis, metabolic cardiomyopathies and NAFLD. Future studies are required to elucidate the effects of selective activators of the other PPAR isoforms (α and δ). In particular, while the actions of agents presumed to be PPARα selective, i.e. the amelioration of the atherogenic lipoprotein profile of insulin resistance and reduction of cardiovascular disease, are established[33–36], little is known about their influence on *in vivo* fatty acid fluxes and metabolic fate. On the relevance of the obese Zucker model to humans, many of the general thiazolidinedione effects reported in Zucker rats are qualitatively similar to effects that have been reported in insulin-resistant patients with type 2 diabetes.

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