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Regulation of adult muscle phenotype by PPARδ

Master Thesis by Ida Gjervold Lunde

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Department of Molecular Biosciences
Faculty of Mathematics and Natural sciences
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Ida Gjervold Lunde

ABSTRACT

A unique characteristic of skeletal muscle is its diversity, reflected in the fibre type composition of muscles and in the heterogeneity of different fibre types. Mammalian skeletal muscle has a remarkable capacity to accommodate to new functional demands, and a high degree of molecular variability is involved in the phenotypic determination of fibre structure, metabolism and contractility. Although this adaptive potential is well established, the signalling pathways linking muscle activity to expression of muscle specific genes, the excitation-transcription coupling, is poorly understood.

This work presents peroxisome proliferator-activated receptor δ (PPARδ) as a possible mediator in the signalling network regulating metabolic and contractile properties of adult skeletal muscle fibres.

PPARs are fatty acid activated transcription factors playing important regulatory roles in development and metabolism. PPARδ is known to regulate β-oxidation of fatty acids in muscle and adipose tissue, but has recently also been implicated in the excitation-transcription coupling by studies in transgenic animals. The aim of this work was to investigate wild type expression patterns of PPARδ and effects of an active PPARδ in skeletal muscles of adult rats, in order to elucidate a possible role for PPARδ in adult muscle adaptation.

In this gain-of-function study, a transgene encoding an intrinsically active fusion protein of a VP16 activation domain and PPARδ (VP16-PPARδ) was transfected into the "fast" *extensor digitorum longus* (EDL) muscle of rat by *in vivo* electroporation. Succinate dehydrogenase (SDH) activity, cross sectional area (CSA) and myosin heavy chain (MyHC) fibre type distribution among the transfected fibres were analysed, and compared to sham transfected and normal controls. In the second part of this study, expression patterns of the wild type PPARδ protein were analysed by immunohistochemistry in normal, untreated *soleus* and EDL muscles.

Overexpression of an active PPARδ in EDL muscle fibres of adult rats resulted in reductions of CSAs and increased SDH activity levels, followed by changes in MyHC expression in slow direction. Immunohistochemical data from normal muscles indicated higher levels of PPARδ in nuclei of slow/oxidative fibres than in fast/glycolytic fibres, which had higher cytosolic levels. These results support the hypothesis of a role for PPARδ in maintaining and transforming muscle fibres in the slow/oxidative direction, for example during endurance training, but also indicate nuclear translocation as a new level of regulation.

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1 INTRODUCTION

The mammalian skeletal muscle is a dynamic system responding to environmental stimuli and has a remarkable capacity to accommodate to new functional requirements. The diversity of muscle fibres reflects this functional flexibility and is related to a high degree of molecular variability involved in the determination of fibre structure, metabolism and contractility. The phenotypic profile of a muscle fibre is ultimately determined by transcription of muscle specific genes, controlled mainly by the electrical activity pattern in the motor neuron. In the adaptive response to altered nervous activity, the expression pattern of contractile and metabolic genes may be modulated, resulting in changes in the phenotypic profile of individual fibres. However, the signalling pathways linking muscle activity to gene transcription are poorly understood, although extensively studied.

This master thesis presents peroxisome proliferator-activated receptor δ (PPARδ) as a possible mediator of metabolic and contractile adaptations of adult skeletal muscle fibres.

1.1 Muscle fibre phenotypes

Skeletal muscles are heterogeneous and consist of structurally and metabolically distinct fibre phenotypes. The composition of fibre phenotypes in a muscle is mainly a reflection of the electrical activity patterns of the motor neurons innervating the muscle, in addition to embryonic origin, mechanical loading, hormonal influence and aging (Pette & Staron, 2000). The molecular diversity of fibre phenotypes is related to the existence of multiple isoforms of proteins involved in contractile and metabolic mechanisms.

Mammalian muscle fibres are often classified according to two major functional characteristics: speed of contraction and aerobic/oxidative or anaerobic/glycolytic production of ATP. Skeletal muscle diversity was realized as early as 1873 when “white” muscles were distinguished from “red” (Ranvier, 1873). Further descriptions of the differences between individual muscle fibres emerged in the following years, and a century later skeletal muscle fibres were divided into three discrete categories based on the pH stability of the myosin ATPase (mATPase): “slow”/oxidative type I, “fast”/oxidative/glycolytic type IIa and “fast”/glycolytic type IIb (Brooke & Kaiser, 1970). Yet another contemporary approach of fibre typing was based upon reference enzymes of aerobic and anaerobic energy metabolism, also resulting in the identification of three major fibre types: “slow” twitch oxidative, “fast” twitch oxidative/glycolytic and “fast” twitch glycolytic (Barnard *et al.*, 1971). These

metabolic properties were associated with type I and type II mATPase activity and fatigability (Edström & Kugelberg, 1968).

The myosin molecule consists of two heavy chains (MyHCs) and four light chains (MyLCs), all influencing on contractile properties of muscle fibres. In the 1980s, the expression of MyHC isoform of individual fibres was shown to correlate with maximum velocity of shortening (Reiser *et al.*, 1985), identifying MyHC fibre types I, IIa and IIb. With the development of immunohistochemical techniques and monoclonal anti-MyHC antibodies, Schiaffino *et al*. (1989) discovered a fourth adult mammalian fibre type, the IIx fibre, expressing MyHC isotype IIx. The IIx fibre was characterised as an intermediate between IIa and IIb fibres in terms of ATPase activity, speed of contraction, metabolic profile and fatigue resistance. 11 different MyHC isoforms encoded by separate genes have been identified in adult mammalian muscles (Pette & Staron, 2000). Normally, adult muscle fibres express only one of these MyHC genes, and MyHC profile is currently the most widely used method for classification of skeletal muscle fibre types.

The MyHC protein exists in four isoforms in limb muscles of adult rodents; type Iβ, IIa, IIx and IIb (table 1.1), while slow type Iβ and fast type IIa and IIx are expressed in human limb muscles (Smerdu *et al.*, 1994). In general terms, slow type I fibres have the slowest speed of contraction due to slow hydrolysis of ATP during cross-bridge cycling, and the best endurance due to high concentrations of mitochondria and oxidative enzymes, ensuring stable and long-term supply of ATP when oxygen is present. Fast type IIb fibres, on the other hand, have the highest speed of contraction and the poorest endurance due to low concentrations of mitochondria and high concentrations of glycogen and glycolytic enzymes, serving as a fast, but short lasting, oxygen independent source of ATP (Schiaffino & Reggiani, 1994). Fibre types IIa and IIx show intermediate physiological properties, as shown in table 1.1.

Nevertheless, none of these fibre types are completely discrete from one another, whether criteria are based on physiological or biochemical characteristics. Hybrid fibre populations, co-expressing two MyHC isoforms, exist, namely type I/IIa, IIa/IIx and IIx/IIb fibres (Pette & Staron, 1990; Schiaffino & Reggiani, 1994). These generally show intermediate physiological properties, lying between their respective “pure” MyHC fibre types, thus reflecting a continuum of contraction speeds (Rivero *et al.*, 1998). The metabolic activity and fatigue resistance of muscle fibres also show high amounts of overlap rather than distinct levels representing each MyHC fibre type. However, significant differences in mean values of enzymatic activity are observed and can be used to separate different fibre types,

although pronounced variations may exist between species, different muscles and even within groups of fibres of the same type.

Table 1.1 Overview of MyHC expression and physical properties of different fibre types of limb skeletal muscles of adult rodents

Fibre type:	MyHC:	Speed of contraction:	Metabolic profile:	Endurance:
I	MyHC Iβ	Slow	Oxidative	Good
IIa	MyHC IIa	Fast	Oxidative-glycolytic	Good-Medium
IIx	MyHC IIx	Faster	Glycolytic-oxidative	Medium-Poor
IIb	MyHC IIb	Fastest	Glycolytic	Poor

Muscle fibre types are determined according to MyHC expression. MyHC expression defines the speed of contraction of individual fibres, while the metabolic profile defines endurance.

1.2 Plasticity of muscle fibre phenotypes

Multiple mechanisms regulate muscle fibre diversification and MyHC gene expression during development. The phenotype of adult muscle fibres can, however, be further modulated in adaptive responses to changes in nerve activity, mechanical loading and unloading, hormonal status and aging (Pette & Vrbova, 1985; Pette & Staron, 1997; Gundersen, 1998; Mercier *et al.*, 1999; Pette & Staron, 2000). These adaptive responses can be quite dramatic and occur in fully differentiated fibres, without prior cell death and regeneration (Gorza *et al.*, 1988). Changes in MyHC isoform expression tend to follow a general scheme of sequential and reversible transitions from fast to slow and slow to fast: MyHC 1β ↔ MyHC IIa ↔ MyHC IIx ↔ MyHC IIb, and transformations often include stages with an increased percentage of hybrid fibres (Pette & Staron, 1997; Windisch *et al.*, 1998; Pette & Staron, 2000).

Some hormones have profound effect on muscle phenotype. Although testosterone may contribute to differences in relative concentrations of MyHC in muscles of males and females (Staron *et al.*, 2000), thyroid hormones appear to have the strongest hormonal influence on adult muscle phenotypes. Hypothyroidism causes fast to slow MyHC transitions while hyperthyroidism elicits transitions in fast direction (Pette & Staron, 1997).

Stretch, mechanical loading (Pattullo *et al.*, 1992) and aging (Larsson & Ansved, 1995) cause fast to slow MyHC transitions in muscle fibres, while mechanical unloading induces a faster phenotype with an increased expression of fast MyHC isoforms (Pette & Staron, 1997, 2000).

The pattern of neuromuscular activity is the primary determinant of phenotypic gene expression during development and in adult muscle fibres (Pette & Vrbova, 1985; Gundersen & Eken, 1992). The impact of neural activity has been demonstrated in denervation experiments showing that in the absence of innervation, slow muscles become faster, and vice versa (Gutmann *et al.*, 1972). A cross-reinnervation model demonstrated that fast muscles turn slow when reinnervated by a slow nerve, and slow muscles turn fast when reinnervated by a fast nerve, following initial denervation (Buller *et al.*, 1960; Pette & Vrbova, 1985). These phenotypic changes relate to the specific patterns of electrical impulses generated by the motor neurons (Lømo *et al.*, 1974). Denervation and direct stimulation of the slow *soleus* (SOL) muscle with a fast electrical stimulation pattern (phasic, high-frequency) using implanted electrodes, resulted in slow to fast transformation (Gorza *et al.*, 1988; Ausoni *et al.*, 1990; Windisch *et al.*, 1998), whereas a slow electrical stimulation pattern (chronic, low-frequency) imposed on the denervated fast *extensor digitorum longus* (EDL) muscle induced fast to slow transformation (Eken & Gundersen, 1988; Ausoni *et al.*, 1990; Schiaffino *et al.*, 1999).

Muscle inactivity or decreased activity tends to shift MyHC expression of muscle fibres in the fast direction and reduces their cross sectional areas (atrophy). Increased activity and functional overload, on the other hand, promote changes in the slow, oxidative direction (Pette & Staron, 2000).

Resistance training is characterized by phasic high-frequency muscle activity. In humans, resistance training has been shown to induce a decrease in type IIx fibres with a following increase in type IIa, whereas in rat the decrease in type IIb is followed by an increase in type IIx fibres (Andersen *et al.*, 2000; Spangenburg & Booth, 2003). At the same time, production of proteins and cross sectional areas of the muscle fibres were increased (hypertrophy) (McCall *et al.*, 1996).

Endurance training is characterized by prolonged low-frequency muscle activity and frequently increases the oxidative metabolism of skeletal muscles (Baldwin *et al.*, 1972; Fitts *et al.*, 1975; Holloszy & Booth, 1976; Dudley *et al.*, 1982; Carter *et al.*, 2001; Koulmann & Bigard, 2006). Endurance training may under extreme conditions induce fast to slow MyHC transitions of muscle fibres depending on the intensity, duration and type of training (Andersen & Henriksson, 1977; Baumann *et al.*, 1987; Fitzsimons *et al.*, 1990; Sullivan *et al.*, 1995; Demirel *et al.*, 1999; Andersen *et al.*, 2000; Allen *et al.*, 2001). Chronic low-frequency electrical stimulation of muscles using implanted electrodes imitates this type of muscular activity, and has been shown to promote similar fast to slow transitions in skeletal muscle

(Pette & Vrbova, 1985; Eken & Gundersen, 1988; Ausoni *et al.*, 1990; Pette & Staron, 1997; Schiaffino *et al.*, 1999). Although these adaptations to muscle activity are observed, the signalling pathways linking muscle excitation to expression of contractile and metabolic genes, are just beginning to be understood.

1.3 Signalling pathways involved in plasticity of muscle fibre phenotypes

Fibre type transformations not only involve changes in MyHC expression, but include changes in the expression of a multitude of contractile and metabolic proteins. Consequently, fibre type transformation and maintenance represent highly coordinated regulations of transcription, translation and post-translational modifications. Even so, published literature suggests that the phenotype of muscle fibres is regulated by multiple signalling pathways rather than a "master" switch or a "master" signalling pathway (Spangenburg & Booth, 2003; Koulmann & Bigard, 2006). A possible model of the excitation-transcription coupling based on existing literature is presented in figure 1.1.

The role of calcineurin (CaN) in muscle adaptation has been a subject of intensive investigation. CaN is a serine/threonine phosphatase consisting of a catalytic and a regulatory subunit and is a major mediator of Ca^{2+} signalling in different cell systems. When activated by binding of Ca^{2+} to calmodulin (CaM), CaN affects expression of target genes by dephosphorylation of substrates, among them the nuclear factor of activated T cells (NFAT) gene family, originally identified in T and B lymphocytes. Dephosphorylated NFATs translocate to the nucleus and bind to promoter regions of target genes (Rao *et al.*, 1997). Since CaN is activated by Ca^{2+}-CaM, intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) is a major determinant of CaN action and hence the expression of its target genes.

Chin *et al.* (1998) found that a CaN dependent transcriptional signalling pathway selectively up-regulated slow-specific gene promoters in cultured myocytes and in mature muscle cells of intact animals, by involvement of proteins from the NFAT and the myocyte enhancer factor 2 (MEF2) families. Their hypothesis was originally based on the finding that the CaN pathway responded preferentially to sustained, low-amplitude elevations of $[Ca^{2+}]_i$ (Dolmetsch *et al.*, 1997) and that the tonic motor neuron activity characteristically innervating slow twitch fibres resulted in such elevations of $[Ca^{2+}]_i$ (Chin & Allen, 1996). Fast fibres, on the other hand, are innervated by nerves with infrequent, phasic firing patterns resulting in $[Ca^{2+}]_i$ transients of insufficient duration to activate CaN. The CaN (Naya *et al.*, 2000) and the CaN-NFAT pathway (McCullagh *et al.*, 2004) have also later been shown to facilitate fast to slow transitions in skeletal muscle *in vivo*.

CaN has also been suggested as a mediator of the slow phenotype expression program through other downstream factors than NFAT. Wu *et al.* (2000) showed that CaN dependent regulation of muscle specific genes could be mediated by MEF2 alone, although greatly enhanced by CaM dependent protein kinase activity (CaMK) and the presence of NFAT. The role of CaMK was further explored in transgenic mice and shown to increase the number of slow type I fibres in the *plantaris* muscle, although it was unclear which form of CaMK mediated these alterations (Wu *et al.*, 2002).

