Pharmacological activation of PPARβ promotes rapid and calcineurin-dependent fiber remodeling and angiogenesis in mouse skeletal muscle

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Recent studies have shown that administration of peroxisome proliferator-activated receptor-β (PPARβ) agonists enhances fatty acid oxidation in rodent and human skeletal muscle and that muscle-restricted PPARβ overexpression affects muscle metabolic profile by increasing oxidative myofiber number, which raises the possibility that PPARβ agonists alter muscle morphology in adult animals. This possibility was examined in this study in which adult mice were treated with a PPARβ agonist, and the resulting changes in myofiber metabolic phenotype and angiogenesis were quantified in tibialis anterior muscles. The findings indicate a muscle remodeling that is completed within 2 days and is characterized by a 1.63-fold increase in oxidative fiber number and by a 1.55-fold increase in capillary density. These changes were associated with a quick and transient upregulation of myogenic and angiogenic markers. Both myogenic and angiogenic responses were dependent on the calcineurin pathway, as they were blunted by cyclosporine A administration. In conclusion, the data indicate that PPARβ activation is associated with a calcineurin-dependent effect on muscle morphology that enhances the oxidative phenotype.

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several genes implicated in myofiber type switching toward oxidative phenotype (37, 42) and angiogenesis (4, 26). We demonstrated that inhibition of calcineurin activity by cyclosporine A (CsA) administration totally prevented both hyperplasic and angiogenic responses to PPARβ agonist treatment, suggesting the involvement of a calcineurin-dependent signaling pathway in PPARβ-promoted muscle remodeling.

MATERIALS AND METHODS

Animals

Animals were maintained in a 12:12-h light-dark cycle and received food (UAR, Villemonais sur Orge, France) and water ad libitum. All experimental procedures were conducted in accordance with the guidelines of the University of Nice-Sophia Antipolis and approved by the Campus Valrose Animal care and ethics committee. PPARβ agonist administration. Ten-week-old male C57BL6J (Janvier, France) were used in the various experiments. GW0742, a PPARβ-specific activator (23), was dissolved in cremophor (Sigma) and injected subcutaneously once a day (9 am) at 1 mg/kg. Control animals received the vehicle at 9 AM. Animals were killed after the indicated times by cervical dislocation, and tibialis anterior muscles were harvested immediately after death. Forty mice were used for histological analysis in a first series of experiments (time points for both treated and control mice: 5, 24, 48, and 96 h; 5 animals per time point and condition). Fifty mice were used for protein analysis in a second series of experiments (time points for both treated and control mice: 2, 5, 24, 48, and 96 h; 5 animals per time point and condition).

PPARβ agonist and CsA coadministration. Twenty 10-wk-old male C57BL6J (five animals per group) were used. CsA (Sigma) treatments were performed twice a day (9 AM and 6 PM) by subcutaneous injections of the compound at 20 mg/kg in cremophor. GW0742 was dissolved in cremophor and injected subcutaneously once a day (2 pm) at 1 mg/kg. Injections of vehicle were performed at 9 AM, 2 PM, and 6 PM (control group); 9 AM and 6 PM (GW group); and 2 PM (CsA group). Animals were killed 48 h after the first injection of GW0742 by cervical dislocation, and tibialis anterior muscles were harvested immediately after death for histological and protein analyses.

Generation and maintenance of PPARβ human skeletal actin-Cre transgenic mice. Animals overexpressing PPARβ specifically in skeletal muscle were generated as described previously (33). Briefly, B6D2 mice harboring a loxP-stop-loxP-PPARβ-hygromycine construction were crossed with B6D2 mice expressing Cre recombinase under human skeletal actin (HSA) promoter (34). All animals were maintained hemizygous for their transgene. The presence of the transgenes was verified by PCR analyses of tail DNA (RedExtractN-Amp Tissue PCR kit, Sigma). Animals harboring the two transgenes were used as PPARβ-overexpressing mice, while animals harboring the HSA-Cre transgene were only used as controls.

Histological Analyses

Tibialis anterior muscles were harvested and frozen in tissue embedding medium (VWR International) immediately after the mouse was killed. Ten-micrometer cryosections were performed from the middle part of muscle, placed on poly-L-lysine coated slides (VWR International), and processed for histological analyses as described below.

SDH staining. SDH activity was revealed by incubation of slides in 50 mM phosphate buffer pH 7.6, 50 mM sodium succinate, and 0.05% nitro blue tetrazolium for 30 min at room temperature. After a wash in sodium chloride 0.9%, the numbers of stained fibers, called SDH-positive myofibers, and unstained fibers, called SDH-negative myofibers, were determined in whole tibialis anterior sections by using Olympus DP-Soft software.

Capillary number determination. Isoclectin B4 was detected using an immunohistochemical method. Briefly, slides were incubated with a biotinylated antibody rose against isoclectin B4 (Vector Laboratories) and signal was revealed using ABC and DAB kits (Vector Laboratories). Capillary numbers were determined in whole tibialis anterior sections by using Olympus DP-Soft software.

