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Thiazolidinediones: metabolic actions in vitro

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Abstract

To unravel the molecular mechanisms and the causal chain of how thiazolidinediones (TZDs) affect glucose homeostasis, it is helpful to analyse their direct influence on isolated specimens of fat, muscle, and liver in vitro. Studies on isolated adipocytes have shown that the nuclear peroxisome proliferator-activated receptor-γ (PPARγ) is an important molecular target for TZDs, through which they trigger adipocyte differentiation and adipose tissue remodelling. It is not clear, however, if the activation of PPARγ in adipose tissue is the cause of all the metabolic actions of TZDs. Based on in vitro studies, two hypotheses have been developed. The first emphasizes PPARγ-mediated actions on adipose tissue, suggesting that insulin sensitization of skeletal muscle and liver is triggered indirectly by changes in circulating concentrations of adipocyte-derived non-esterified fatty acids and peptide hormones. The second states that TZDs improve glucose homeostasis independently from adipose tissue actions by the direct interaction with muscle and liver. This hypothesis is supported by direct TZD actions on fuel metabolism of skeletal muscle and liver in vitro, which seem to be independent from PPARγ signalling. Major progress has been made in understanding the mechanisms involved in the effects of TZDs on adipose tissue but the causal chain responsible for their antihyperglycaemic action is still not clear. The involvement of other molecular targets in addition to PPARγ, of adipocyte-derived messengers, and of direct interaction with skeletal muscle and liver have yet to be clarified. [Diabetologia (2002) 45:1211–1223]

Keywords Thiazolidinedione, peroxisome proliferator-activated receptor-γ, in vitro, adipose tissue, skeletal muscle, liver.

Thiazolidinediones (TZDs) are increasingly important in the treatment of Type II (non-insulin-dependent) diabetes mellitus. Troglitazone was the first TZD approved for treating Type II diabetes but it was withdrawn from the market because of its detrimental hepatic side effects [1, 2]. Other TZDs, rosiglitazone and pioglitazone, are now available and are not toxic to the liver [3, 4]. At present, additional compounds of this class are under preclinical evaluation.

The structural characteristic common to all TZDs is a thiazolidinedione ring, to which divergent molecular moieties are attached (Fig. 1). Because different TZDs as well as compounds with structures resembling the thiazolidinedione ring (e.g., isoxazolidinediones, oxadiazolidinediones; Fig. 1) carry comparable antidiabetic potentials, the thiazolidinedione ring is assumed to relate to antihyperglycaemic TZD action, whereas the substituted moieties seem to modulate pharmacokinetic and pharmacodynamic properties.

The glucose lowering action of TZDs depends on the presence of insulin. Insulin-deficient rats treated

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Abbreviations: C/EBP, CCAAT/enhancer binding protein; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; RXR, retinoid X receptor; SPPARM, selective peroxisome proliferator-activated receptor modulator; TZD, thiazolidinedione

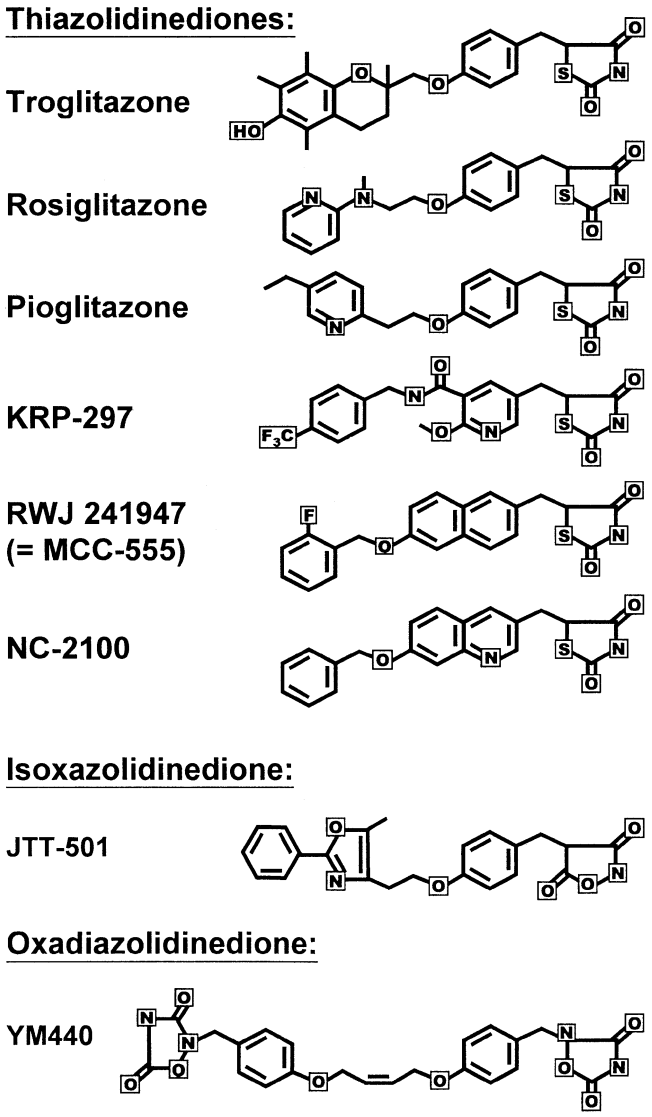

Fig. 1. Structures of TZDs and related compounds. All depicted compounds carry similar antidiabetic potentials. TZDs share a thiazolidinedione ring structure, while JTT-501 and YM440 structurally have very similar isoxazolidinedione and oxadiazolidinedione rings, respectively (right parts of the molecules). Major similarities in structure suggest that a common molecular mechanism underlies their antidiabetic action

with TZDs have persistent hyperglycaemia as long as insulin is absent, but respond better to exogenous insulin administration [5, 6]. This implies that TZDs amplify the action of insulin on target tissues and, therefore, they have been categorized as insulin sensitizers.