Interestingly, Wu *et al.* (2002) reported increased mRNA levels of PPARγ coactivator 1α (PGC-1α) in the transgenic animals overexpressing and active form of CaMK IV, a regulator of adaptive, *non*-shivering thermogenesis, mitochondrial biogenesis and oxidative metabolism *in vivo* (Lehman *et al.*, 2000; Puigserver & Spiegelman, 2003). PGC-1α was later shown to be preferentially expressed in slow muscles and to induce the formation of slow twitch fibres in transgenic animals (Lin *et al.*, 2002). However, PGC-1α does not bind to DNA itself, but rather works through interactions with transcription factors. In cultured muscle cells, PGC-1α activated transcription in co-operation with MEF2 and served as a target for CaN signalling, thus integrating Ca^{2+} signalling, mitochondrial biogenesis and myofibrillar protein regulators.

Myogenin is a basic helix-loop-helix (bHLH) transcription factor primarily expressed in slow fibre types (Voytik *et al.*, 1993). Hughes *et al.* (1999) found that transgenic mice overexpressing myogenin had an increased oxidative capacity of fast muscles and reduced fibre sizes compared to wild type muscles, but reported no change in MyHC expression. These effects were also observed in muscle fibres of adult mice after somatic myogenin DNA transfer (Ekmark *et al.*, 2003). Myogenin is proposed to be linked to slow motor neuron activity through CaN (Friday *et al.*, 2000).

The Ras-mitogen activated protein kinase (MAPK) pathway has also been implicated in the nerve activity dependent differentiation of slow muscle fibres (Murgia *et al.*, 2000; Koulmann & Bigard, 2006).

MyoD, on the other hand, has been proposed as a regulator of a fast fibre phenotype. MyoD is a bHLH transcription factor primarily expressed in fast fibre types (Voytik *et al.*, 1993). Seward *et al.* (2001) showed that MyoD knock-out mice expressed low levels of MyHC IIb mRNA compared to muscles of wild type mice. Similar effects have been seen after somatic MyoD DNA transfer, implicating that MyoD plays a role in the regulation of a fast fibre phenotype (Ekmark *et al.*, unpublished).

The Six and Eya pathway is another mechanism proposed to be involved in the establishment and maintenance of a fast twitch muscle phenotype (Grifone *et al.*, 2004).

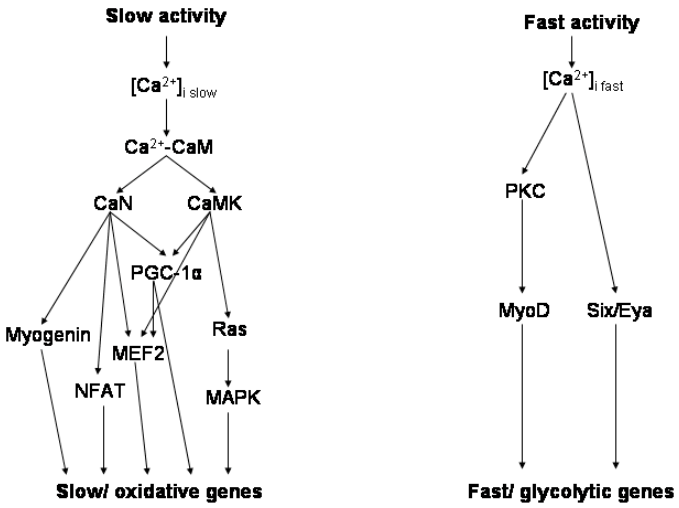

Figure 1.1 Overview of signalling pathways proposed to be involved in the excitation-transcription coupling in skeletal muscle
Patterns of electrical activity in motor neurons induce expression of muscle specific genes through activation of intracellular mediators, defining the phenotype of individual muscle fibres.

In addition to the pathways illustrated in figure 1.1, recent data suggest that PPARδ might be involved in regulating muscle phenotype (see section 1.5).

1.4 Peroxisome proliferator-activated receptors (PPARs)

The nuclear receptor family of peroxisome proliferator-activated receptors (PPARs) was originally named for the ability of the first identified member to induce hepatic peroxisome proliferation in mice in response to xenobiotic stimuli (Issemann & Green, 1990). However, later studies have revealed that PPARs are nuclear receptors functioning as fatty acid activated transcription factors playing important regulatory roles in development, inflammation, glucose and lipid metabolism (Schmidt *et al.*, 1992; Xu *et al.*, 1999; Willson *et al.*, 2000; Blaschke *et al.*, 2006).

The PPARs belong to a subset of nuclear receptors functioning as heterodimers with 9-*cis* retinoid X receptors (RXRs) (Kliewer *et al.*, 1992; Mangelsdorf & Evans, 1995). PPARs are activated by free fatty acids (FFAs) and their metabolites (Keller *et al.*, 1993; Forman *et al.*, 1997; Xu *et al.*, 1999), however they are rather promiscuous as to ligand partners (Ferre, 2004). As an activated complex, RXR-PPAR binds to PPAR responsive elements (PPREs) within the promoter region of target genes. The RXR-PPAR complex can be activated by the ligand of either receptor, and the activation state may be modified by phosphorylation (Gilde & Van Bilsen, 2003; Diradourian *et al.*, 2005; Gelman *et al.*, 2005) or binding of cofactors (McKenna *et al.*, 1999; Berger & Moller, 2002; Krogsdam *et al.*, 2002; Gilde & Van Bilsen, 2003).

Three closely related mammalian subtypes encoded by separate genes have been identified: α, γ, and β/δ (Dreyer *et al.*, 1992; Kliewer *et al.*, 1994). They all share a common domain structure typical of nuclear receptors and a common mechanism of action (Desvergne & Wahli, 1999; Ferre, 2004; Nagy & Schwabe, 2004), shown in figure 1.2.

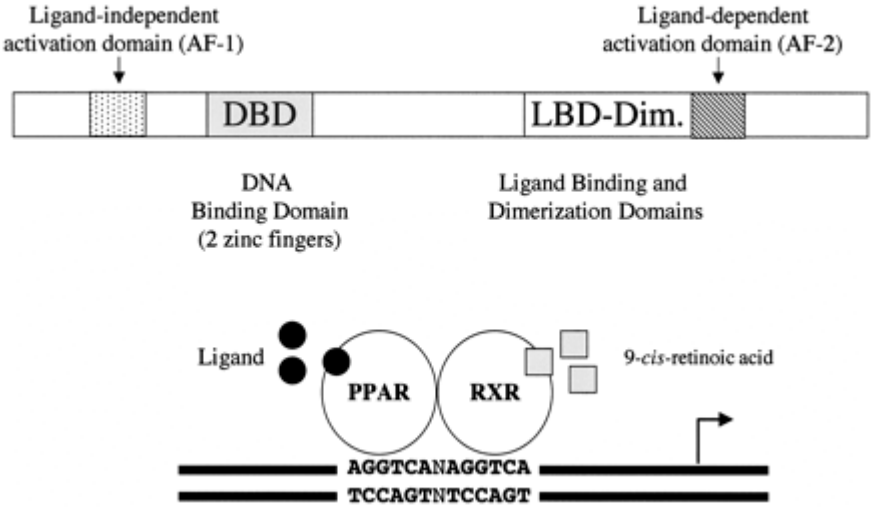

Figure 1.2 General structure and mechanism of action of PPARs
PPAR isoforms share a highly conserved, common domain structure and mechanism of action (figure copied from Ferre, 2004).

The PPAR subtypes exhibit distinct patterns of tissue expression and overlapping, yet distinct biological activities (Kliewer *et al.*, 1994; Jones *et al.*, 1995; Braissant *et al.*, 1996; Escher *et al.*, 2001; Berger & Moller, 2002; Gilde & Van Bilsen, 2003).

PPARα is expressed in metabolically active tissues including liver, heart, kidney and skeletal muscle (Braissant *et al.*, 1996). It is implicated in fatty acid catabolism mainly by regulating hepatic β- and ω-oxidation. PPARα is the molecular target of fibrates, a class of lipid-lowering drugs (Guerre-Millo *et al.*, 2000; Berger & Moller, 2002; Berger *et al.*, 2005).

PPARγ has the most limited expression pattern of the PPARs; it is found predominantly in white and brown adipose tissue, macrophages, colon and placenta (Braissant *et al.*, 1996). PPARγ plays a central role in adipogenesis and is the target of thiazolidinediones, insulin sensitizers (Rosen *et al.*, 2000; Willson *et al.*, 2000; Berger & Moller, 2002; Berger *et al.*, 2005; Semple *et al.*, 2006).

PPARδ is ubiquitously expressed, although highly expressed in metabolically active tissues (Kliewer *et al.*, 1994; Braissant *et al.*, 1996). Until very recently its roles were unclear, but it has now been established as a regulator of β- and ω-oxidation of fatty acids (Oliver *et al.*, 2001; Muoio *et al.*, 2002; Wang *et al.*, 2003).

Altogether, these lipid "sensors" regulate a large spectrum of homeostatic functions, including development, inflammation, lipid and glucose metabolism. A current view on their integrated metabolic actions is shown in figure 1.3.

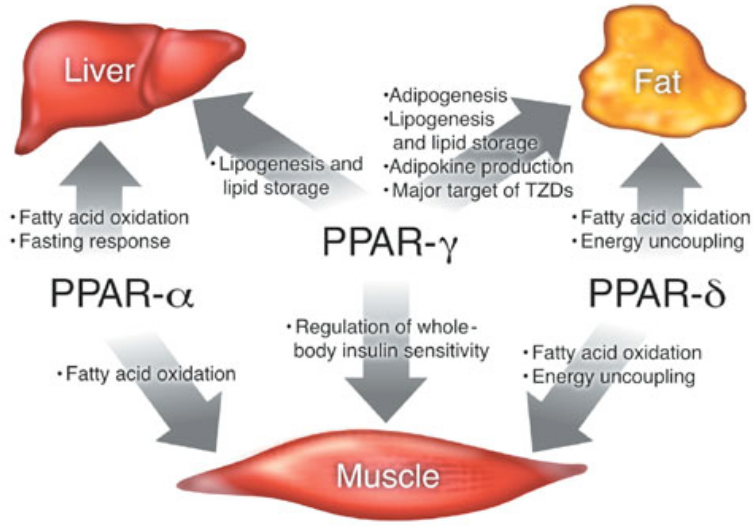

Figure 1.3 Metabolic integration by PPARs (figure copied from Evans *et al.*, 2004)
The three PPAR isoforms regulate lipid and glucose homeostasis through co-ordinated activities in muscle, liver and adipose tissue.

1.5 PPARδ and skeletal muscle

During the last few years, knowledge about the physiological functions of PPARδ has increased considerably. Strong evidence suggests that PPARδ is an important and central regulator of fatty acid oxidation (FAO) in several tissues, such as heart, adipose and particularly skeletal muscle tissue. In skeletal muscle, which is one of the major sites of lipid catabolism and utilization, PPARδ expression is several fold higher than that of PPARα and PPARγ (Braissant *et al.*, 1996; Escher *et al.*, 2001; Muoio *et al.*, 2002; Gilde & Van Bilsen, 2003; Wang *et al.*, 2004).

As shown in PPARα knock-out mice by Muoio *et al.* (2002), PPARδ was capable of inducing multiple pathways co-operatively promoting FAO in skeletal muscle, a function previously assigned to PPARα. Furthermore, treatment of rat or human cultured myotubes with a PPARδ agonist, GW742, resulted in increased FAO and induced expression of several lipid regulatory genes. To directly assess the role of PPARδ in skeletal muscle cells, the responses to natural and synthetic agonists were investigated in C2C12 myotubes overexpressing the receptor and in dominant-negative mutants (Holst *et al.*, 2003). The response was an induction of genes involved in lipid metabolism and an increase of FAO. Overexpression enhanced these effects, while the opposite was observed in the dominant-negative mutant. Moreover, PPARδ expression was reported to be regulated by nutritional changes. mRNA levels were drastically up-regulated in mouse *gastrocnemius* muscles after a

24h starvation period and restored to control level upon refeeding. These changes were accompanied by parallel alterations in expression of genes involved in lipid metabolism. Tanaka *et al*. (2003) demonstrated that in L6 myotubes, PPARδ controlled FAO by regulating a large panel of genes involved in FA transport, β-oxidation and mitochondrial respiration, while Dressel *et al*. (2003) showed that in C2C12 myotubes, PPARδ was the main PPAR isotype involved in FAO.

In vivo experiments have confirmed the implication of PPARδ in the regulation of fatty acid catabolism, suggested by the *in vitro* experiments described above. Tanaka *et al.* (2003) demonstrated that in wild type mice, administration of PPARδ agonist GW501516 for 3-4 weeks increased fatty acid β-oxidation in skeletal muscle. Furthermore, agonist treatment of mice fed on a high-fat diet ameliorated diet-induced obesity and insulin resistance by enhancing FAO, inducing mitochondrial biogenesis and reducing intramuscular fat depots. Additionally, agonist treatment markedly improved plasma glucose and blood insulin levels in genetically obese *db/db* mice, also supported by Lee *et al*. (2006).

To investigate the roles of PPARδ in lipid metabolism more precisely, transgenic mice models were developed. Wang *et al.* (2003) described a transgenic mouse model in which a constitutively active form of PPARδ (VP16-PPARδ) was selectively overexpressed in adipose tissue. An up-regulation of genes involved in FA catabolism and energy uncoupling was observed, accompanied by a decrease in adiposity of animals both fed on normal and on high-fat diet. Moreover, this type of overexpression and treatment with GW501516 prevented development of obesity in *db/db* mice, shown in figure 1.4. These results implicated PPARδ as an important regulator of fat burning *in vivo*, and therefore also as a potential therapeutic target in the treatment of obesity and associated disorders.

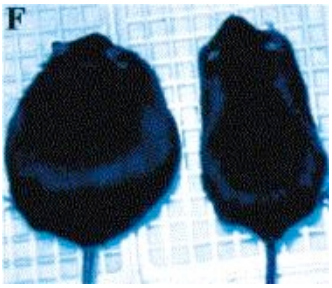

Figure 1.4 Activation of PPARδ in adipose tissue protects against obesity in a genetically fat mouse model (figure copied from Wang *et al.*, 2003)
Left: *db/db* mouse
Right: *db/db* / VP16-PPARδ in adipose tissue

Another transgenic mouse model was constructed using a cre/lox recombination approach (Luquet *et al.*, 2003). Muscle specific overexpression of PPARδ increased the oxidative capacity of *soleus, tibialis anterior* and *plantaris* muscles, illustrated by an increase in succinate dehydrogenase (SDH) activity level. These changes were accompanied by a net reduction in body fat content related to a decrease in adipocyte diameter. The transgenically

induced muscle phenotype observed was reminiscent of that promoted by endurance exercise; however no changes in muscle cross sectional area (CSA) or MyHC expression were observed. Luquet and co-workers speculated that PPARδ could be involved in exercise promoted muscle adaptation, and indeed; a 2.7 fold increase in PPARδ protein content was seen in the *plantaris* muscle of wild type mice after 6 weeks of training, compared to *non*-trained controls. Wang *et al.* (2004) continued to explore the functions of PPARδ by transgenic expression of VP16-PPARδ in skeletal muscle. Remarkably, this transgenic overexpression of an active PPARδ induced a significantly increased number of type I fibres in the muscles, compared to controls. Spectacularly, the transgenic mice were capable of continuous running of up to twice the distance of a wild type littermate. Gene expression typical of oxidative type I fibres and mitochondrial biogenesis was induced, and similar changes were also observed in GW501516 agonist treated wild type mice. The transgenic mice even showed resistance to obesity when fed on a high-fat diet. These results collectively showed that muscle specific activation of PPARδ induced a fibre phenotype reminiscent of that seen after endurance exercise, implicating a role for PPARδ in the excitation-transcription coupling in skeletal muscle.

Furthermore, Wang *et al*. (2004) investigated the protein levels of endogenous PPARδ in homogenates from different wild type mice muscles, and found it to be higher in the slow muscle SOL than in the mixed muscle *gastrocnemius* and the fast EDL. These results supported the hypothesis of a role for PPARδ in the regulation of a slow muscle phenotype, although the precise distribution of the protein within muscles and among fibre types has not been investigated (Gilde & Van Bilsen, 2003), and was one of the aims of this work.