Protein Expression Analyses

Total proteins from tibialis anterior muscles were extracted in a buffer containing 50 mM Tris-HCl, pH 6.8, 10% glycerol, 10% SDS, 10 mM dithiothreitol, and Protease Inhibitor Complete Cocktail (Roche Molecular Biochemicals). Fifty micrograms of total protein were analyzed by SDS-PAGE and blotted on polyvinylinide fluoride membranes (Amersham Pharmacia Biotech). The antibodies used were as follows: Ms-351 (LabVision Neomarkers) for vascular endothelial growth factor-A (VEGF-A), sc-28188 (Santa Cruz Biotechnologies) for platelet endothelial cell adhesion molecule 1 (PECAM-1), and sc-302 (Santa Cruz biotechnologies) for Myf-5 and Ms-273 (LabVision Neomarkers) for MyoD1. Signals for the ubiquitously expressed TATA binding protein (TBP) were detected using sc-2718 antibody (Santa Cruz Biotechnologies) and used for loading normalization. Signals were detected with horseradish peroxidase conjugated rabbit or mouse polyclonal antibody (Promega), using Uptlight chemilumescence detection spray (Interchim) and quantified by digital imaging (Fuji LAS3000).

Statistical Analyses

All values are presented as means ± SD. Two-way ANOVA tests were performed for comparisons between groups and duration of treatment. When significant changes were observed in ANOVA tests, Fisher’s paired least significant difference post hoc test was applied to locate the source of significant differences. Analyses were performed with Stat View Abacus Concept version 5. No significant differences were observed for any variable among control groups at the different time points.

RESULTS

PPARβ Pharmacological Activation Increases Oxidative Myofiber Number in Tibialis Anterior Muscles

To characterize the effects of PPARβ activation on myofiber composition of tibialis anterior muscles, adult C57Bl6J male mice received a daily subcutaneous injection of a specific PPARβ agonist, GW0742 (1 mg/kg). Cross-sections around the midportion of muscles were prepared at various times and treated for in situ staining of SDH activity, a marker of mitochondrial complex II content. This method allows for the distinction between oxidative fibers, i.e., rich in mitochondria and appearing in dark, and glycolytic fibers, i.e., poor in mitochondria and remaining unstained. In control animals, the tibialis anterior muscle contains almost equal amounts of SDH-negative and SDH-positive myofibers (Fig. 1A). The number of both oxidative and glycolytic fibers remained unchanged in mice treated up to 24 h with the PPARβ agonist. By contrast, after 48 h of treatment, the total myofiber number was significantly increased (+600 fibers; P < 0.05; n = 5). This increment in myofiber number is predominantly related to an increased oxidative fiber number (+500; P < 0.05; n = 5), whereas the SDH-negative fiber number was not significantly changed (Fig. 1, B and C).

PPARβ activation also promoted an important and time-dependent reduction of the diameter of both SDH-positive (Fig. 2A) and SDH-negative fibers (Fig. 2C). Fiber diameter
was unchanged up to 24 h and reduced by ~25% after 48 h of treatment in both fiber types. Fiber size remained unchanged for longer treatment. Interestingly, the analysis of fiber size distribution after 48 h of treatment revealed that the reduction of mean diameter affects the totality of the SDH-positive (Fig. 2B) and SDH-negative (Fig. 2D) fibers.

We next examined the effects of PPARβ activation on the expression levels of regulatory transcription factors implicated in myogenesis, MyoD1 and Myf5 (20). Western blot analyses revealed that PPARβ activation led to a transient accumulation of both myogenic markers in tibialis anterior muscles (Fig. 3). The effect on Myf5 was already detectable after 2 h, maximal after 5 h (1.7-fold), returned to the control level at 24 h, while longer treatments led to important reduction of the protein content (2-fold decrease after 48 h of treatment). PPARβ activation led to a more marked (~3-fold) and delayed effect on MyoD1 protein accumulation. Upregulation of MyoD1 protein was detectable at 5 h, persisted up to 48 h, and returned to control value after 96 h of chronic treatment.

**PPARβ Pharmacological Activation Promotes Muscle Angiogenesis**

Endurance exercise training that leads to myofiber transition toward a more oxidative phenotype is also characterized by increased muscle vascularization. To determine the effects of PPARβ activation on capillary density, we performed in situ staining of isolecitin B4, a glycoprotein expressed in endothelial cell membranes, in cross-sections around the midportion of tibialis anterior muscles from adult mice receiving or not daily subcutaneous injections of GW0742 at 1 mg/kg. Typical pictures of isolecitin B4 detection are shown for tibialis anterior from untreated (Fig. 4A) and 48 h GW0742-treated (Fig. 4B) animals. Quantification of the capillary number in whole muscle sections revealed a significant increase in the capillary number after 24 h in GW0742-treated animals. After 48 h, the capillary number per muscle section was increased by 1.5-fold and did not significantly change for longer periods of treatment (Fig. 4C).