Euglycaemic-hyperinsulinaemic clamp tests on Type II diabetic patients and insulin resistant rodents showed that oral TZD treatment improves insulin-stimulated glucose disappearance [7, 8, 9, 10, 11, 12, 13, 14, 15], which is reflected by increased glucose transport into insulin-stimulated skeletal muscle [16, 17, 18] and fat [5, 6, 8, 19] ex vivo. In parallel to TZD-induced peripheral insulin sensitization, an improved ability of insulin to suppress glucose appearance, which is basically a function of the liver, was observed in most [7, 8, 9, 10, 11] but not all [12, 13, 15] clamp studies. Hence, TZD treatment amplifies the effects of insulin on the most important tissues of carbohydrate metabolism including muscle, liver, and fat. Skeletal muscle, being the primary target tissue for insulin-stimulated glucose disposal [20, 21], plays the quantitatively predominant role in TZD-dependent glucose lowering [7, 12, 13, 15, 17, 22].

Although metabolic changes caused by prolonged TZD treatment have been thoroughly described in vivo and ex vivo, clinical TZD therapy still relies on limited knowledge as to the early cascade of molecular and metabolic events that are the basis for antihyperglycaemic TZD action. A major difficulty in understanding the mechanisms of TZD action is to separate events that are causal for antidiabetic action from other humoral and metabolic phenomena that arise secondarily to glucose lowering or are unrelated to antidiabetic action. In this context, studies on the direct interaction of TZDs with isolated cells and tissues in vitro are important, because such an approach allows distinguishing the direct interaction of TZDs with a target tissue from events related to TZD-induced changes in ambient hormones and metabolites. This review summarizes available knowledge regarding metabolic TZD action on fat, muscle, and liver in vitro, and discusses to which extent in vitro studies have provided a basis for understanding the mechanisms of antidiabetic action in vivo.

Adipose tissue

Direct TZD action on isolated fat. The observation that in vitro, TZD compounds are specific and potent agonists of peroxisome proliferator-activated receptor-γ (PPARγ) [23, 24] was a breakthrough in the search of molecular targets. PPARγ is a member of the nuclear hormone receptor family, it is abundantly expressed in adipose tissue [25, 26, 27], and forms heterodimers with retinoid X receptor-α (RXRα) [28, 29]. Like other nuclear receptors, PPARγ is considered to act exclusively by modulating gene expression. Any binding of an agonistic ligand thus triggers structural reconformation of the PPARγ-RXRα heterodimer with the release of corepressors and the recruitment of coactivators [30, 31]. The activated receptor-cofactor complex modulates transcription patterns due to interacting with peroxisome proliferator response elements (PPREs) located in the promotor regions of many genes, most of which are important for lipid metabolism [32].

The major physiologic role of PPARγ is its involvement in adipocyte differentiation. PPARγ is increasingly expressed during the early phase of the development of preadipocytes into mature fat cells and acts together with CCAAT/enhancer binding proteins

(C/EBPs) to stimulate full differentiation [33, 34]. In vitro, the adipogenic activity of PPARγ is shown, for example, by the increased expression of PPARγ which stimulates cultured fibroblasts to develop into mature fat cells [35], and by the ectopic expression of PPARγ which together with C/EBPα can trigger myoblasts with no inherent adipogenic potential to transdifferentiate into adipocytes [36].

PPARγ-agonistic potencies and efficacies of TZDs are routinely determined by means of reporter assays, which quantify in vitro the transcriptional rates of genes provided with PPREs (e.g. [37, 38, 39]). As functional PPARγ agonists, TZDs strongly induce the differentiation of many cell types into mature adipocytes in vitro [23, 40, 41, 42, 43, 44, 45, 46]. Of note, during the TZD-induced transition of a stem cell into a mature adipocyte, the cell increasingly acquires the ability to respond to insulin and progressively expresses genes important for glucose metabolism, like glucose transporters [40, 45, 47] or uncoupling proteins [48, 49, 50]. Whereas the induction of a set of genes required for insulin responsiveness and carbohydrate handling seem to be part of the normal adipogenic program, other attributes seem specific for fat cell differentiation under TZD exposure. Thus, TZDs favour the development of brown rather than white fat cells [48, 49], and specifically address preadipocytes from subcutaneous fat with little influence on preadipocytes obtained from an omental depot [41].

The role of PPARγ in adipocyte differentiation as well as the potential of TZDs to trigger differentiation of adipocytes via PPARγ has been established and summarized in a number of other reviews (e.g. [31, 32, 51, 52, 53, 54]). It is less clear, however, in what way and to what extent PPARγ-activation and direct interaction with fat cells are the initial events that ultimately lead to antidiabetic TZD action in vivo.