PPARδ gene disruption is lethal at early stages for almost all the embryos due to a placental defect. The surviving knock-out animals are smaller than control littermates and exhibit striking reductions of adiposity in all types of fat tissue, skin defects and alterations of myelinisation. As this was not the case in mice with an adipose-specific deletion, the fat reduction probably was a reflection of peripheral PPARδ functions on systemic lipid metabolism (Peters *et al.*, 2000; Barak *et al.*, 2002). Moreover, Wang *et al*. (2004) found that the few surviving null mice could sustain only a third of the running time and distance of age- and weight matched wild type counterparts, further suggesting a role for PPARδ in enhancement of physical endurance.

Taken together, these *in vitro* and *in vivo* observations strongly implicate PPARδ as an important regulator of fatty acid catabolism in skeletal muscle and adipose tissue. Data presented in this chapter also indicate that PPARδ and its ligands might constitute a key

molecular switch in the regulation of muscle fibre type and oxidative capacity, as seen in transgenic animals (Luquet *et al.*, 2003; Wang *et al.*, 2004). On the other hand, the observed effects in the transgenic animals may reflect developmental effects only, as the transgenic product is present from early embryonic development. The transgenic effects may also reflect systemic effects, and therefore be related only to an altered metabolic state of the animal as a whole. Consequently, the findings might have no bearing on the importance of PPARδ in the regulation of skeletal muscle phenotype in adult animals.

This study investigates the role of an active PPARδ in individual skeletal muscle fibres of adult animals in relation to the mechanisms underlying determination of fibre phenotype, with experiments precluding developmental effects, genetic disposition, and more global and complex effects of physical activity on the organism as a whole. The aim was to identify distinct molecular mechanisms operating in the muscle cells themselves, mediating adaptive phenotypic changes in adult muscle that can be linked to alterations in muscle activity.

1.6 Aim of the study

1. **Will PPARδ activity induce phenotypic changes in individual skeletal muscle fibres of adult animals?**

To address this question, VP16-PPARδ was transfected into muscle fibres of the fast EDL of adult rats by *in vivo* electroporation. MyHC expression, SDH activity level and CSA of the transfected fibres were analysed five or fourteen days after transfection and compared to that of sham transfected and normal *non*-transfected control fibres.

2. **What are the expression patterns of PPARδ protein within wild type muscles and among different fibre types?**

To answer this, normal untreated fast EDL and slow SOL muscles of adult rats were cryosectioned and neighbouring cross sections were stained for PPARδ expression, SDH activity and MyHC fibre type.

2 MATERIALS AND METHODS

2.1 Overview

In the first part of this study, EDL muscles of adult rats were transfected with two plasmids; one encoding a constitutively active form of PPARδ and one encoding the reporter protein β-galactosidase, used to identify transfected fibres. The right leg EDL was transfected with both plasmids while the left leg EDL was transfected only with the reporter plasmid, serving as sham control. The transfer of plasmid DNA into the muscle fibres was facilitated by *in vivo* electroporation of the muscle. Five or fourteen days after the transfection and electroporation procedure, muscles were excised and cryosectioned. Transverse serial sections were histochemically stained for β-galactosidase and SDH activity, and fibre types were determined using monoclonal antibodies against MyHCs. On the basis of the histochemical analysis of neighbouring sections, muscle fibres from the different experimental groups were compared to each other and to normal *non*-transfected, randomly selected fibres from the same muscles.

In the second part of this study, EDL and SOL muscles of normal, *non*-treated rats were excised and cryosectioned. Neighbouring sections were histochemically stained for PPARδ expression, SDH activity and MyHC fibre types in order to describe the expression patterns of the wild type protein under normal conditions.

2.2 Animals

Twenty male WISTAR rats, 200-250 g body weight, were used in this study. The rats were delivered by the Norwegian Institute of Public Health, and kept in cages at the animal research facilities of the University of Oslo. The air temperature was kept at 22ºC with humidity ranging from 50-60 %, and the light was regulated at 12/12 hours cycles. Food and water were given *ad libitum*.

All animal procedures were reviewed and approved by the Norwegian Animal Research Authority and were conducted in accordance with the Norwegian Animal Welfare Act of December 20th, 1974, no. 37, chapter VI, sections 20-22, and the Regulation of Animal Experimentation of January 15th, 1996.

2.3 Surgical procedures

Each animal was initially anaesthetized with an intraperitoneal injection of 5 µl/g Equithesin (Sykehusapoteket Rikshospitalet, Norway; Appendix A, 5.1.1). The effect of the anaesthetics was controlled by checking for absence of withdrawal reflex when pinching the metatarsus region, and if necessary, additional anaesthetics were administrated. After deep anaesthesia was induced, hair was removed from the front part of the lower leg using an electric shaver and hair removal cream (Veet, Reckitt and Coleman). The rat was laid on its back, and one leg was fixed onto a styrofoam bloc by pinning it into a locked position. The *extensor digitorum longus* muscle was surgically exposed and 100 µl DNA solution (appendix A, 5.1.2, 5.1.3) was injected into the interstitium in the centre of the muscle, before transfected into the muscle fibres during electroporation. Following the surgical procedure, the wound was closed with sutures.

Five or fourteen days after transfection, animals were re-anaesthetized and the EDL muscles surgically excised. The animals were sacrificed by neck dislocation while still under deep anaesthesia.

For the excision of wild type muscle from normal, *non*-treated rats, the surgical procedure consisted of administration of anaesthetics as described above, exposure and excision of EDL or SOL muscles, before the animals were sacrificed as previously described.

2.4 Plasmids

To induce overexpression of PPARδ, a pCMX plasmid (Umesono *et al.*, 1991) encoding the intrinsically active VP16-PPARδ fusion protein was transfected into the muscle fibres of EDL (figure 2.1B). The DNA sequence encoding the PPARδ protein was inserted into the HindIII/ BamHI sites of the 4.5 kb pCMX plasmid (Andrews & Faller, 1991). To generate the VP16-PPARδ transgene, a VP16 domain from the herpes simplex virus (HSV) was fused in frame N terminally to the PPARδ gene, using the HindIII restriction sites. The 78 amino acid long VP16 domain is a strong transcriptional activator of early viral genes. VP16 insertion resulted in a constitutively active transgene driven by a cytomegalovirus (CMV) promoter. The pCMX-VP16-PPARδ plasmid was kindly donated by Ronald M. Evans for use in these experiments.

A reporter plasmid, pAP-lacZ, was co-transfected with the pCMX-VP16-PPARδ plasmid into the muscle cells for identification of transfected fibres, and serving as sham control when transfected into muscle fibres alone (figure 2.1A). The 7.8 kb pAP-lacZ plasmid encode the *Escherichia Coli* β-galactosidase sequence driven by a Rouse sarcoma virus (RSV) promoter, in addition to an origin of replication driven by a simian virus (SV) 40 promoter (Kisselev *et al.*, 1995).

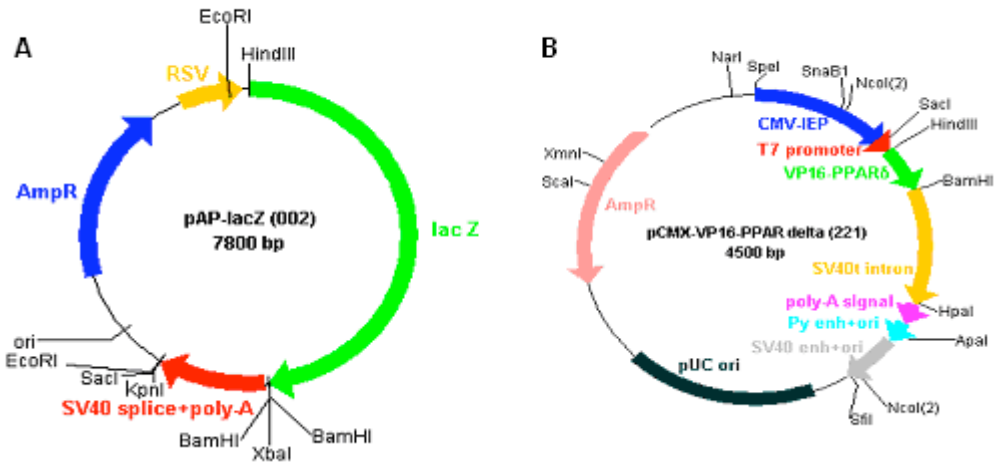

Figure 2.1 Expression plasmids

A. The 7.8 kb reporter plasmid, pAP-lacZ, encoding β-galactosidase

B. The 4.5 kb experimental plasmid, pCMX-VP16-PPARδ, encoding VP16-PPARδ.

2.5 Transfection of plasmids

2.5.1 Transfection in tissue culture

To confirm the actual transcription and translation of the VP16-PPARδ transgene into a functional protein, human embryonic kidney cells (HEK-293) were transfected with the expression plasmid pCMX-VP16-PPARδ, using a Lipofectamine2000 kit from Invitrogen. A sham control group was transfected only with the reporter plasmid, pAP-lacZ.

Proteins were extracted from the cells as described by Laemmli (1970) and 30 μg of protein from the two groups was run on SDS-PAGE according to Burnette (1981), followed by Western blotting (BIO-RAD protocol (1999)). The VP16-PPARδ protein was visualized by application of a specific rabbit anti-VP16 IgG primary antibody (1:1000, SIGMA, V4388) and a goat horse radish peroxidase (HRP) conjugated anti-rabbit IgG secondary antibody (1:1000, Abcam, ab6721), followed by the use of an ECL Western Blotting Detection kit (Amersham). Visualization by application of a rabbit anti-PPARδ IgG primary antibody (1:500, Abcam, ab8937) was tested, but not successful due to extensive background staining.

2.5.2 *In vivo* electroporation

In vivo electroporation of muscle fibres was performed as previously described by Mathiesen (1975), and is shown in figure 2.2. The electroporation procedure permeates the cell membrane and facilitates somatic transfer of DNA into muscle cells.

Following surgical exposure of EDL, 100 μl of DNA solution (appendix A, 5.1.2, 5.1.3) was injected into the interstitium in the centre of the muscle from the distal end, using a U-100 insulin BD Micro-Fine™ syringe. Subsequently, five trains of 1000 symmetrical bipolar pulses (200 μs in each direction) with a peak to peak voltage of 50 V were run across the muscle by two 1 mm thick/2 cm long silver electrodes, placed approximately 3-5 mm apart. The pulses were generated by a pulse generator (Pulsar 6bp-a/s, Fredrick Haer & Co), and the electrical charge was registered by an analogue oscilloscope (03245A, Gould Advance).

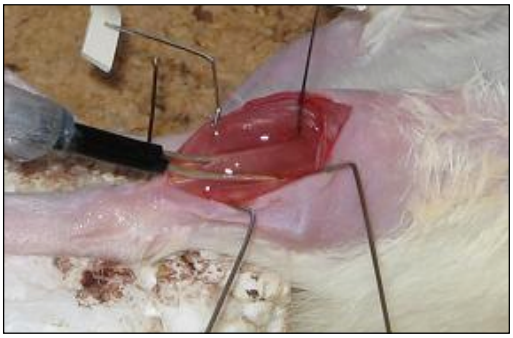

Figure 2.2 *In vivo* electroporation
Two silver electrodes create an electrical field across the EDL muscle, previously injected with DNA solution, to facilitate the somatic transfer of DNA into muscle fibres.

A DNA solution containing a mix of the two plasmids was injected into the right leg EDL (appendix A, 5.1.3), whereas a DNA solution containing only the reporter plasmid was injected into the left leg EDL (appendix A, 5.1.2), serving as sham control. As previously shown by Rana *et al.* (2004), nearly 100 % co-expression results when two separate plasmids are co-transfected into muscle fibres by *in vivo* electroporation. As a result of the differential transfection of muscle fibres in the right and left leg EDL, two experimental groups were formed; the VP16-PPARδ transfected fibres (hereafter called the PPARδ transfected fibres) and the lacZ transfected fibres (hereafter called the sham transfected fibres).

Additionally, an equivalent number of randomly selected normal, *non*-transfected fibres from the same muscles constitute yet another experimental group, serving as internal controls (hereafter called the normal fibres). To ensure an unbiased material, these were always selected as the nearest fibre down to the left from the transfected fibres. The normal fibres from the PPARδ transfected and the sham transfected muscles are presented as one experimental group throughout this study when no significant differences were found between the groups and statistical calculations without pooling the data, yielded the same result (see section 2.9 for statistical details). One exception was fibre type distribution fourteen days after transfection, where the number of normal type I fibres was higher in the PPARδ transfected (n=14) than in the sham transfected (n=2) muscles. For simplicity reasons, this material was still presented as one group in table 3.1, section 3.2.

All the three experimental groups presented in this study consisted of pooled data from several animals, as no systematic interanimal variations were observed.

The three different experimental groups of this study are presented in table 2.1.

Table 2.1 Overview of experimental groups, expression vectors and overexpressed proteins

Experimental group:	Expression vectors:	Overexpressed proteins:
Normal fibres	-	-
Sham transfected fibres	pAP-lacZ	β-galactosidase
PPARδ transfected fibres	pCMX-VP16-PPARδ pAP-lacZ	VP16-PPARδ β-galactosidase

2.6 Histochemistry

2.6.1 Excision and freezing of muscles

The transfected EDL muscles were surgically excised in the experimental animals five or fourteen days after the electroporation procedure, and the SOL and EDL muscles excised from normal, untreated rats. The muscles were slightly stretched between two pins attached to a double layer of thin wax (Tenax wax, S. S. White Manufacturing), and subsequently frozen in melting isopentane (-160ºC) and liquid nitrogen (-196ºC). The muscles were stored in 2 ml microtubes (SARSTEDT) at -80ºC for further analysis.

2.6.2 Preparation of transverse muscle serial sections

The frozen muscles were mounted in Tissue Tek optimal cutting temperature compound (Sakura Finetechnical Company) and cryosectioned at 10 µm in the cryotome (HM560M Microme). The temperature of the muscle tissue was adjusted to -18ºC and the knife to -24ºC. Transverse serial sections were mounted on SuperFrost Plus slides (Menzel-Gläser) and stored at -80ºC for further histochemical analysis.

2.6.3 Staining for β-galactosidase activity

β-galactosidase was used as a reporter protein in the transfection experiments to identify transfected fibres (Lojda, 1970; Sanes *et al.*, 1986). β-galactosidase activity was determined histochemically in a colour reaction by the addition of the enzyme's substrate, 5-bromo-4-chloro-3-indolyle-β-D-galactoside (X-gal) to the muscle sections (appendix A, 5.1.4). β-galactosidase hydrolyses X-gal to a colourless product named indoxyle. Indoxyle, in turn, dimerises and creates insoluble blue crystals that can be visualized under the microscope, thereby identifying the transfected fibres, as shown in figure 2.3.

To exclude the possibility of *non*-specific staining, the staining procedure was performed without the X-gal substrate on a test section. This negative control did not result in any positively stained blue fibres.

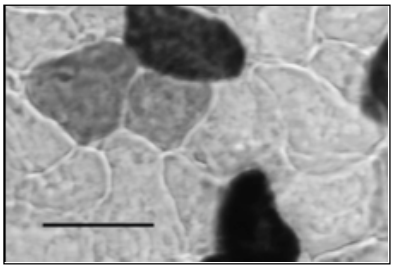

Figure 2.3 Staining for β-galactosidase activity
EDL cross section stained for β-galactosidase activity by the addition of the substrate X-gal. LacZ transfected fibres were identified by blue staining (here seen in grey/black). Scale bar: 50 µm.

2.6.4 Staining for succinate dehydrogenase activity

SDH is a flavoprotein catalyzing the oxidation of succinate to fumarate, and is the only membrane-bound enzyme in the citric acid cycle in mitochondria of cells. Accordingly, the level of SDH activity may be used as an indicator of the oxidative capacity and of the mitochondrial content of muscle fibres.

The colour reaction reflecting the SDH activity of a muscle fibre quantitatively, was performed as described by Bancroft (1975) (appendix A, 5.1.5). The staining method is based on the reduction and protonation of tetrazolium to formazan, catalyzed by the SDH enzyme, when succinate is added as substrate to the sections. Tetrazolium is colourless and insoluble while formazan is crystalloid and has a blue-purple colour. Consequently, the amount of colouring reflects the SDH activity level of the respective fibre, serving as a quantitative measurement of its oxidative capacity.

Staining without the substrate succinate on a test section yielded no positively stained fibres, serving as negative control.

2.6.5 Staining for myosin heavy chain isoform

Monoclonal antibodies against MyHC subtypes were used to determine muscle fibre type, kindly provided by Stefano Schiaffino. Secondary antibodies were conjugated to fluroescein or cyanine in order to visualize specific binding of primary antibody to individual muscle fibres on the cross sections (table 2.2, appendix A, 5.1.6). When fluorescein (FITC) or cyanine is illuminated with blue-green (λ=485 nm) or green (λ=546 nm) light, respectively, fluorescence is emitted, and can thereby be used to identify the positively stained fibres.

Negative control sections stained only with secondary antibodies gave no fluorescence for any of the four primary antibodies used.

Table 2.2 Overview of antibodies used to identify MyHC subtype expression in muscle fibres

MyHC:	Primary antibody:	Secondary antibody:
I	BA-D5	Rabbit anti-mouse IgG, FITC conjugated (SIGMA, F-9137)
IIa	SC-71	Rabbit anti-mouse IgG, FITC conjugated (SIGMA, F-9137)
***Non*-IIx**	BF-35	Rabbit anti-mouse IgG, FITC conjugated (SIGMA, F-9137)
IIb	BF-F3	Goat anti-mouse IgM, Cyt 3 (J115-165-020, Jackson ImmunoResearch Lab.)

An example of histochemical analysis of serial sections is shown in figure 2.4, showing β-galactosidase, SDH and anti-MyHC staining in a PPARδ transfected EDL muscle. MyHC fibre type, SDH activity and cross sectional area of the transfected and the randomly selected normal fibres were determined as shown in the stained neighbouring sections.

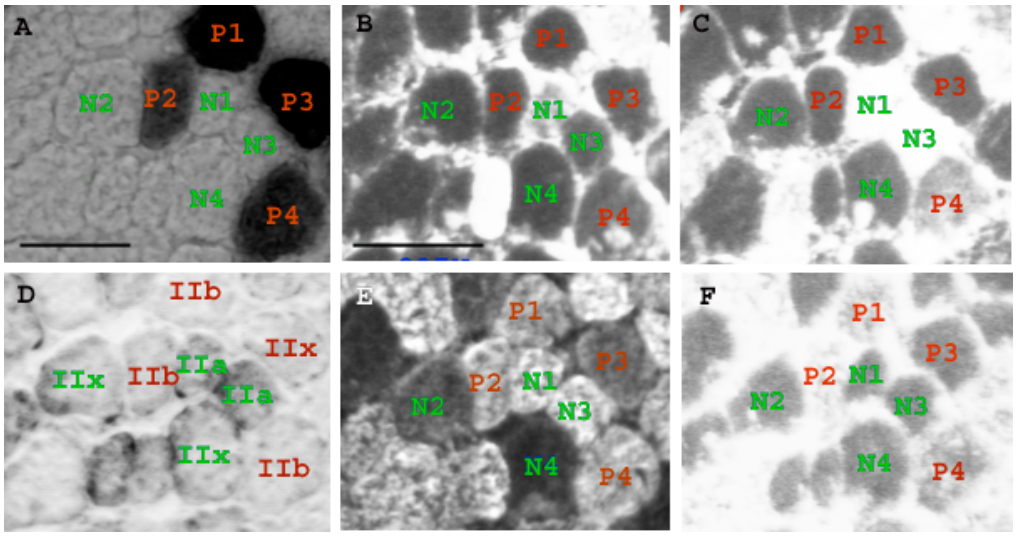

Figure 2.4 A-F Example of serial cross section staining in a VP16-PPARδ transfected EDL muscle

Serial cross sections stained for β-galactosidase activity (A), MyHC I (B), MyHC IIa (C), SDH activity (D), MyHC *non*-IIx (E) and MyHC IIb (F). Transfected fibres are β-galactosidase positive and appear dark (P1-4, labelled red). Randomly selected, normal *non*-transfected fibres are β-galactosidase negative and appear bright (N1-4, labelled green). Fibre types were determined as presented in the SDH image (D). The differential strength of SDH staining reflects differences in oxidative capacity among fibres. Scale bars: A, D: 50 µm; B, C, E, F: 50 µm.

2.6.6 Staining for VP16 expression

Staining for VP16 expression in VP16-PPARδ transfected fibres was performed to confirm the identification of transfected fibres from the β-galactosidase staining and to further confirm the actual translation of the transgene (appendix A, 5.1.7).

A primary monoclonal rabbit IgG antibody against the VP16 domain from the herpes simplex virus (SIGMA, V4388) was applied to the sections, followed by a goat anti-rabbit IgG fluorescein secondary antibody (Vector Laboratories Inc., FI-1000). The staining pattern was compared to neighbouring sections stained for β-galactosidase activity.

Negative controls stained only with secondary antibody yielded no fluorescence.

2.6.7 Staining for PPARδ expression

Staining for PPARδ expression was performed on EDL and SOL muscle sections from normal rats to analyse wild type expression patterns, and on sections from VP16-PPARδ transfected muscles to confirm the identification of transfected fibres from the β-galactosidase staining and the actual translation of the transgene (appendix A, 5.1.8).

A rabbit polyclonal primary anti-PPARδ antibody (Santa Cruz Biotechnology Inc., Sc-7197) was applied to the sections, followed by a goat anti-rabbit IgG fluorescein secondary antibody (Vector Laboratories Inc., FI-1000). The fluorescence detected was used to illustrate the level of PPARδ expression.

Negative controls stained only with secondary antibody yielded no fluorescence.

2.6.8. Staining for localization of nuclei

Staining for localization of nuclei was performed on cross sections of EDL and SOL muscles from normal *non*-treated rats and on VP16-PPARδ transfected muscles (appendix A, 5.1.9).

A UV-excitable (λ=400 nm) nucleic acid stain was applied to the sections (Hoechst 33342, Molecular Probes). The positively stained areas represent nuclei and were compared to neighbouring sections from the same area of the same muscle, stained for VP16 or PPARδ expression, in order to determine possible nucleic localization of the VP16-PPARδ fusion protein or the wild type PPARδ protein.

2.7 Imaging

2.7.1 Bright field imaging

Bright field images of muscle cross sections stained for β-galactosidase and SDH activity were taken using a CCD video camera (C2400, Hamamatsu) connected to a microscope (BX50W1, Olympus). The stained sections were mounted in glycerine gel and photographed in a dark room using 4x or 10x water immersion objectives (UMFPlanF1, Olympus). The images were digitalized through an image-processing unit (Argus-20, Hamamatsu) prior to the transferral to a Power Macintosh G3 computer and further processing in Photoshop 7.0 (Adobe).

2.7.2 Fluorescence imaging

Muscle cross sections stained with fluorescein or cyanine conjugated secondary antibodies were imaged using a SIT video camera (C2400-08, Hamamatsu) connected to a microscope (BX50W1, Olympus). The sections were photographed in a dark room using a 20x water immersion objective (UMFPlanF1, Olympus). Three filter cubes (Omega Optical) were used to illuminate the sections with blue-purple (XF11), green (XF37) or blue-green (XF22) light. The blue-purple filter was used for the nucleic acid staining, the green filter for staining where a cyanine conjugated secondary antibody was used, and the blue-green filter for staining where a fluorescein conjugated secondary antibody was applied to the sections. Also here the images were digitalized through an image-processing unit (Argus-20, Hamamatsu) prior to the transferral to a Power Macintosh G3 computer, and further processing in Photoshop 7.0 (Adobe).

2.8 Quantitative histochemistry

2.8.1 Quantification of SDH activity

SDH activity was measured in muscle fibres overexpressing VP16-PPARδ, sham transfected fibres and in the randomly selected normal fibres within the same areas of the muscles, in addition to in fibres from normal, untreated muscles.

SDH activity was quantified by the measurement of grey tone of manually encircled muscle fibres in ImageJ (NIH). The mean grey value was presented on a scale ranging from 0 (white) to 255 (black). The scale was standardized for all sections before images were taken in order to be able to compare fibres from different muscles. In addition, the mean grey value of normal IIb fibres from each section was set to 0, while that of normal IIa fibres was set to 1, and the SDH activity level of other fibres from the same section were calculated in relation to these set points. SDH activity level of analysed fibres was thus presented as a relative value in order to account for potential differences in staining or calibration of light upon imaging of the individual sections.

2.8.2 Calculation of cross sectional area

CSA was measured in muscle fibres overexpressing VP16-PPARδ, sham transfected fibres and in randomly selected normal fibres within the same areas of the muscles.

CSA was measured by manually encircling individual muscle fibres in ImageJ (NIH), and the number of pixels was later converted to μm^2 by calibration against a μm scale.

2.9 Statistical analysis

For statistical comparison of SDH activity and CSA of VP16-PPARδ transfected, sham transfected and normal *non*-transfected fibres, a one-way Anova with a Bonferroni post-test was performed. The level of significance was set to 5 %. As this test is based on a Gaussian distribution of the overall population of values, a normality test was run (Kolmogorov-Smirnov (KS) test) prior to the one-way Anova. For $p>0.10$, the population was concluded to be approximately Gaussian and suitable for one-way Anova.

For statistical comparison of fibre type distribution among fibres transfected with pCMX-VP16-PPARδ, pAP-lacZ and normal controls, a Fisher's exact test was used. The level of significance was set to 5 %.

The statistical analysis was performed in GraphPad Prism 4.

3 RESULTS

3.1 Expression of the VP16-PPARδ fusion protein

To confirm the actual expression and translation of the VP16-PPARδ transgene, HEK-293 cells were transfected with the experimental plasmid, pCMX-VP16-PPARδ. A sham group was transfected with the reporter plasmid, pAP-lacZ (2.5.1). Following protein extraction and SDS-PAGE, a Western blot was run. The resulting blot is presented in figure 3.1, showing a VP16 positive band of about 53 kDa, the expected size of the VP16-PPARδ fusion protein, in the pCMX-VP16-PPARδ transfected cells, but not in the sham transfected cells.

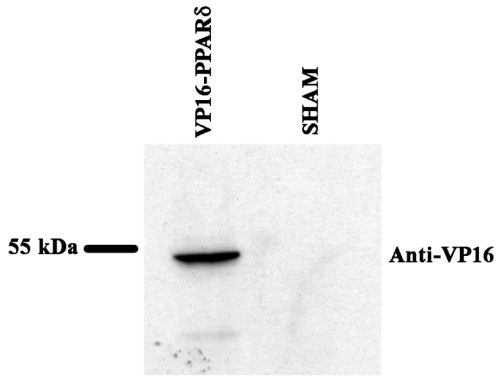

Figure 3.1 Expression of VP16-PPARδ in tissue culture
Western blot of protein extracts from HEK-293 cells transfected with the sham plasmid pAP-lacZ (SHAM) or the experimental plasmid pCMX-VP16-PPARδ (VP16-PPARδ). The band represents the 53 kDa VP16-PPARδ protein, visualized by application of a specific VP16 antibody (SIGMA).

Cross sections from VP16-PPARδ transfected EDL muscles were histochemically stained for VP16 (figure 3.2) and PPARδ (figure 3.3) expression to further confirm the expression of the transgene. The stained sections were compared to neighbouring sections stained for β-galactosidase activity to confirm co-expression following co-transfection.

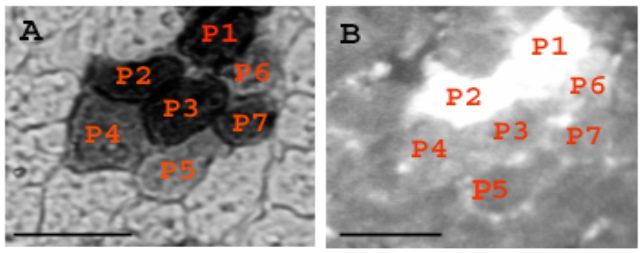

Figure 3.2 A, B Co-transfection is reflected in co-expression of β-galactosidase and VP16
Serial cross sections of a VP16-PPARδ transfected EDL muscle stained for β-galactosidase activity (A) and VP16 expression (VP16 antibody, SIGMA) (B), showing co-expression following co-transfection in fibres marked P1-7 (labelled in red). Scale bars: 50 μm.

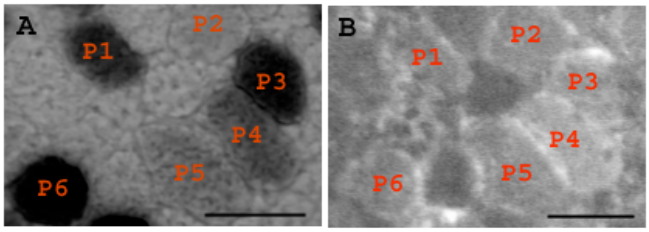

Figure 3.3 A, B Co-transfection is reflected in co-expression of β-galactosidase and PPARδ

Serial muscle sections of a VP16-PPARδ transfected EDL muscle stained for β-galactosidase activity (A) and PPARδ expression (PPARδ antibody, Santa Cruz Biotech. Inc.) (B), showing co-expression following co-transfection in fibres marked P1-6 (labelled in red). Scale bars: 50 μm.

As can be seen from figures 3.2 and 3.3, the VP16-PPARδ transgene was expressed and translated in the β-galactosidase identified PPARδ transfected fibres, with the protein localized to both nuclei and cytoplasm. The observed strength of the VP16 staining seemed to correlate well with the strength of the β-galactosidase staining, however interpretation of the PPARδ staining was confounded by the presence of the endogenous PPARδ protein.

Altogether, these *in vitro* and *in vivo* results demonstrated that the transgene product was of the right size and was expressed in fibres co-transfected with the lacZ reporter, an important pre-requisite for further experiments using these plasmids.

3.2 Fibre type distribution

The distribution of fibre types in the three experimental groups was calculated by counting the number of different fibre types identified by anti-MyHC staining five and fourteen days after transfection. Their distribution is shown in table 3.1/figure 3.4.

The composition of fibre types in normal EDL observed in this study (table 3.1/figure 3.4) was in accordance with previous observations of fibre type distribution in rat EDL, consisting of approximately 45 % type IIb, 29 % type IIx, 23 % type IIa and 3 % type I (Windisch *et al.*, 1998), or 60 % IIb, 27 % IIx, 10 % IIa and 3 % type I (Demirel *et al.*, 1999).

As to hybrid fibres, positively staining for two or more subtypes of MyHC (Pette & Staron, 1990; Schiaffino & Reggiani, 1994; Windisch *et al.*, 1998), fibre types were defined according to the relatively highest strength fluorescence (I/IIa hybrids). However, the lack of a IIx specific antibody (only a *non*-IIx antibody) made it impossible to detect IIa/IIx and IIx/IIb hybrids, the most important hybrids in EDL and in this study.