Adipogenic TZD action in vivo. An essential role of PPARγ for the development of adipocytes not only in vitro, but also under physiologic conditions in vivo, is implicated by PPARγ knock out mice in which white and brown adipose tissue can develop only from stem cells with at least one intact allele for PPARγ [55, 56]. Furthermore, adipogenic action of oral TZD treatment is indicated by increases in adiposity and body weight which accompany the therapeutic effects on glucose homeostasis [57, 58, 59]. Closer inspection shows, however, that TZDs do not simply induce growth of fat mass but trigger adipose tissue remodelling in a sophisticated manner. This is in line with the observation that TZD stimulation in vitro leads to the development of adipocytes with specific attributes. In rodents, TZDs stimulate the development of small adipocytes and the apoptosis of large adipocytes, which results in adipose tissue predominantly composed of small cells [57, 60, 61]. Furthermore, considerable growth of subcutaneous adipose mass without a change, or even with a decrease, in visceral fat is seen in humans treated with troglitazone [62, 63, 64] which obviously reflects the superior effect of TZDs on subcutaneous versus omental adipocytes as shown in vitro [41].

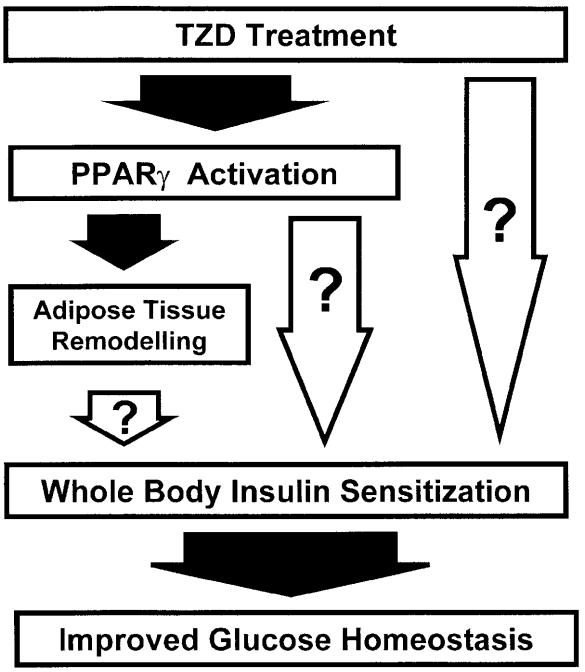

Fig. 2. Possible modes of antidiabetic TZD action. It is undisputed that TZDs trigger adipose tissue remodelling via PPARγ and that insulin sensitization is essential for antihyperglycaemic TZD action (*full arrows*). Several modes of action are under discussion for the causal chain of events leading to insulin sensitization (*open arrows*). Whether PPARγ activation and/or direct effects on adipose tissue are the sole cause of improvement of glucose homeostasis is not yet clear

Hence, evidence suggests that direct and PPARγ-dependent effects on adipocytes documented in vitro are responsible for adipose tissue remodelling in vivo. How far the insulin sensitizing and antidiabetic activities of TZDs are secondary to stimulation of PPARγ and/or to adipose tissue remodelling, however, has yet to be established. Three modes of antidiabetic TZD action seem possible (Fig. 2): (i) whole body insulin sensitization is secondary to PPARγ-mediated adipogenesis and adipose tissue remodelling; (ii) both adipogenesis and insulin sensitization are induced via PPARγ, but different pathways are involved distal to receptor activation; and (iii) insulin sensitization, in contrast to adipogenesis, is mediated via a mechanism that does not involve PPARγ. Although efforts to pinpoint to what extent each of these mechanisms is responsible for antidiabetic TZD action have not brought forth a final answer, it is worth while to critically analyse this question on the basis of presently available knowledge.

PPARγ and antidiabetic TZD action. Studies using PPARγ-deficient mice could be very helpful in deter-

mining, whether PPARγ activation as documented in vitro is essential for antidiabetic TZD action in vivo but unfortunately such animals are hardly viable [55, 65]. Although changes in insulin action characterizing heterozygous knock-out mice (*PPARγ*$^{+/-}$) [65] and humans with mutations or polymorphisms in the PPARγ gene [66, 67] suggest PPARγ-dependent regulation of glucose homeostasis in vivo, there is disagreement as to whether available data supports or challenges the hypothesis that increased PPARγ activity relates to higher insulin sensitivity [22, 68].

The most important observation supporting a causal interdependence between TZD-induced PPARγ activation and the improvement of glucose homeostasis is the association of PPARγ agonistic activity in vitro with antidiabetic action in vivo, which characterizes numerous TZDs [16, 37, 69, 70, 71, 72], and also non-TZD PPARγ agonists belonging to the classes of isoxazolidinediones [73, 74], tyrosine derivatives [75, 76, 77], and phenylacetic or phenoxyacetic acid derivatives [37]. Although the maximal antihyperglycaemic potentials of many TZDs and other PPARγ agonists are similar, oral dose requirements for half-maximal glucose lowering in diabetic mice differ considerably and correlate strongly with PPARγ binding activities and transactivating potentials of the respective compound in vitro [37, 69, 71]. Furthermore, antidiabetic action has also been ascribed to agonistic ligands of RXRα, the heterodimeric partner of PPARγ [78, 79]. Against this background it is tempting to relate the glucoregulatory effects of both PPARγ and RXRα agonists to the stimulation of the PPARγ-RXRα heterodimer and thus to the same molecular pathway.