Table 3.1 Distribution of fibre types in EDL 5 and 14 days after VP16-PPARδ transfection and in control groups

Time from transfection to muscle excision:		**5 days:**		**14 days:**	
Fibre type:	**Experimental group:**	**n**	**%:**	**n**	**%:**
I	Normal	11	2.5	16	2.5
	Sham transfected	4	1.4	4	1.6
	PPARδ transfected	3	1.9	17	4.4
IIa	Normal	72	16.4	79	12.5
	Sham transfected	46	16.2	28	11.5
	PPARδ transfected	19	12.3	98	25.3*
IIx	Normal	111	25.3	193	30.6
	Sham transfected	79	27.8	76	31.3
	PPARδ transfected	45	29.2	98	25.3
IIb	Normal	244	55.7	343	54.4
	Sham transfected	155	54.6	135	55.6
	PPARδ transfected	87	56.5	175	45.1*
Total number of analysed fibres:		876		1262	

5 days after transfection, there were no significant differences in fibre type distribution between any of the experimental groups. However, there was a significant increase in the proportion of IIa fibres ($p<0.0001$) and a significant decrease in the proportion of IIb fibres ($p=0.0045$) among the PPARδ transfected fibres 14 days after transfection, compared to the control groups; the *non*-transfected and the sham transfected fibres (*).

Five days after transfection, 876 fibres from six EDL muscles were analysed (table 3.1/figure 3.4). There were no significant differences in fibre type distribution between the PPARδ transfected, sham transfected and normal controls, nor between the sham transfected and the normal fibres ($p>0.05$).

Fourteen days after transfection, 1262 fibres from twelve EDL muscles were analysed (table 3.1/figure 3.4). There were significant differences in fibre type distribution when comparing PPARδ transfected fibres to normal controls; a 9.3 % decrease in the proportion of IIb fibres ($p=0.0045$) and a 12.8 % increase in the proportion of IIa fibres ($p<0.0001$), representing a doubling in the proportion of IIa fibres. Also when comparing PPARδ transfected fibres to sham transfected fibres, there were significant differences in fibre type distribution; a decrease in the number of IIb fibres ($p=0.0113$) and an increase in IIa fibres ($p<0.0001$). However there were no significant differences in fibre type distribution between sham transfected and normal controls ($p>0.05$).

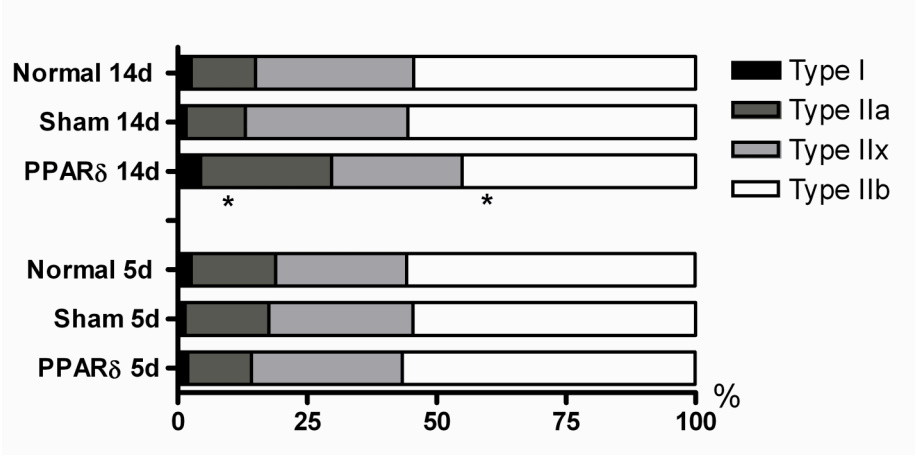

Figure 3.4 Distribution of fibre types in EDL 5 and 14 days after VP16-PPARδ transfection and in control groups

Fibre types were defined according to MyHC expression. 5 days after transfection, there were no significant differences in fibre type distribution among the PPARδ transfected fibres and the control groups. However, 14 days after transfection, there was a significant increase in the proportion of IIa fibres ($p<0.0001$) and a significant decrease ($p=0.0045$) in the proportion of IIb fibres among the PPARδ transfected fibres compared to the control groups; the normal *non*-transfected and the sham transfected fibres (*). There were no significant differences between the control groups ($p>0.05$).

3.3 Cross sectional area

CSAs of muscle fibres from the three experimental groups were measured five and fourteen days after transfection and are presented in mean ± SEM for each fibre type in table 3.2/figure 3.5.

Normally, the hierarchy of CSA of fibre types in EDL and mixed muscles in general is IIb>IIx>IIa>I (Rivero *et al.*, 1998; Nakatani *et al.*, 1999), also confirmed by this study (table 3.2/figure 3.5).

Table 3.2 Cross sectional areas of EDL muscle fibres 5 and 14 days after VP16-PPARδ transfection and in control groups

Time from transfection to muscle excision:			**5 days:**		**14 days:**
Fibre type:	**Experimental group:**	**n**	**Cross sectional area, mean ± SEM, μm^2:**	**n**	**Cross sectional area, mean ± SEM, μm^2:**
I	Normal	11	809 ± 35	16	1026 ± 12
	Sham transfected	4	852 ± 35	4	996 ± 9
	PPARδ transfected	3	727 ± 14	17	1010 ± 24
IIa	Normal	72	1062 ± 16	79	1087 ± 8
	Sham transfected	46	1081 ± 14	28	1097 ± 9
	PPARδ transfected	19	929 ± 36*	98	1015 ± 13*
IIx	Normal	111	1178 ± 12	193	1233 ± 7
	Sham transfected	79	1209 ± 11	76	1229 ± 9
	PPARδ transfected	45	1070 ± 32*	98	1129 ± 11*
IIb	Normal	244	1359 ± 11	343	1499 ± 7
	Sham transfected	155	1376 ± 9	135	1493 ± 8
	PPARδ transfected	87	1223 ± 30*	175	1354 ± 10*
Total number of analysed fibres:		876		1262	

CSAs are presented in mean ± SEM for each fibre type. PPARδ transfected IIa, IIx and IIb fibres had significantly reduced CSAs compared to sham transfected and normal controls both 5 and 14 days after transfection ($p<0.001$) (*). There were no significant differences between the control groups ($p>0.05$).

There were too few type I fibres to perform statistical tests on, and consequently they were therefore not analysed further.

Five days after transfection, 876 fibres from six EDL muscles were analysed and their CSAs are presented in table 3.2/figure 3.5 (hatched columns). PPARδ transfected IIa fibres had significantly smaller CSAs than sham transfected ($p<0.001$) and normal IIa controls ($p<0.001$), shown by a mean reduction of 13 % compared to normal IIa fibres (figure 3.5A, hatched columns). CSAs of PPARδ transfected IIx fibres were significantly reduced by 9 % compared to normal ($p<0.001$) and sham transfected IIx fibres ($p<0.001$) (figure 3.5B, hatched columns). PPARδ transfected IIb fibres showed an average 10 % reduction in CSA

compared to normal IIb controls ($p<0.001$) and sham transfected IIb fibres ($p<0.001$) (figure 3.5C, hatched columns). There were no significant differences in CSAs between sham transfected and normal IIa, IIx and IIb controls ($p>0.05$).

Fourteen days after transfection, 1262 fibres from eleven EDL muscles were analysed and their CSAs are presented in table 3.2/figure 3.5 (open columns). PPARδ transfected IIa fibres showed a mean 7 % reduction in CSA compared to normal ($p<0.001$) and sham transfected IIa controls ($p<0.001$) (figure 3.5A, open columns). PPARδ transfected IIx fibres showed a mean 8 % reduction in CSA compared to normal IIx fibres ($p<0.001$) and sham transfected controls ($p<0.001$) (figure 3.5B, open columns). PPARδ transfected IIb fibres showed a mean 10 % reduction in CSA when compared to normal ($p<0.001$) and sham transfected IIb controls ($p<0.001$) (figure 3.5C, open columns). There were no significant differences in CSAs between sham transfected and normal IIa, IIx and IIb fibres ($p>0.05$).

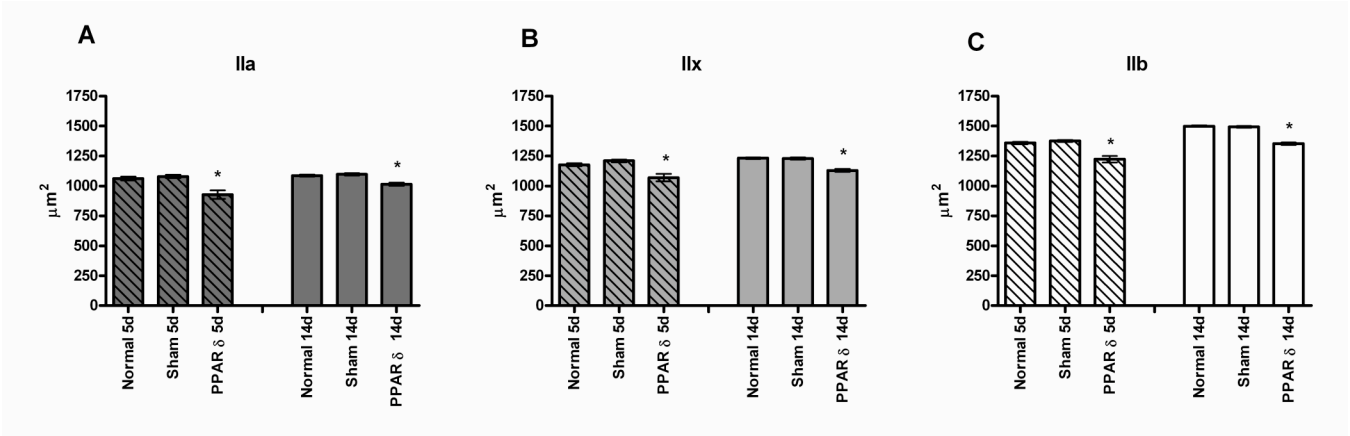

Figure 3.5 A, B, C Cross sectional area of IIa, IIx and IIb fibres 5 and 14 days after VP16-PPARδ transfection and in control groups

CSA values are presented in mean ± SEM (μm^2). PPARδ transfected IIa, IIx and IIb fibres had a significantly reduced CSAs compared to sham transfected and normal controls both 5 (hatched columns) and 14 days (open columns) after transfection ($p<0.001$) (*). There were no significant differences between the control groups ($p>0.05$).

3.4 SDH activity

Relative SDH activity levels of muscle fibres from the three experimental groups were measured both five and fourteen days after transfection and data are presented in mean ± SEM for each fibre type in table 3.3/figure 3.6.

Normally, the hierarchy of SDH activity level among fibre types in the EDL and mixed muscles in general is I>IIa>IIx>IIb (Rivero *et al.*, 1998; Nakatani *et al.*, 1999), also confirmed by this study (table 3.3/figure 3.6).

Table 3.3 Relative SDH activity level of EDL muscle fibres 5 and 14 days after VP16-PPARδ transfection and in control groups

Time from transfection to muscle excision:		5 days:		14 days:	
Fibre type:	**Experimental group:**	**n**	**Relative SDH-activity, mean ± SEM:**	**n**	**Relative SDH-activity, mean ± SEM:**
I	Normal	11	1.278 ± 0.063	16	1.248 ± 0.068
	Sham transfected	4	1.223 ± 0.020	4	1.490 ± 0.067
	PPARδ transfected	3	1.406 ± 0.085	17	1.290 ± 0.059
IIa	Normal	72	1.000 ± 0.037	79	1.006 ± 0.020
	Sham transfected	46	1.044 ± 0.029	28	1.043 ± 0.028
	PPARδ transfected	19	1.170 ± 0.058	94	1.196 ± 0.022*
IIx	Normal	111	0.472 ± 0.024	187	0.336 ± 0.012
	Sham transfected	79	0.410 ± 0.019	76	0.278 ± 0.013
	PPARδ transfected	45	0.520 ± 0.078	94	0.824 ± 0.029*
IIb	Normal	244	0.004 ± 0.015	336	$-2.1*10^{-6}$ ± 0.014
	Sham transfected	155	-0.020 ± 0.019	135	-0.080 ± 0.008
	PPARδ transfected	87	0.145 ± 0.038*	171	0.401 ± 0.035*
Total number of analysed fibres:		876		1237	

Relative SDH values are presented in mean ± SEM for each fibre type. 5 days after transfection, PPARδ transfected IIb fibres had a significantly increased SDH activity level compared to sham transfected and normal controls ($p<0.001$) (*). 14 days after transfection, PPARδ transfected IIa, IIx and IIb fibres had a significantly increased SDH activity level compared to that of sham transfected and normal controls ($p<0.001$).

The number of type I fibres was too small to perform statistical analysis on, and consequently they were excluded from further analysis.

Five days after transfection, 876 fibres were analysed and their relative SDH activity levels are presented in table 3.3/figure 3.6 (hatched columns). PPARδ transfected IIb fibres showed a significant increase in SDH activity level compared to normal IIb fibres ($p<0.001$) and sham transfected IIb fibres ($p<0.001$). On average, the SDH activity of PPARδ transfected IIb fibres increased to 0.145 when that of normal IIb fibres was defined as 0 and that of normal IIa fibres as 1 (figure 3.6C, hatched columns). There were no significant differences between sham transfected and normal IIb fibres ($p>0.05$), nor between PPARδ

transfected, sham transfected and normal IIa and IIx fibres ($p>0.05$) (figure 3.6A, B, hatched columns).

Fourteen days after transfection, 1237 fibres were analysed and their relative SDH activity levels are presented in table 3.3/figure 3.6 (open columns). PPARδ transfected IIa fibres showed a significant increase in SDH activity level compared to normal IIa fibres ($p<0.001$) and sham transfected IIa fibres ($p<0.01$). On average, the SDH activity of PPARδ transfected IIa fibres increased to 1.196 when that of normal IIa fibres was set to 1 and that of normal IIb fibres to 0 (figure 3.6A, open columns). The SDH activity of PPARδ transfected IIx fibres on average increased to 0.705 compared to normal IIx fibres ($p<0.001$), having a mean SDH activity level of 0.336, and sham transfected IIx fibres ($p<0.001$) (figure 3.6B, open columns). The SDH activity level of PPARδ transfected IIb fibres increased significantly compared to normal IIb fibres ($p<0.001$) and sham transfected IIb fibres ($p<0.001$). On average, the SDH activity level of PPARδ transfected IIb fibres increased to 0.401 when that of normal IIa fibres was set to 1 and that of normal IIb fibres to 0 (figure 3.6C, open columns). There were no significant differences in SDH activity between the controls; the sham transfected and the normal IIa, IIx and IIb fibres ($p>0.05$).

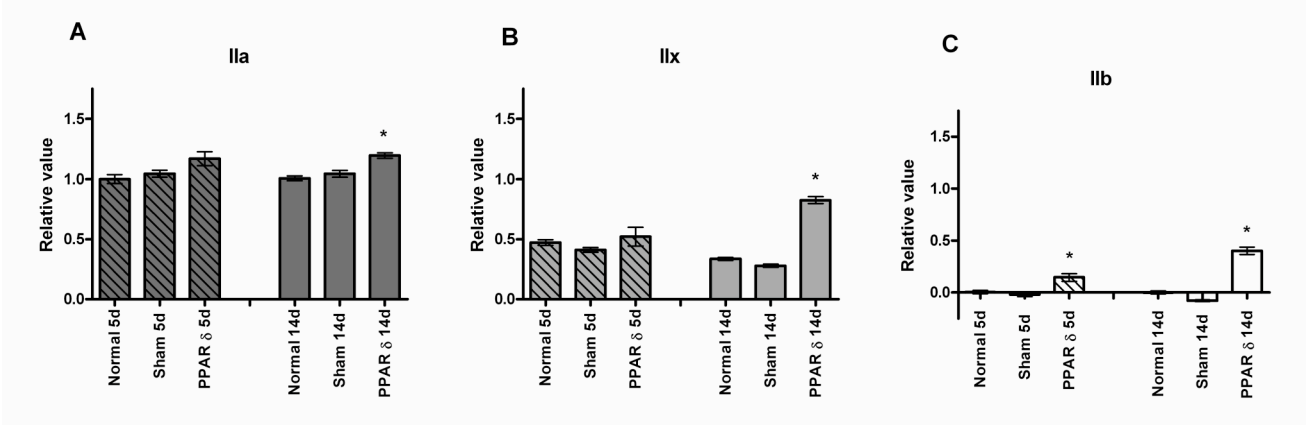

Figure 3.6 A, B, C Relative SDH activity levels of IIa, IIx and IIb fibres 5 and 14 days after VP16-PPARδ transfection and in control groups

Relative SDH levels are presented in mean ± SEM. 5 days after transfection, PPARδ transfected IIb fibres had a significantly increased SDH activity level compared to controls ($p<0.001$) (*) (hatched columns). 14 days after transfection, PPARδ transfected IIa, IIx and IIb fibres had significantly increased SDH activity level compared to that of sham transfected and normal controls ($p<0.001$) (open columns). There were no significant differences between the control groups ($p>0.05$).