All these reports on qualitative and quantitative correlations of receptor activation with improvement of glucose homeostasis led to a broad acceptance of the hypothesis that TZD-induced insulin sensitization and amelioration of hyperglycaemia are secondary to PPARγ activation. Other evidence suggests, however, that the mechanisms underlying metabolic TZD action could be more complex. The new TZDs RWJ241947 (MCC-555), NC-2100, and PAT5A, and also the antidiabetic oxadiazolidinedione YM440 (molecular structures in Fig. 1), carry antihyperglycaemic and insulin sensitizing potentials at least equal to those of established TZDs [38, 39, 80, 81, 82], although they have markedly lower efficacies for PPARγ affinity, PPARγ transactivation, and adipocyte differentiation in vitro [38, 39, 80, 81]. In addition, no increases in fat and body weight which characterize most TZDs [57, 58, 59] are found in response to treatment with NC-2100 or YM440 [38, 81], which suggests little adipogenic action in vitro and in vivo. Dissociation of adipogenic and metabolic TZD action has also been reported for isolated adipocytes transfected with a PPARγ dominant-negative mutant, in which TZDs and other agonists maintained their ability to stimulate glucose transport, albeit their adipogenic potential was severely impaired [83].

The failure of several TZDs to show PPARγ activation and adipogenic action proportionate to their glucose-lowering potentials implicates that increased PPARγ activity alone is not sufficient to explain both adipogenic and antidiabetic properties of all TZDs. Apart from the option that metabolic TZD action could in part rely on molecular targets other than PPARγ, an alternative concept referred to as the selective PPAR modulator (SPPARM) model can explain different actions via the same receptor. Basically, the SPPARM model holds that the repertoire of target genes addressed via PPARγ depends on the individual ligand, a mode of action that is established for other nuclear receptors [84]. In vitro, structural reconfirmation and cofactor recruitment by PPARγ differ between TZD versus non-TZD ligands and also between individual TZD compounds [85, 86] suggesting that PPARγ is indeed capable of ligand-specific signalling. Adding to the complexity, PPREs located in the promotor regions of different genes are not entirely identical and availability of cofactors could be based on tissue-specific expression, which in turn could vary depending on the physiologic or pathophysiologic state (e.g. fasted vs fed, lean vs obese, healthy vs diabetic). Hence, the structure of receptor-ligand-cofactor-PPRE-complexes that arise to modulate gene transcription can depend on a number of factors including the specific PPARγ ligand, the tissue site, and the metabolic state.

Challenging traditional views about the agonistic or antagonistic character of a ligand, the SPPARM model could explain, how each individual TZD addresses a specific set of genes, elicits a unique pattern of gene transcription, and triggers a unique biologic response without requiring another primary target in addition to PPARγ. This implies that a partial or context-dependent agonism of PPARγ could be sufficient for antidiabetic action as long as the ligand affects a specific and yet unidentified gene (or set of genes) responsible for insulin sensitization.

With respect to the hypothesis of PPARγ being the only relevant target of TZDs, the consequences of such hypothetical ligand-specific signalling are manifold. The SPPARM model invalidates the argument that an antidiabetic compound lacking major PPARγ activity in a reporter assay must act via an other molecular target. On the other hand, the model challenges the basic assumption that the potential of a given compound to trigger insulin sensitization and glucose lowering in vivo can be estimated from its PPARγ-agonistic activity in vitro. The latter point questions the usefulness of PPARγ reporter assays in search for promising compounds and also weakens the argument that PPARγ agonism in vitro correlates with antidiabetic action in vivo [37, 69, 71]. Hence, the extent to which metabolic action of TZDs is caused by PPARγ signalling is difficult to assess and further research will be necessary to differentiate the contribution of

this receptor and other targets to the antidiabetic action of TZD compounds.

Adipose tissue and antidiabetic TZD action. The causal relevance of PPARγ-mediated effects on adipose tissue for antidiabetic TZD action can not be evaluated only by weighing the evidence for and against PPARγ-dependency of glucose lowering but also by analysing if and in what way adipose tissue is essential. In this context, TZD-induced amelioration of hyperglycaemia in patients and mice with lipodystrophy (i.e. with a distinct shortage in adipose tissue mass) has been argued to indicate a mode of action that does not require direct interaction with fat [87, 88]. Opposing results from another lipodystrophic mouse model [89], however, give alternative interpretations such that TZD action could depend on an interaction with residual fat or that fatty livers, as characteristic of such syndromes, could functionally replace adipose mass. Thus so far, studies on TZD action in lipodystrophy failed to clarify whether adipose tissue is the primary site of the antidiabetic effects of TZDs [90].

Of note, the hypothesis stating that TZDs act exclusively via fat contrasts somewhat with the minor contribution that adipose tissue makes to whole body glucose metabolism [20, 21]. Actually, the predominant role in TZD-dependent glucose lowering is unanimously attributed to increased insulin-stimulated glucose disposal into skeletal muscle [7, 12, 13, 15, 17, 22] with many, but not all, reports suggesting a contribution of reduced hepatic glucose output ([7, 8, 9, 10, 11, 15] vs [12, 13, 14]). To interpret antidiabetic TZD action as mediated by adipose tissue, it is therefore necessary to hypothesize a TZD-induced metabolic or humoral signal, which is released from fat and acts on skeletal muscle and probably on the liver (Fig. 3). Identification of this hypothetical TZD-induced messenger would be crucial to strengthen the contention of fat-dependent TZD action.

Prerequisites essential for such a putative messenger are (i) that its output from fat changes under TZD treatment and (ii) that it modulates glucose metabolism of skeletal muscle and perhaps the liver. At present, non-esterified fatty acids (NEFA) are regarded as the main candidate, because TZD treatment reduces plasma NEFA [5, 11, 71, 72, 82, 91, 92] and because NEFA are established modulators of insulin-stimulated glucose transport and glycogen synthesis in skeletal muscle in vitro and in vivo [93, 94, 95, 96]. The causal sequence would be that adipogenic TZD action on fat supports triglyceride storage and, hence, the withdrawal of NEFA from the circulation which improves muscle insulin sensitivity and glucose homeostasis [97]. However, the simple association of decreased plasma NEFA with antidiabetic TZD action is not sufficient to confirm such hypothesized sequence of events. Actually, no evidence has yet been provided

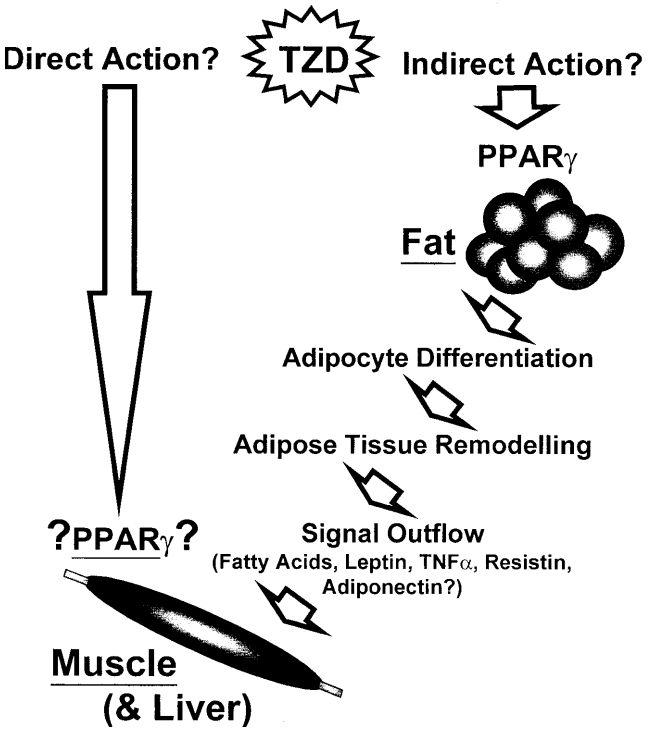

Fig. 3. Mode of TZD action on muscle and liver: two hypotheses. Amelioration of insulin resistance of skeletal muscle (and probably also of the liver) is an essential step in the cascade of events leading to antihyperglycaemic TZD action. At present, it is not known, to what extent TZDs address muscle and liver via a PPARγ-induced messenger from the adipocyte and to what extent they act directly via PPARγ and/or other molecular targets

to confirm that the TZD-induced reduction in plasma NEFA precedes muscle insulin sensitization rather than being a secondary phenomenon.

Beside being a source of NEFA, adipose tissue is increasingly recognized as an endocrine organ that produces glucoregulatory hormones like leptin, tumour necrosis factor-α (TNF-α), resistin, and adiponectin. Since TZDs modulate their adipose expression, these secretory peptides have also been referred to as candidates for TZD-induced fat-to-muscle signalling (Fig. 3) [58, 60, 98, 99, 100, 101, 102, 103]. Hard evidence, however, for a crucial role of fat-derived peptides is scarce. Leptin can hardly be held responsible for antidiabetic TZD action, because the vast majority of animal experiments documenting antihyperglycaemic effects have used *ob/ob* mice, *db/db* mice, or *fa/fa* rats, which are incapable of leptin signalling because of mutations in the genes encoding either for leptin or the leptin receptor [104, 105]. The ability of resistin to act on skeletal muscle and liver has not been evaluated yet, whereas TNF-α modulates insulin action in a cultured muscle cell line [106] but fails to influence glucose metabolism of native skeletal muscle specimens in vitro [107, 108]. The most plausible peptide candidate seems to be adiponectin, which is increasingly produced under TZD treatment

[98, 99], exhibits glucose-lowering activity [98, 109, 110], and modulates fuel metabolism of hepatocytes as well as of native and cultured muscle in vitro [109, 110]. Nevertheless, it remains to be shown that adponectin is essential for the metabolic action of TZDs.

Conclusion, adipose tissue. TZDs thus have a major direct impact on isolated adipocytes in vitro, which is attributable to PPARγ-mediated modulation of gene transcription leading to cellular differentiation. The mechanisms characterized in vitro are obviously responsible for structural and functional changes in adipose tissue during oral TZD administration in vivo. It is not clear, however, to what extent these direct effects on fat also cause TZD-induced improvement of glucose homeostasis, which is mainly a function of skeletal muscle. To understand whether TZD actions on muscle and liver are secondary to signal output from adipose tissue, it needs to be clarified, if changes in circulating NEFA are the cause or the result of insulin sensitizing TZD action and if fat-derived peptides have the potential to act as TZD-induced messengers to skeletal muscle and liver.