3.5 Wild type PPARδ expression

Histochemical staining for PPARδ expression was performed on muscle cross sections from normal *non*-treated rats in order to visualize potential differences in expression patterns between one typical fast leg muscle, EDL, and one typical slow leg muscle, SOL. Expression patterns were compared to SDH activity level, MyHC expression and localization of nuclei by histochemical staining of neighbouring sections.

3.5.1 Wild type PPARδ expression in normal rat EDL

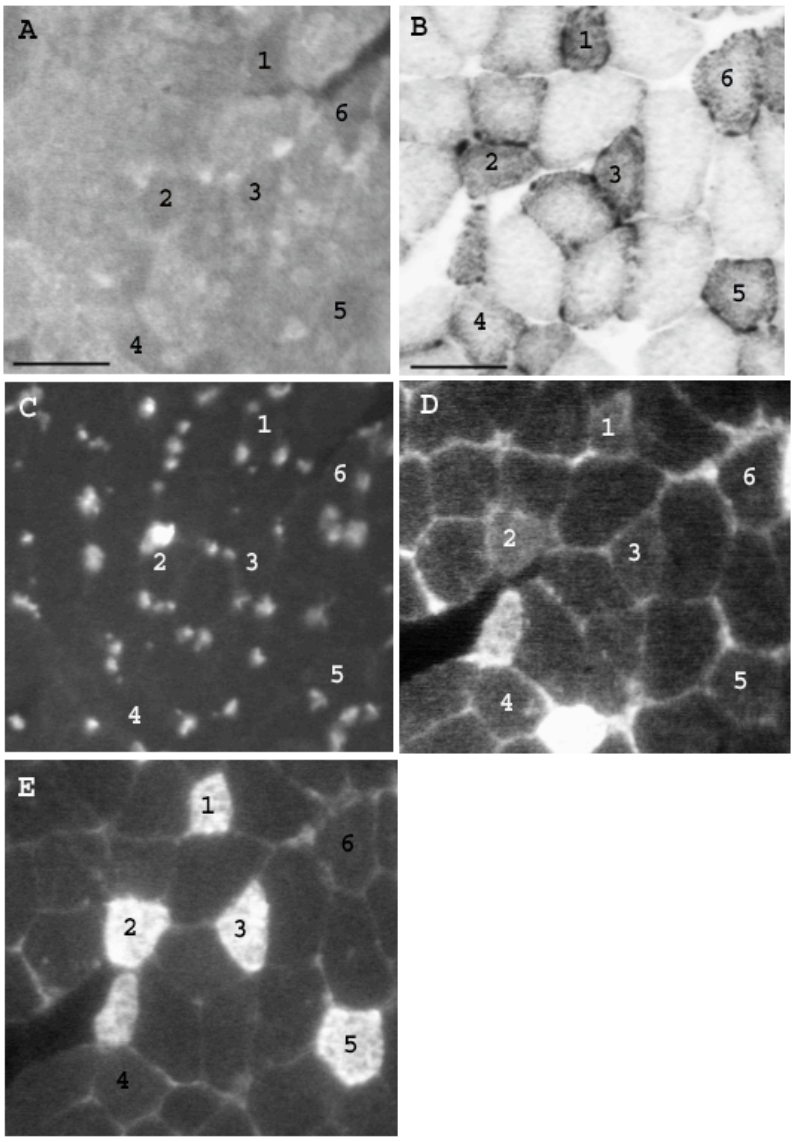

Figure 3.7 Serial sections of normal rat EDL stained for PPARδ, SDH activity, nuclei, MyHC I and IIa
Serial cross sections stained for PPARδ expression (A), SDH activity level (B), localization of nuclei (C), MyHC I (D) and MyHC IIa (E). Cytosolic PPARδ expression was uniform and weak, although some fibres, such as 1-6 were distinguished by even weaker expression. These showed nucleic PPARδ expression (A/C), had the highest SDH activity level (B) and 1-3, 5 expressed MyHC IIa (E). Scale bars: 50 µm.

As can be seen from figure 3.7, there was a relatively uniform and weak cytosolic PPARδ signal in normal rat EDL. Seven muscles were stained and this pattern was confirmed in all, in addition to in two muscles from NMRI mice (data not shown).

On close examination, some fibres showed distinctly weaker cytosolic PPARδ expression than the majority; all having a small CSA and a high SDH activity; characteristics of type I and IIa fibres in EDL, for instance fibres 1, 2, 3 and 5 in figure 3.7. Some of these fibres expressed PPARδ in nuclei, such as for example fibres 2 and 3 in figure 3.7.

3.5.2 Wild type PPARδ expression in normal rat SOL

The SOL muscle is a typical slow muscle exclusively composed of oxidative fibres. MyHC fibre type composition of WISTAR rat SOL has previously been reported to be 72 % type I, 25 % type IIa, 3 % type IIx and 0 % type IIb. As opposed to in EDL, the SDH activity of IIa fibres in SOL is higher than that of type I fibres, and type I fibres have the largest CSA (Nakatani *et al.*, 1999), also confirmed by this study.

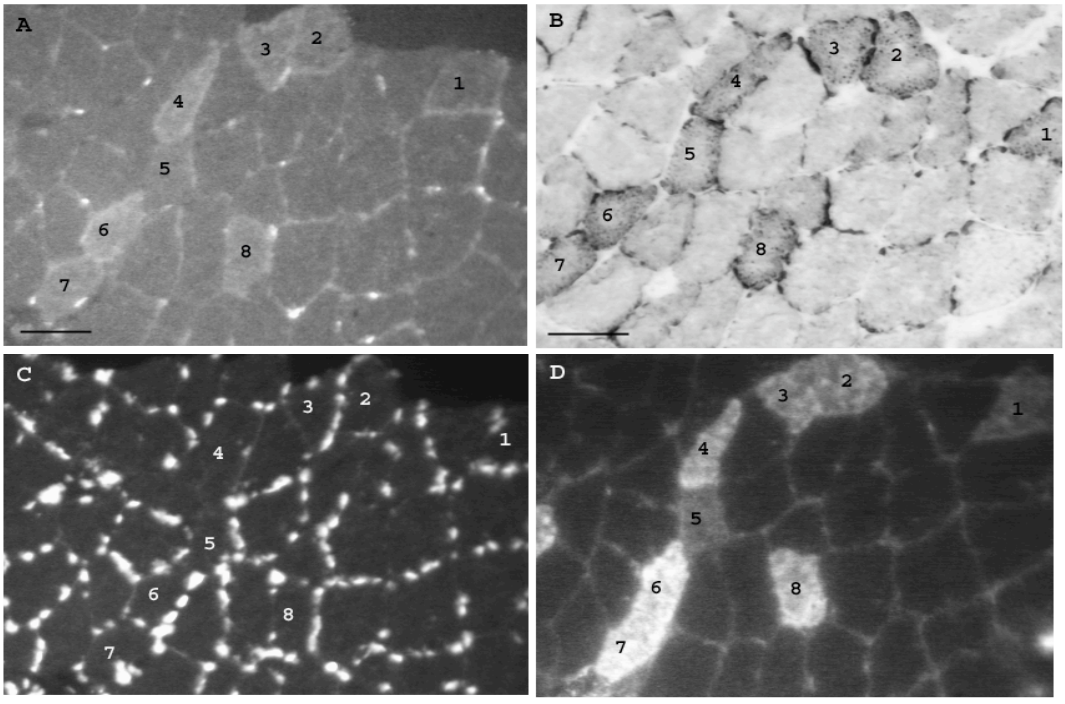

Figure 3.8 Serial sections of normal rat SOL stained for PPARδ, SDH activity, nuclei and MyHC IIa
Serial cross sections stained for PPARδ expression (A), SDH activity level (B), localization of nuclei (C) and MyHC IIa (D). Fibres 1-8 had the highest SDH activity level (B), expressed MyHC type IIa (D) and expressed PPARδ at the highest cytosolic level (A). Other fibres were type I and expressed PPARδ in nuclei. Scale bars: 50 μm.

As can be seen from figure 3.8, there was a strong correlation between cytosolic PPARδ expression and SDH activity level in muscle fibres from normal rat SOL. Fibres having the highest SDH activity levels (fibres 1-8) also expressed PPARδ at the highest cytosolic levels (fibres 1-8), and also in nuclei. Fibres 1-8 were all identified as type IIa, shown in figure 3.8D. Additionally, there was a consistency between the observed strength of cytosolic PPARδ expression, SDH activity level and MyHC expression. For example, fibre number 5 had a relatively low cytosolic PPARδ expression, a somewhat lower SDH activity level and was probably a MyHC IIa/IIx hybrid. Apart from fibres 1-8, other fibres seen in figure 3.8 were all type I fibres, and expressed PPARδ only in the nuclei. No IIx fibres were observed.

100 % of fibres positive for cytosolic PPARδ expression, similar to fibres 1-8 in figure 3.8A, expressed MyHC type IIa. These were also the fibres with the highest SDH activity levels observed both in previous studies and in this. A total of 289 cytosolic PPARδ positive fibres from two muscles were analysed; all being type IIa and all being the ones with the relatively highest SHD activity.

The expression pattern of PPARδ in EDL and SOL is summarized in table 3.4.

Table 3.4 Cytosolic and nucleic localization of wild type PPARδ protein as observed in normal rat EDL and SOL

Muscle:	**Subcellular localization of PPARδ:**	**I**	**IIa**	**IIx**	**IIb**
EDL	Nucleic	+	+	-	-
SOL	Nucleic	++	+	-	-
EDL	Cytosolic	-	-	+	+
SOL	Cytosolic	-	++	NA	NA

Scale: - (no detectable signal), + (signal), ++ (strong signal),

NA (not available; no IIx and IIb fibres were observed in SOL)

4 DISCUSSION

The present study show that overexpression of an intrinsically active PPARδ (VP16-PPARδ) in EDL muscles of adult rats affect MyHC expression, SDH activity level and CSA of individual fibres in a slow and oxidative direction. SDH activity and fibre size were affected already after five days, while changes in MyHC fibre type were detected after fourteen days, when a significantly higher proportion of IIa fibres and a significantly lower proportion of IIb fibres, was observed. Furthermore, immunohistochemical data from wild type muscle indicated higher levels of PPARδ in nuclei of slow/oxidative fibres than in fast/glycolytic fibres. The findings that nucleic PPARδ level is highest in slow/oxidative fibres and that PPARδ has the ability to activate a slow gene program, suggest that PPARδ might function as a mediator of adult muscle plasticity. Surprisingly; IIa, IIx and IIb fibres expressed PPARδ in the cytosol. This variability in subcellular distribution might indicate that not only the level, but also the translocation of PPARδ, could be regulated.

4.1 PPARδ and its effect on MyHC expression of skeletal muscle fibres

Contractile proteins are among the most stable of all proteins. MyHC expression has previously been shown to be regulated mainly at the transcriptional level (Izumo *et al.*, 1986), and to have a relatively slow turnover rate (Martin *et al.*, 1977). However, the rates of synthesis have been shown to be faster for MyHC I and IIa than for IIx and IIb in male WISTAR rat EDL, and MyHC IIb has been shown to have the most stable expression (Maier *et al.*, 1988). As reported by Maier *et al.* (1988), MyHC type IIa has a turnover period of 6-10 days, MyHC IIx 14-21 days and type IIb 10-14 days. Furthermore, Maier and co-workers observed that an initial increase in type IIa fibres (6-10 days) was accompanied by a moderate decrease in type IIb fibres in rat EDL after chronical slow stimulation using implanted electrodes, followed by an extensive decrease in type IIb fibres 14-21 days after the onset of stimulation. These results are consistent with the results from this study, also observing fast to slow transformation in the same muscle, rat strain and gender. The effect on type IIa fibres was significant; a doubling in the proportion of IIa was observed fourteen days after VP16-PPARδ transfection. Moreover, no effect was seen on the IIx population, and the increase in IIa fibres was accompanied by a decrease in type IIb fibres. As could have been predicted from the turnover rates, no effect was observed on fibre type transformation five days after transfection.

Fibre type transformations have previously been shown to include stages in which two MyHC subtypes are co-expressed (Maier *et al.*, 1988; Pette & Staron, 1997; Windisch *et al.*, 1998). The far most frequent hybrid fibres in the EDL would be the IIa/IIx and IIx/IIb fibre types, but these are not possible to detect by certainty as there to date is no available IIx antibody (only a *non*-IIx antibody). Consequently, we were unable to detect a possible and likely increase in hybrid fibres after PPARδ transfection. Furthermore, we may have overestimated the number of "pure" IIa (possibly IIa/IIx hybrids) and IIb fibres (possibly IIx/IIb hybrids). In the view of this, our results represent a conservative estimate of the transformations caused by overexpression of active PPARδ.

Endurance training may induce fast to slow MyHC transitions in muscle fibres (Andersen & Henriksson, 1977; Baumann *et al.*, 1987; Fitzsimons *et al.*, 1990; Sullivan *et al.*, 1995; Demirel *et al.*, 1999; Mercier *et al.*, 1999; Andersen *et al.*, 2000; Allen *et al.*, 2001). As PPARδ was shown to induce similar effects, PPARδ is a potential candidate mediating adaptation to endurance training in adult skeletal muscle. Indeed, an increase in the PPARδ protein content following endurance training in mice and humans has been reported (Luquet *et al.*, 2003; Mahoney *et al.*, 2005). This regulation of the expression level of PPARδ itself has been confirmed in our group by micro array analysis of mRNA levels in EDL and SOL, and in the same muscles subjected to slow and fast electrical stimulation, respectively (Rana *et al.*, unpublished). The signal intensity had the following order: SOL WT>EDL slow stimulated>EDL WT>SOL fast stimulated; showing that a slow electrical activity pattern up-regulated PPARδ expression level. Moreover, treatment of wild type rodents with a synthetic PPARδ agonist has been shown to increase the expression of genes involved in the slow twitch contractile apparatus, such as myoglobin and troponin I slow, but not MyHC, and to decrease the expression of troponin I fast (Wang *et al.*, 2004; Barish *et al.*, 2006). These results, in addition to the results from our study, indicate that activation of PPARδ in adult animals may induce changes in contractile properties of skeletal muscle fibres in slow direction.

4.2 PPARδ and its effect on cross sectional area of skeletal muscle fibres

Cross sectional area of muscle fibres is associated with MyHC expression and oxidative state. In EDL and mixed muscles in general, type I fibres normally have the smallest CSA, type IIb fibres the largest and type IIa and IIx fibres intermediate CSAs. Furthermore, there is an inverse relationship between CSA and SDH activity level for the different fibre types (Rivero *et al.*, 1998; Nakatani *et al.*, 1999). These correlations were also observed in

this study, and may be explained by the fact that slow twitch fibres with high oxidative capacity and good endurance are in need of constant supply of oxygen and nutrition from the blood, in part ensured by short diffusion distances.