Skeletal muscle

Direct TZD action on isolated muscle. Although indirect action of TZDs via a fat-derived mediator is a plausible concept, antidiabetic action could, alternatively or additionally, also relate to direct interaction with skeletal muscle (Fig. 3). Direct action on muscle could explain why the antidiabetic efficacy of an individual TZD does not necessarily correlate with its adipogenic and antihyperlipidaemic potentials [38, 39, 89]. Aiming to differentiate direct from indirect effects, the examination of TZD action on isolated skeletal muscle in vitro, and hence in the absence of any signal from other tissues, is an important experimental approach.

In many studies, TZDs were found to stimulate glucose transport into skeletal muscle in vitro [16, 70, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120] but the accompanying changes in intracellular glucose fluxes were rather inconsistent, ranging from stimulation to inhibition of both glucose oxidation and glycogen synthesis [16, 70, 111, 112, 113, 114, 121]. The heterogeneity of effects on intracellular glucose routing is obviously related to the diversity in experimental settings. Studies varied with regard to the structure and concentration of the TZD used, the period of exposure, and the muscle preparations which included perfused rat hindlimb [120], freshly isolated rat soleus muscle [16, 111, 112, 113, 121], pre-cultured human muscle biopsies [114, 115, 116], and permanently cultured muscle cell lines [70, 117, 118, 119]. Comparing different studies is further complicated by the lipophilic character of TZDs that strongly bind to protein [122], which implies a modulation of the bioavailable TZD concentration in vitro by the quantity and quality of protein and detergent added to the incubation or perfusion medium. In spite of such differences in the applied experimental protocols, cautious analysis of available information leads to interesting conclusions.

TZD-induced glucose transport in vitro has been reported to be independent of concomitant insulin stimulation [16, 111, 112, 114, 115, 116, 117, 118, 119] and to occur very rapidly within 30 to 90 min not only in skeletal muscle [16, 111, 112, 113, 115, 120], but also in cardiomyocytes [123] and adipocytes [124]. This led some authors to attribute an acute "insulin-mimetic" potential to TZDs [115, 124] but such interpretation needs to be applied with caution. Whereas insulin is a storage signal directing intracellular glucose flux mainly into glycogen [111, 125], glucose transport into short-term TZD-exposed muscle increases in association with a very marked increase of the glycolytic flux and the depletion of glycogen [16, 111, 112, 113, 120]. Thus insulin-mediated glucose uptake relates to transport and glycogen synthesis, which should not be mistaken for glucose transport without concomitant glycogen storage. The substrate handling of muscle acutely exposed to TZDs in vitro resembles the metabolic response to contractions or to anoxia [125, 126] rather than the metabolic response to insulin.

Although acute stimulation of total glycolysis in isolated muscle is seen over a broad range of TZD concentrations, distinction of aerobic versus anaerobic glucose utilization suggests that different mechanisms are involved at low versus high concentrations. Whereas low TZD concentrations inhibit the conversion of glucose into CO_2 (Fig. 4A) [112, 113], concentrations above those prevailing in the circulation of treated patients trigger very distinct increases in both aerobic and anaerobic fuel catabolism (Fig. 4B) [16, 112]. A catabolic effect on glucose metabolism as induced by high TZD concentrations (Fig. 4B) is likewise triggered by very high concentrations of other compounds [125, 127] and therefore could represent a rather unspecific response to stress. These considerations emphasize how misleading it can be to attribute an insulin-mimetic potential to TZDs without any evidence, apart from the acute stimulation of glucose transport.

A specific mechanism probably underlies the acute effect of low micromolar TZD concentrations on muscle glucose handling, which can be described as a TZD-induced shift of glycolytic flux from the aerobic towards the anaerobic pathway (Fig. 4A) [112, 113]. Under these conditions, CO_2 production from both glucose and NEFA is markedly reduced and is accompanied by a distinct increase in lactate production, whereby increased glucose uptake and glycogen depletion obviously compensate for the lower yield of ATP from anaerobic than aerobic substrate utilization (Fig. 4A) [112, 113]. Such changes in muscle glucose