Endurance training may induce reductions in CSA of individual skeletal muscle fibres, previously observed in endurance trained rats and fast rat muscles subjected to slow electrical stimulation (Brown *et al.*, 1976; Tamaki, 1987; Demirel *et al.*, 1999). As an active PPARδ was shown to promote similar CSA reductions, the effects on CSA seen after endurance training in adult animals might be mediated by PPARδ. Another transcription factor linked to slow activity, myogenin, has also been shown to induce reduced CSAs of type II fibres when overexpressed in EDL of mice (Ekmark *et al.*, 2003). However, when ligand dependent PPARδ was overexpressed in skeletal muscle using a cre/lox approach, no effect was seen on the CSAs of the different muscles (Luquet *et al.*, 2003). The CSA of individual fibres was not measured by Luquet and co-workers, only that of the whole muscle, and the receptor was not activated by exogenous ligands or in an intrinsically active form, possibly explaining the lack of observed effect of PPARδ overexpression on muscle CSA.

4.3 PPARδ and its effect on oxidative capacity of skeletal muscle fibres

Results from this study show that reductions in cross sectional area are accompanied by increased SDH activity levels of PPARδ transfected EDL fibres. This is in accordance with the inverse relationship between SDH activity level and CSA observed by others (Rivero *et al.*, 1998; Nakatani *et al.*, 1999). The results also show that changes in CSA and oxidative capacity of individual fibres precede changes in MyHC expression.

Endurance training frequently increases the oxidative metabolism of skeletal muscles without inducing MyHC transitions (Baldwin *et al.*, 1972; Fitts *et al.*, 1975; Holloszy & Booth, 1976; Dudley *et al.*, 1982; Demirel *et al.*, 1999; Carter *et al.*, 2001). Holloszy & Booth (1976) suggested that the increase in oxidative capacity following endurance exercise was a result of an increased number of, increased size of, or altered quality of mitochondria. This hypothesis has later been confirmed in several studies (Hoppeler *et al.*, 1973; Kiessling *et al.*, 1974; Katsuta *et al.*, 1988). As an active PPARδ was shown to increase the SDH activity of individual muscle fibres, effects of endurance training on oxidative capacity might be mediated by PPARδ, just as another slow transcription factor, myogenin, has been shown to induce increased SDH activity of type II fibres when overexpressed in mice EDL (Ekmark *et al.*, 2003). However, an increase in the SDH activity level of IIb fibres was detected before

that of IIa and IIx fibres, already five days after PPARδ transfection. This might be explained by the low starting level, and hence greater potential for increase, of IIb fibres in EDL.

Further support for a role of PPARδ in the regulation of oxidative metabolism and adaptation to endurance training has been found by looking at the expression patterns of possible PPARδ target genes. Luquet *et al*. (2003) observed an up-regulation of three oxidative enzymes; SDH, citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (BOAC) after transgenic overexpression of PPARδ in skeletal muscle, using a cre/lox approach. Conversely, activities of glycolytic enzymes remained unchanged. Two other proteins involved in fatty acid oxidation were also up-regulated: uncoupling protein 2 (UCP-2) and heart fatty acid binding protein (H-FABP). In the same study they observed a considerable increase in the number of SDH positive fibres in the fast *tibialis anterior* of double transgenic mice, when analyzing histological sections. Although no effect was observed on MyHC expression or CSA of muscles, overexpression of PPARδ resulted in changes in the muscles reminiscent of adaptations promoted by endurance training. Wang *et al*. (2004) reported an up-regulation of several genes involved in oxidative metabolism and mitochondrial biogenesis after transgenic overexpression of the intrinsically active VP16-PPARδ in skeletal muscle, and mitochondrial DNA was increased significantly in *gastrocnemius* of the transgenic mice. Treatment of wild type mice with a specific agonist even induced the similar target genes, among them PGC-1α, further supporting the hypothesis of a role for PPARδ in the regulation of oxidative capacity of muscle fibres.

4.4 Expression patterns of PPARδ in wild type skeletal muscle

It has previously been shown that PPARδ mRNA and protein levels are higher in homogenates from the slow SOL than fast EDL muscle (Wang *et al.*, 2004). However, we are the first to investigate the precise fibre type distribution and the subcellular localization of the PPARδ protein in muscle fibres.

The distinct subcellular localization patterns of PPARδ under normal conditions imply PPARδ in the regulation of a slow and oxidative phenotype in adult skeletal muscle, and might indirectly link level of expression and subcellular localization to muscle activity. Observations from histological staining of normal SOL and EDL muscles were summarized in table 3.4 (section 3.5.2). Fibre types I and IIa had PPARδ localized to nuclei while fibre types IIx and IIb had PPARδ localized to cytosol. However, type IIa fibres of SOL additionally expressed PPARδ in the cytosol, i.e. all type II fibres expressed PPARδ in the cytosol, while type I fibres showed only nucleic expression. As PPARδ functions in the

nucleus, and has been implicated in the regulation of a slow and oxidative phenotype of adult muscle fibres in the first part of this study, the high levels of nucleic staining found in slow/oxidative type I and IIa fibres were expected, and further supported the hypothesis of a role for PPARδ in the regulation of a slow and/or oxidative phenotype. Furthermore, by observing the stained sections, we got the impression that the total level of PPARδ protein in SOL and EDL was consistent with previous findings from muscle homogenates; SOL>EDL (Wang *et al.*, 2004), also implying PPARδ in the regulation of a slow/oxidative phenotype.

The observation of high cytosolic PPARδ levels, however, was surprising. Shown by Berger *et al*. (2000), the nuclear import of PPARs is not dependent on binding of ligand, but rather a constitutive shuttle of translated protein from the cytoplasm to the nucleus. However, the nucleo-cytoplasmic shuttling of PPARγ has been shown to be influenced by bacterial infection (Kelly *et al.*, 2004), and activity dependent translocation of the slow transcription factor NFAT has recently been shown in skeletal muscle (Tothova *et al.*, 2006). Based on the latter publications and the observation in this study that fast/glycolytic fibre types show cytosolic expression whereas slow/oxidative fibres show nucleic expression, we hypothesize that translocation of PPARδ to the nucleus might be mediated by slow activity. This would constitute a completely new mechanism of action and level of regulation of PPARδ and of the PPAR family as a whole. PPARδ activity has previously been shown to be regulated at the transcriptional level (Luquet *et al.*, 2003; Mahoney *et al.*, 2005) and at the level of ligand or dimerization partner activation (Kliewer *et al.*, 1992; Keller *et al.*, 1993; Xu *et al.*, 1999; Wang *et al.*, 2004), but also by cofactors (Berger & Moller, 2002; Krogsdam *et al.*, 2002; Gilde & Van Bilsen, 2003; Wang *et al.*, 2003) and possibly by phosphorylation (Gilde & Van Bilsen, 2003; Diradourian *et al.*, 2005; Gelman *et al.*, 2005). The proposed activity dependent translocation might involve Ca^{2+} dependent phosphorylation prior to translocation, with Ca^{2+} transient levels being the initial mediator of slow activity. This potential regulation of nuclear translocation in adult animals might also constitute a new target mechanism for the pharmaceutical industry in pharmacological treatment of metabolic diseases.

4.5 PPARδ's role in the excitation-transcription coupling in skeletal muscle

Previous publications have hypothesized that PPARδ is a mediator in the excitation-transcription coupling in skeletal muscles, based on results from transgenic models (Luquet *et al.*, 2003; Wang *et al.*, 2004). This work shows that PPARδ is a probable mediator in these signalling pathways also in adult animals, and indicate a possible link to slow activity and endurance training which previously have been shown to promote similar transformations.

While endurance training might induce changes in the expression levels of PPARδ (Luquet *et al.*, 2003; Mahoney *et al.*, 2005), another hypothesis is that changes in the levels of FFAs and FA metabolites accompanying muscle activity itself, serve as signals for changes in muscle phenotype by activating PPARδ. Physiological conditions associated with a rise in the concentration of FFAs and increased FAO are long-time fasting and endurance exercise. Treatment of wild type rodents with a synthetic PPARδ agonist has, as mentioned, been shown to increase the expression of genes involved in the slow twitch contractile apparatus (Wang *et al.*, 2004; Barish *et al.*, 2006), supporting the hypothesis of ligands serving as internal signals for muscle adaptation to slow activity. Furthermore, the level of PPARδ protein seems to be under nutritional control, being dramatically up-regulated in *gastrocnemius* muscle of mice after a 24 hour starvation period, and restored to control levels upon refeeding (Holst *et al.*, 2003). An up-regulation of fatty acid translocase/CD36 (FAT/CD36) and H-FABP in *gastrocnemius* after the starvation period was also reported, paralleling mRNA levels of PPARδ, also these levels being restored to normal upon refeeding. These results support the hypothesis of ligands serving as internal signals for adaptation to fasting, another physiological condition in which the muscle adapts to lipid catabolism. Possibly functioning as a lipid "sensor", PPARδ is able to induce genes involved in oxidative metabolism and mitochondrial biogenesis, ensuring supplies of ATP when levels of carbohydrates are low.

A model of the mechanism of action of PPARδ in muscle adaptation to endurance exercise and long-time fasting is shown in figure 4.1.

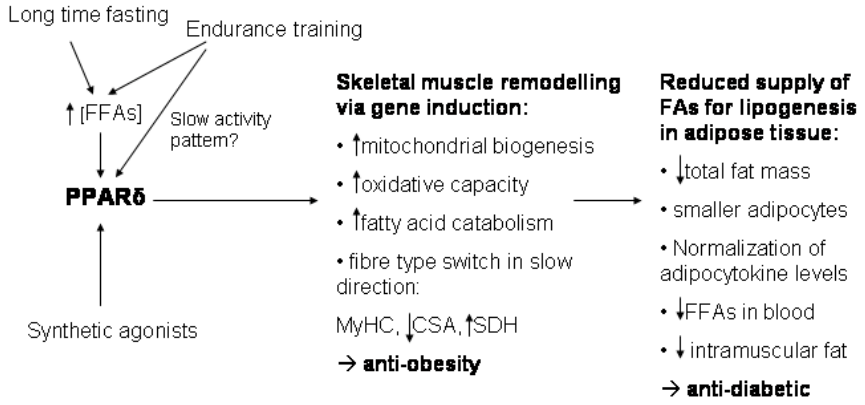

Figure 4.1 Proposed mechanism of action for PPARδ in muscle adaptation to endurance exercise and long-time fasting, and in the prevention of development of obesity and diabetes II

Activation of PPARδ by endogenous or synthetic ligands is proposed to induce a skeletal muscle remodelling in slow direction and increasing oxidation of fatty acids, resulting in an anti-obesity effect. The resulting reduced supply of free fatty acids for lipogenesis in adipose tissue gives smaller adipocyte sizes, a normalization of adipocytokine levels and an insulin sensitizing effect in peripheral tissue (anti-diabetic effect).

However, there are different hypotheses concerning the role of PPARδ in the excitation-transcription signalling network. Several lines of evidence support an interaction between PPARδ and co-activator PGC-1α (Wu *et al.*, 1999; Lehman *et al.*, 2000; Puigserver & Spiegelman, 2003). Tanaka *et al.* (2003) showed that activation of PPARδ by the selective agonist GW501516 induced PGC-1α mRNA levels *in vitro* and *in vivo*, an effect accompanied by increased mitochondrial biogenesis, and identified a highly conserved PPRE in the PGC-1α promoter. Wang *et al*. (2003) identified an *in vivo* association between PPARδ and PGC-1α in nuclear extracts from *gastrocnemius* muscle, suggesting that the thermogenic function of PGC-1α might be mediated by PPARδ and that PGC-1α might be an activator of PPARδ in muscle. Lin *et al*. (2002) showed that overexpression of PGC-1α in transgenic mice induced the formation of slow twitch fibres of skeletal muscle, with increased myoglobin content and induction of genes involved in oxidative metabolism; essentially the same muscle remodelling observed when the intrinsically active PPARδ was overexpressed in transgenic mice by Wang *et al*. (2004). Furthermore, the results from this study and from Luquet *et al*. (2003) support the hypothesis of a role for PPARδ in the formation and maintenance of oxidative, slow twitch fibres in skeletal muscle. Endurance training has previously been shown to increase both the level of PGC-1α (Goto *et al.*, 2000) and PPARδ (Luquet *et al.*, 2003; Mahoney *et al.*, 2005) in rodents and humans. Collectively, these results support a role for both PPARδ and PGC-1α in the excitation-transcription coupling in skeletal muscle, probably working in concert mediating a slow and oxidative phenotype. However, Wang *et al*. (2004) has also implicated PPARδ as a potential downstream transcription factor in the CaN or CaMK signalling pathways. A possible model of the role of PPARδ in the excitation-transcription coupling is presented in figure 4.2.

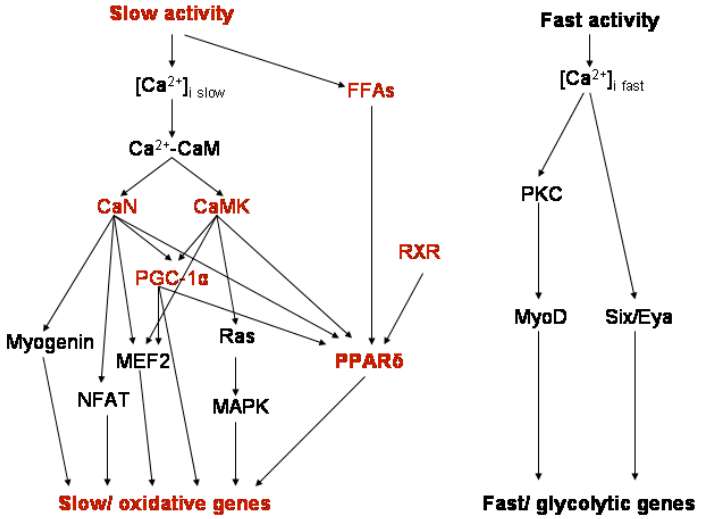

Figure 4.2 Proposed role of PPARδ in the excitation-transcription coupling in skeletal muscle

PPARδ is suggested as a mediator of slow activity, influencing on the expression of slow and oxidative muscle specific genes.

4.6 Roles of PPARδ in health and disease

"Metabolic syndrome", typified by diabetes II, obesity, hyperlipidaemia and cardiovascular diseases, has reached epidemic proportions in western societies. These pathologies are associated with a combination of excessive lipid intake and lack of physical exercise, yet the actual molecular mechanisms leading to the metabolic syndrome have not been fully clarified (Fredenrich & Grimaldi, 2004; Berge *et al.*, 2005; Eckel *et al.*, 2005; Grimaldi, 2005; Grundy, 2005; Sarti & Gallagher, 2006). The risk factors, however, are directly influenced by diet, metabolism and physical activity.

Skeletal muscle is a major mass peripheral tissue and accounts for more than 30% of the energy expenditure of the body. Muscle is the primary tissue of insulin stimulated glucose uptake, disposal and storage, and also influences metabolism by modulation of lipid flux (Smith & Muscat, 2005; Barish *et al.*, 2006). Fat accumulation in adipose tissue and especially in muscle is known to predispose for insulin resistance (Perseghin *et al.*, 1999; Unger & Orci, 2000; Fasshauer & Paschke, 2003; Luquet *et al.*, 2003; Luquet *et al.*, 2004; Perseghin, 2005). Consequently, skeletal muscle metabolism plays a significant role in insulin sensitivity, blood lipid profile, obesity and physical fitness.

Metabolism is largely regulated by nuclear hormone receptors functioning as transcription factors (Beaven & Tontonoz, 2006), among these PPARδ. PPARδ was in this study shown to affect oxidative capacity and MyHC fibre type of adult skeletal muscle, and to possibly be regulated at the level of nuclear translocation.

These results may have useful therapeutic applications as muscle fibre type composition previously has been shown to be associated with obesity, weight loss and diabetes II. Skeletal muscles of obese and diabetic II patients have been shown to have reduced oxidative capacity, increased glycolytic capacity and a decreased percentage of type I fibres (Abou *et al.*, 1992; Hickey *et al.*, 1995; Tanner *et al.*, 2002). Furthermore, single nucleotide polymorphisms (SNPs) in the PPARδ gene have also been associated with obesity. Out of nine SNPs identified, five were significantly associated with obesity (Shin *et al.*, 2004). Vänttinen *et al*. (2005) found associations between muscle insulin sensitivity and three SNPs, while an association between one SNP and concentrations of HDL and LDL cholesterol was found by Skogsberg *et al.* (2003).

Treatment with synthetic PPARδ agonists has been shown to improve the lipid profile of mice and monkeys and to reverse diet-induced obesity and insulin resistance of mice (Leibowitz *et al.*, 2000; Oliver *et al.*, 2001; Tanaka *et al.*, 2003; Wang *et al.*, 2003; Wang *et*

al., 2004; Grimaldi, 2005; Staels & Fruchart, 2005). Experimental evidence coming from both *in vitro* and *in vivo* approaches has indicated that both adipose tissue and skeletal muscle play important roles in the lipid-lowering and anti-diabetic actions of PPARδ activators, affecting mainly plasma lipid levels, total adiposity and adipocytokine production (Unger & Orci, 2000; Fasshauer & Paschke, 2003; Evans *et al.*, 2004; Luquet *et al.*, 2004).

As with any drug anticipated in the treatment of diseases, safety issues concerning possible side effects of *non*-tissue-specific PPARδ agonists, have been raised. Particular attention has focused on a potential connection to carcinogenesis, as PPARδ is expressed at a high level in colon tumours and has been ascribed an oncogenic function after being identified as a direct transcriptional target of β-catenin (He *et al.*, 1999). This hypothesis was further reinforced by the demonstration that colon cancer cells in which PPARδ had been knocked out, failed to form tumours in mice (Park *et al.*, 2001). However, carcinogenic function was not confirmed by Barak *et al*. (2002) nor by Harman *et al*. (2004), with the latter group actually showing that PPARδ attenuates colon carcinogenesis. The implication of PPARδ in carcinogenesis is still a matter of debate, however, a *non*-tissue-specific PPARδ agonist is in these days undergoing clinical trials in obese and diabetic patients (GlaxoSmithKline) (Martindale, 2004). The potential regulated nuclear translocation in muscle, implicated by this study, may have possible applications in the development of a muscle specific drug to avoid potential adverse side effects.

4.7 Future perspectives

To possibly link regulation of the PPARδ protein to specific patterns of activity, protein levels in muscles subjected to different patterns of electrical stimulation could be measured on Western blots or visualized on histological sections. As to the potential regulation of nuclear translocation of PPARδ, it would be interesting to histochemically stain sections from other wild type muscles than SOL and EDL, like the fast *tibialis anterior* and mixed *gastrocnemius* muscle, and sections from electrically stimulated muscles. To further investigate this possible activity mediated nuclear translocation of PPARδ, PPARδ-GFP fusion proteins similar to what has been done for NFAT, could be made (Tothova *et al.*, 2006). Muscles could be subjected to different patterns of stimulation and transfected fibres directly observed by *in vivo* imaging (Bruusgaard *et al.*, 2003; Rana *et al.*, 2005).

Based on the effects on MyHC expression seen in the overexpression study fourteen days after transfection, it would be interesting to extend the time period from transfection to muscle excision. As observed by Maier *et al*. (1988), transformation of type IIx and IIb fibres

in rat EDL after chronic slow stimulation was greatest 14-21 days after the onset. An increased observation period was not possible because of immune responses to β-galactosidase or bacterial antigens from the expression system (McMahon *et al.*, 1998), however promising DNA purification procedures might allow longer periods of exposure (Chen *et al.*, 2005).

Another interesting gain-of-function approach than the one performed in this study, would be to activate endogenous PPARδ *in vivo* by delivery of agonists locally in the muscle. This could be done by implanting mini-osmotic pumps, delivering naturally occurring lipids, the synthetic metabolically active fatty acid TTA or the specific agonist GW501516. Results could indirectly link PPARδ to endurance activity, a condition in which the concentration of FFAs is naturally high.

Conceptually, loss-of-function experiments are more powerful than overexpression studies in determining the relative importance of a molecular mechanism. As most PPARδ null mice die (Peters *et al.*, 2000; Barak *et al.*, 2002) and the few surviving null mice have developmental impairments, a better approach might be to look at phenotypic properties of individual muscle fibres where the PPARδ gene is knocked out. Muscle specific single cell "knock-outs" could be made by injecting siRNA *in vivo*, using electroporation as described by Golzio *et al.* (2005). This method is essentially the same as the one used in the overexpression studies of this work. Muscle specific single cell "knock-outs" could also be made by using a cre/lox approach combined with intracellular injections and electroporation of cre expression vectors.

Promoter analysis of fibre type specific genes could be another approach to establish the role of PPARδ in the excitation-transcription coupling. By examining promoter regions of potential target genes, one can possibly find out whether or not PPARδ mediates activity effects by direct or indirect transcriptional control of target genes. More specific, this work would include the search for conserved PPREs upstream of typical slow or oxidative genes, such as troponin I slow, PGC-1α, MyHC I/IIa or SDH. Furthermore, ChIP assays (Chromatin immunoprecipitation) could be performed in order to determine if these genes are direct targets of PPARδ.

4.8 Conclusions

1. Overexpression of an intrinsically active PPARδ (VP16-PPARδ) in the EDL muscle of adult rats induced significant phenotypic changes in slow and oxidative direction, shown by changes in MyHC expression from fast to slow isotype, reduction in CSA and increased SDH activity of transfected fibres, compared to controls.

2. There is a differential expression pattern of the wild type PPARδ protein among different muscles and fibre types with respect to level of expression and subcellular localization. High levels of PPARδ in cell nuclei were correlated to an oxidative and slow phenotype, while high cytosolic levels were correlated to a fast and glycolytic phenotype. These differences in subcellular localization could reflect that nuclear translocation is regulated and imply PPARδ in the regulation of a slow and/or oxidative phenotype.

These findings suggest a role for PPARδ in the excitation-transcription coupling in adult skeletal muscle, regulating a slow and oxidative phenotype. This PPARδ signalling pathway may be important during muscle adaptation to endurance exercise in adult animals.

5 APPENDICES

5.1 Appendix A

5.1.1 Equithesin (1 ml)

(Sykehusapoteket ved Rikshospitalet, Norway, catalogue number 601284)

Chloralhydrate	42.5 mg
Magnesiumsulphateheptahydrate	21.0 mg
Pentobarbital	9.7 mg
Ethanol (96 %)	76.0 mg
Propyleneglycol	428.0 mg
dH_20	

5.1.2 pAP-lacZ DNA solution (100 µl)

pAP-lacZ in H_2O (2 µg/µl)	50 µl
4 M NaCl	4 µl
dH_20	46 µl

5.1.3 pCMX-VP16-PPARδ and pAP-lacZ DNA solution (100 µl)

pCMX-VP16-PPARδ in H_2O (2 µg/µl)	25 µl
pAP-lacZ in H_2O (2 µg/µl)	25 µl
4 M NaCl	4 µl
dH_20	46 µl

5.1.4 Staining for β-galactosidase activity

1. Thaw the sections to room temperature
2. Make the fixation solution:

(Para)Formalaldehyde (Electron Microscopy Sciences)	2.0 g
Gluteraldehyde (Electron Microscopy Sciences)	400 µl
10x PBS (pH 7.1)	10.0 ml
dH_20	69.2 ml

Solve the formalaldehyde in dH_2O (60ºC), adjust volume to 100 ml and pH to 7.1.

3. Fixate the sections at 4ºC for 20 minutes by encircling the sections using a hydrophobic pen (H-4000, Vector) and applying a large drop of solution
4. Wash the sections in PBS (pH 7.1), 3x 5 minutes
5. Make the β- galactosidase staining solution:

10x PBS (pH 7.1)	150 µl
0.2 M Potassium Ferrocyanide	30 µl
0.2 M Potassium Ferricyanide	30 µl
1 M $MgCl_2$	3 µl
dH_20	1260 µl
X-gal (50 mg in DMSO) (Promega)	30 µl

6. Stain over night at 37ºC
7. Wash the sections in PBS (pH 7.1), 3x 5 minutes
8. Mount the sections in glycerine gel:

Gelatine (PROLABO)	15 g
Glycerol (Invitrogen)	100 ml
dH_2O	100 ml

5.1.5 Staining for SDH activity

1. Make the 0.2 M phosphate buffer (pH 7.4):

Sodium phosphate (MERCK)	22.72 g
Potassium phosphate (MERCK)	5.40 g
dH_2O	1000 ml

Solve the salts in the water and adjust pH to 7.4

2. Make the 0,2 M sodium succinate substrate solution:

Sodium succinate (FLUKA Chemika)	16.2 g
dH_2O	500 ml

Solve the sodium succinate in the water.

3. Make the SDH staining solution:

0,2 M phosphate buffer	5 ml
0,2 M sodium succinate solution	5 ml
Nitro Blue Tetrazolium (NBT)	10 mg

4. Incubate the sections with staining solution for 45 minutes at 37ºC, after having encircled the sections with a hydrophobic pen
5. Make the fixation solution described in appendix A, 5.1.4
6. Fixate the sections at 4ºC for 20 minutes
7. Wash the sections in PBS (pH 7.4), 3x 5 minutes
8. Mount the sections in glycerine gel as described in appendix A, 5.1.4

5.1.6 Staining for MyHC isoform

Staining for MyHC I, MyHC IIa and MyHC *non*-IIx:

1. Use a hydrophobic pen to encircle the muscle sections
2. Dilute the primary antibody 1:2000 in 1 % BSA in PBS (pH 7.4)
3. Incubate the sections with the primary antibody for 60 minutes in room temperature
4. Wash the sections in PBS (pH 7.4), 3x 5 minutes
5. Dilute the secondary antibody 1:200 in 0.5 % BSA in PBS (pH 7.4)
6. Incubate the sections with the secondary antibody for 30 minutes at 37ºC
7. Wash the sections in PBS (pH 7.4), 3x 5 minutes

Staining for MyHC IIb:

1. Use a hydrophobic pen to encircle the muscle sections
2. Dilute the primary antibody 1:2000 in 0.5 % BSA in PBS (pH 7.4)
3. Incubate the sections with the primary antibody for 45 minutes at 37ºC
4. Wash the sections in PBS (pH 7.4), 3x 5 minutes
5. Dilute the secondary antibody 1:300 in 0.5 % BSA in PBS (pH 7.4)
6. Incubate the sections with the secondary antibody for 45 minutes at 37ºC
7. Wash the sections in PBS (pH 7.4), 3x 5 minutes

MyHC:	Primary antibody:	Secondary antibody:
I	BA-D5	Rabbit anti-mouse IgG, FITC conjugated (SIGMA, F-9137)
IIa	SC-71	Rabbit anti-mouse IgG, FITC conjugated (SIGMA, F-9137)
***Non*-IIx**	BF-35	Rabbit anti-mouse IgG, FITC conjugated (SIGMA, F-9137)
IIb	BF-F3	Anti-mouse IgM, Cyt 3 (J115-165-020, Jackson ImmunoResearch Laboratories)

5.1.7 Staining for VP16 expression

1. Use a hydrophobic pen to encircle the muscle sections
2. Dilute the primary antibody 1:300 in 1 % BSA in PBS (pH 7.4)
3. Incubate the sections with the primary antibody for 60 minutes in room temperature
4. Wash the sections in PBS (pH 7.4), 3x 5 minutes
5. Dilute the secondary antibody 1:500 in 0.5 % BSA in PBS (pH 7.4)
6. Incubate the sections with the secondary antibody for 30 minutes at 37ºC
7. Wash the sections in PBS (pH 7.4), 3x 5 minutes

Primary antibody:	**Secondary antibody:**
Rabbit IgG anti-VP16 (SIGMA, V4388)	Goat fluorescein anti-rabbit IgG (H+L) (FI-1000, Vector Laboratories Inc.)

5.1.8 Staining for PPARδ expression

1. Use a hydrophobic pen to encircle the muscle sections
2. Dilute the primary antibody 1:100 in 1 % BSA in PBS (pH 7.4)
3. Incubate the sections with the primary antibody for 60 minutes in room temperature
4. Wash the sections in PBS (pH 7.4), 3x 5 minutes
5. Dilute the secondary antibody 1:500 in 0.5 % BSA in PBS (pH 7.4)
6. Incubate the sections with the secondary antibody for 30 minutes at 37ºC
7. Wash the sections in PBS (pH 7.4), 3x 5 minutes

Primary antibody:	**Secondary antibody:**
Rabbit polyclonal IgG anti-PPARδ (Sc-7197, Santa Cruz Biotechnology Inc.)	Goat fluorescein anti-rabbit IgG (H+L) (FI-1000, Vector Laboratories Inc.)

5.1.9 Staining for localization of nuclei

1. Use a hydrophobic pen to encircle the muscle sections
2. Dilute the nucleic acid stain solution 1:1000 in PBS (pH 7.4)
3. Apply a drop of the diluted stain solution directly to the sections and incubate for 5-10 seconds
4. Wash in PBS (pH 7.4) for 1 minute

Nucleic acid stain solution:
Hoechst (33342, Molecular Probes, catalogue nr. H-3570)

5.2 Appendix B

5.2.1 Abbreviations

ATP	Adenosine triphosphate
bHLH	Basic helix-loop-helix
BOAC	β-hydroxyacyl-CoA dehydrogenase
CaM	Calmodulin
CaN	Calcineurin
CaMK	Calmodulin dependent protein kinase
ChIP	Chromatin immunoprecipitation
CMV	Cytomegalovirus
CS	Citrate synthase
CSA	Cross sectional area
DNA	Deoxyribonucleic acid
EDL	*musculus extensor digitorum longus*
FA	Fatty acid
FAO	Fatty acid oxidation
FAT/CD36	Fatty acid translocase/CD36
FFA	Free fatty acid
FITC	Fluorscein
GFP	Green fluorescent protein
HDL	High density lipoprotein
HEK-293	Human embryonic kidney cells 293
H-FABP	Heart fatty acid binding protein
HRP	Horse radish peroxidase
HSV	Herpes simplex virus
Ig	Immunoglobin
KS	Kolmogorov-Smirnov
LDL	Low density lipoprotein
MAPK	Mitogen activated protein kinase

mATPase	Myosin adenosine triphosphate –ase
MEF2	Myocyte enhancer factor 2
mRNA	Messenger ribonucleic acid
MyHC	Myosin heavy chain
MyLC	Myosin light chain
NFAT	Nuclear factor of activated T cells
PGC-1α	PPAR gamma coactivator 1 alpha
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PPARα	Peroxisome proliferator-activated receptor alpha
PPARδ	Peroxisome proliferator-activated receptor delta
PPARγ	Peroxisome proliferator-activated receptor gamma
PPRE	Peroxisome proliferator-activated receptor responsive element
RSV	Rouse sarcoma virus
RXR	9-*cis*-retinoid X receptor
SDH	Succinate dehydrogenase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
SEM	Standard error of the mean
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SOL	*musculus soleus*
SV	Simian virus
TTA	Tetradecylthioacetic acid
UCP-2	Uncoupling protein 2
V	Volt
VP16	Herpes simplex virus transactivator protein 16
X-gal	5-bromo-4-chloro-3-indolyle-β-D-galactoside

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