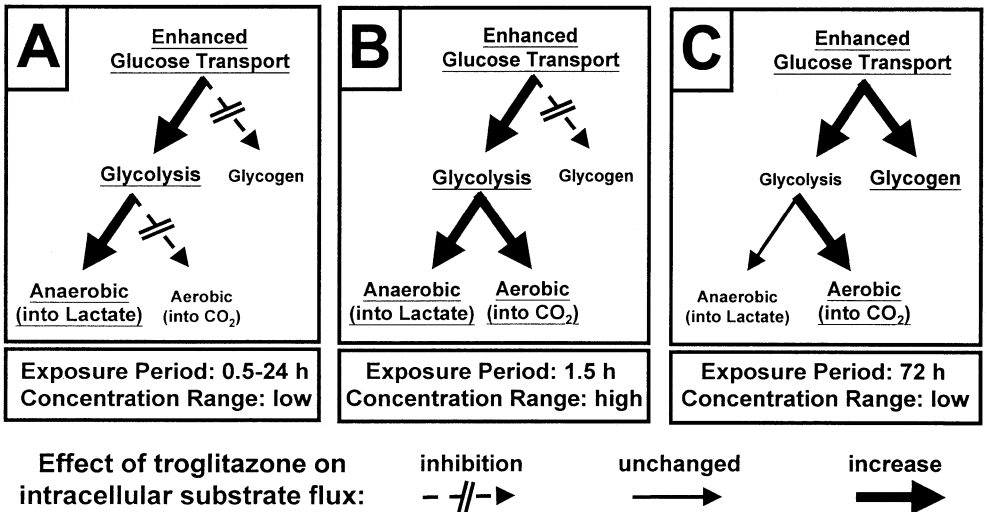

Fig. 4A–C. Troglitazone action on isolated rat soleus muscle. Depending on the concentration and exposure period, troglitazone stimulates glucose transport into the isolated rat soleus muscle in association with divergent effects on intracellular glucose routing. **A** Short-term exposure to troglitazone concentrations in the low micromolar range inhibits glucose oxidation and shifts glycolytic flux into lactate production which, presumably due to higher substrate demands for anaerobic ATP production, is associated with glycogen depletion ("anoxia-like" response); **B** short-term exposure to troglitazone concentrations in the high micromolar range triggers aerobic and anaerobic glycolysis, and glycogen depletion, which might be an unspecific stress response ("contraction-like" response); and **C** long-term exposure to troglitazone concentrations in the low micromolar range increases insulin-stimulated glycogen storage and glucose oxidation ("insulin-like" or "insulin-sensitizing" response). Adapted from references [111, 112, 113, 121]

handling are in line with inhibitory action of TZDs on mitochondrial respiration, a mechanism which is believed to contribute to the action of the antidiabetic agent metformin [128, 129].

Evidence for direct and acute TZD action on the mitochondrium in vivo, however, is scarce. Although a troglitazone-induced increase of insulin-stimulated glucose disposal within 20 min and amelioration of hyperglycaemia within 2.5 h have been reported in rodents [5, 130], studies on humans point to a delayed action on glucose homeostasis. However, such delay in antidiabetic action does not exclude the involvement of an acute mitochondrial mechanism, because short-term stimulation of glycolysis by regular bouts of exercise will, in the long-term, also result in insulin sensitization.

In contrast to glycogen depletion by short-term TZD exposure of freshly isolated muscle specimens, long-term TZD exposure increases glycogen synthase activity and glycogen synthesis in muscle-derived cell cultures [114, 115, 116, 131]. In agreement with these findings from cell cultures, the acute glycogenolytic effect of troglitazone on isolated rat soleus muscle turns into a glycogenic response after a prolonged exposure for 72 h (Fig. 4C) [121]. Under the latter conditions, an insulin-dependent increase in glycogen synthesis is accompanied by an insulin-independent increase in glucose oxidation [121], which is a pattern of changes as in soleus muscle specimens prepared from orally TZD-treated obese rodents [16, 18, 91]. Although parallel responses to prolonged TZD treatment in vitro and in vivo support the idea that direct action on muscle could be important for therapeutic TZD action, any conclusion from long-term incubation of isolated muscle is hampered by the fact that the regulation of glucose metabolism could change considerably in muscle cells devoid of their physiologic environment. Therefore, it is important to be cautious when extrapolating findings from long-term incubated or cultured muscle to the physiologic situation.

Molecular targets of TZDs in skeletal muscle. Whereas the undisputed mediator of a large number of TZD effects on adipocytes is PPARγ, an involvement of this receptor in the direct actions on skeletal muscle is not established. In muscle, mRNA encoding for PPARγ is much lower than in fat [25, 26, 27] but PPARγ protein has been described to be relatively more abundant, reaching 31 to 100% of adipose concentrations [132]. However, not a single response of skeletal muscle could yet be clearly attributed to PPARγ activation and the functional relevance of PPARγ-signalling is still not clear in this tissue.

In contrast to the lack of evidence for PPARγ-signalling there is evidence for PPARγ-independent actions of TZDs on skeletal muscle which are obviously responsible for the acute inhibition of mitochondrial fuel oxidation in isolated muscle specimens [113]. PPARγ-mediated actions depend on rather slow processes, including gene expression and protein synthesis. However, TZDs diminish muscular CO_2 production in vitro within 30 min, which can not be inhibited by blockers of transcription or translation [113].

Conclusion, skeletal muscle. In addition to their influence on isolated adipocytes, TZDs directly and PPARγ-independently affect glucose metabolism of isolated skeletal muscle in vitro, which includes insulin-independent stimulation of glycolysis and, at low concentrations, inhibition of mitochondrial CO_2 production. While major progress has been made in understanding PPARγ-mediated gene expression, adipose tissue remodelling, and signal output from fat, the relevance of the direct TZD action on skeletal muscle for the therapeutic effects of TZD administration is still not clear. To fully understand the mode of antidiabetic TZD action, it will be essential to differentiate the contribution of PPARγ-dependent versus PPARγ-independent mechanisms, and of direct versus indirect actions on skeletal muscle.

Liver

Direct TZD action on isolated liver. Increased glucose disposal into skeletal muscle is very important for TZD-induced glucose lowering but, although not confirmed in all studies [12, 13, 14], a contribution of reduced hepatic glucose output seems very likely [7, 8, 9, 10, 11, 15]. As for muscle, TZD action on the liver might be due to direct effects as well as to indirect actions via fat-derived messengers (Fig. 3).

In perfused rat livers as well as in isolated hepatocytes, TZDs were reported to acutely reduce the rate of glucose release, which was mainly a function of reduced gluconeogenesis from lactate in most settings [133, 134, 135, 136, 137]. Like TZD-induced glucose transport into isolated muscle, acute suppression of hepatic glucose output in vitro did not depend on concomitant insulin stimulation, and was therefore interpreted as "insulin-mimetic" TZD action [133, 134, 135]. At variance to what should be expected for full insulin-mimetic action, the reduction of glucose production by TZDs was associated with a considerable stimulation of the glycolytic pathway and with an increased net lactate release [135, 136, 138]. Although the rates of glycogen synthesis and glucose oxidation were not determined in liver, acute effects on the isolated liver seem to resemble those seen in isolated skeletal muscle. In both tissues, TZDs shift substrate flux into glycolysis and, hence, away from alternative routes like glucose production and glycogen synthesis.

At variance to the glycolytic effect of short-term TZD exposure, long-term exposure increased glycogen synthase activity in cultured hepatocytes [131, 139] which correlates with findings on long-term TZD-exposed cultured muscle [115, 116]. Whether increased activity of this enzyme is accompanied by increased glycogen storage in TZD-treated liver cells, however, can not be known without appropriate data on substrate fluxes and glycogen content.

Molecular targets for TZDs in liver. The healthy liver resembles skeletal muscle in that it hardly expresses PPARγ [25, 26] and in that a regulatory role of PPARγ has not yet been clearly shown. In isolated muscle, rapid TZD stimulation of glycolysis does not seem to depend on PPARγ signalling [113] and a comparable glycolytic effect on isolated liver as well as data on troglitazone-induced inhibition of phosphoenolpyruvate carboxykinase gene expression [140] suggest that TZDs address hepatocytes, like muscle cells, via PPARγ-independent pathways.

Whereas PPARγ-independent mechanisms seem to account for the acute metabolic actions of TZD compounds, the development of hepatic steatosis could render the liver responsive for PPARγ-mediated adipogenic TZD action [90, 141, 142, 143]. Steatotic hepatocytes resemble adipocytes not only with respect to triglyceride accumulation, but also in that they abundantly express PPARγ and several other genes which characterize fat cells [90, 141, 142]. Increased abundance of PPARγ in fatty livers could explain, why TZDs fail to induce lipid accumulation in livers of healthy animals, but exacerbate pre-existing steatosis and the expression of adipocyte-specific genes in livers of lipoatrophic and obese mice [90, 141, 143]. Nevertheless, worsening of lipid accumulation, which could be indicative of hepatic PPARγ-signalling, has only been described in mouse liver, whereas TZDs were reported to ameliorate hepatosteatosis in rats and humans [64, 72, 144, 145].

Nuclear receptors other than PPARγ could also be hepatic targets for at least some TZDs. In vitro, the TZDs KRP-297 and pioglitazone, as well as the isoxazolidinedione JTT-501 (Fig. 1) transactivate both PPARγ and PPARα [72, 73, 146]. PPARα is mainly expressed in the liver and is regarded as the mediator of the antihyperlipidaemic action of fibrates [147]. As pure PPARα agonists can elicit insulin-sensitization [7, 148, 149, 150], this receptor could contribute jointly with PPARγ to the antidiabetic action of dual agonists. Of note, activation of both subtypes might even be preferable to pure PPARγ-agonism, because PPARα activity could increase the antihyperlipidaemic efficacy of a TZD [72, 151] and could attenuate PPARγ-induced adiposity and weight gain [150].

In vitro, the TZD troglitazone also transactivates human pregnane X receptor (PXR) at concentrations required for the stimulation of PPARγ [152]. The potential of this hepatic nuclear receptor to mediate antidiabetic action is not clear but PXR activation by troglitazone has been suggested to contribute to the improvement of glucose homeostasis in dexamethasone-treated rats [153].

Conclusion, liver. The liver resembles skeletal muscle in that acute TZD exposure in vitro leads to an insulin-independent increase in glycolysis, and in that there is a lack of any evidence for functional PPARγ signalling (except for severely steatotic livers of specific mouse

models). At variance to muscle, however, nuclear receptors other than PPARγ are expressed in the liver which provide targets for at least some TZDs in vitro, and therefore could contribute to their metabolic effects in vivo. Thus, TZDs are basically capable to directly modulate hepatic glucose metabolism in vitro, but the precise contribution of direct effects on the liver to antidiabetic TZD action in vivo is not yet clear.

Concluding remarks

Major progress has been made in understanding the mechanisms that causally underlie the metabolic actions of TZD compounds. Antihyperglycaemic action of TZDs is predominantly based on the amelioration of insulin resistance, with skeletal muscle as the quantitatively most important tissue for insulin-induced glucose lowering. In vitro, TZDs activate PPARγ and stimulate adipocyte differentiation, which provides the mechanism for TZD-induced adipose tissue remodelling in vivo and seems to be associated with the output of adipose-derived signals that might modulate glucose metabolism of other tissues.

Beyond these established findings, however, we are still not able to fully understand the causal chain leading to metabolic TZD action. Direct and PPARγ-independent effects on glucose metabolism of isolated skeletal muscle and liver suggest that the involvement of other molecular mechanisms, in addition to PPARγ-activation in adipose tissue, can not be excluded. To further elucidate the mechanisms of antidiabetic TZD action, it will be necessary to sort out, if the predominant role presently ascribed to PPARγ activation in fat tissue holds true, or if we are biased by the overwhelming amount of research focusing on this receptor. Therefore, it will be important to establish the interdependence of adipogenic and antidiabetic TZD action and to elucidate the relative share of direct and indirect TZD actions on skeletal muscle, liver, and adipose tissue.

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