To further investigate this angiogenic response to PPARβ activation, we quantified by Western blot the expression levels of VEGF-A, a potent angiogenic peptide that elicits mitogenic action on endothelial cells (18), and PECAM-1, a typical endothelial marker, in tibialis anterior muscles from animals treated for various times with the PPARβ agonist. As shown in Fig. 5, these experiments revealed that the effects of PPARβ activation on VEGF-A protein amounts were detectable after 2 h, peaked at 5 h (8-fold induction), and remained valuable up to 24 h. The induction was reduced for longer periods of treatment, and after 96 h, the VEGF-A signal was significantly reduced (3-fold decrease) compared with untreated animals. PPARβ activation also exerted a potent and fast action on PECAM-1 protein content (~8-fold induction after 8 h). PECAM-1 amounts were reduced for longer periods of treatment but remained significantly higher than in control animals up to 96 h of treatment.

Collectively, these results clearly indicate that pharmacological activation of PPARβ promotes a fast and impressive enhancement of the oxidative phenotype of myofibers that involves upregulation of myogenic markers and an angiogenic response. In that respect, treatment of wild-type animals with the PPARβ agonist leads to a muscle remodeling that is reminiscent of that induced by regular physical training.
Calcineurin Signaling is Implicated in PPARβ-Promoted Muscle Remodeling

Given the fact that activation of calcineurin pathway has been implicated in the transition process toward a more oxidative phenotype in skeletal muscle (2, 10, 32), we examined the effects of alteration of the calcineurin signaling on muscle remodeling promoted by PPARβ pharmacological activation. To that purpose, adult animals were treated with GW0742 in the presence or the absence of CsA at a concentration known to block the calcineurin signaling pathway (9). The numbers of SDH positive and negative myofibers and the capillary number were determined after 48 h of coadministration of the compounds (Fig. 6). These analyses established that CsA had no significant effect on both the SDH-positive and SDH-negative myofiber numbers and on capillary number in tibialis anterior muscles from GW0742-untreated mice. By contrast, CsA administration totally prevented the muscle remodeling induced by GW0742 treatment, i.e., the increases in SDH-positive fibers, in total myofiber number (Fig. 6A) and in capillary density (Fig. 6B).

We next investigated the effects of CsA administration on the expression levels of MyoD1 and VEGF-A proteins in tibialis anterior muscles from mice treated or not with GW0742 for 48 h. As shown in Fig. 6C, CsA administration totally abolished the PPARβ-promoted accumulation of myogenic and angiogenic markers in muscles.

These observations indicated that the calcineurin pathway is necessary for PPARβ-promoted muscle remodeling.

Muscle-Specific PPARβ Overexpression Promotes Angiogenesis

We have previously shown that muscle-targeted PPARβ overexpression promoted an increase in the number of oxidative myofibers in various mouse muscles (31). To compare the muscle remodeling induced by PPARβ pharmacological activation or muscle-specific PPARβ overexpression, we determined the capillary number and total myofiber number in tibialis anterior muscles from 10-wk-old double transgenic mice (harboring both HSA-Cre and Stop-PPARβ transgenes) and their control littermates (animals harboring the HSA-Cre transgene only). These data are reported in Table 1 together with the values obtained for adult C57Bl6J wild-type mice treated for 2 days with GW0742. In accordance with our previous observations (31), PPARβ overexpression in skeletal muscle promoted an increase of 37% of total myofiber number. Despite a significantly reduced myofiber number in untreated C57Bl6J mice compared with control B6D2 mice, PPARβ pharmacological activation led after 48 h to a similar 37% increase in total myofiber number. By contrast, PPARβ overexpression clearly appeared less effective than PPARβ activa-
tion (25 vs. 55% increase in capillary number, respectively) in promoting angiogenesis.

**DISCUSSION**

In this study, we demonstrate that treatment of adult mice by a specific PPAR\(\beta\)/H9252 agonist leads to a very fast remodeling of tibialis anterior muscles. Our data indicate that administration of GW0742 to adult mice induces a 1.37-fold increment of total myofiber number largely accounted for by an increase in SDH-positive myofibers (Fig. 1; Table 1) associated with a 1.55-fold increase in capillary density (Fig. 4; Table 1). Interestingly, this muscle remodeling took place very rapidly, was complete after 2 days of treatment and did not change thereafter despite continued PPAR\(\beta\) agonist administration (Figs. 1 and 4). A time-course study of the molecular events in response to PPAR\(\beta\) activation confirmed the histological observations and showed rapid but transient upregulation of both myogenic (Myf5 and MyoD1) and angiogenic markers (VEGF-A and PECAM-1) in the tibialis anterior muscle (Figs. 3 and 5).

These observations indicate that PPAR\(\beta\) agonist treatment promotes histological and biochemical changes of skeletal muscle that are reminiscent of those taking place during exercise-induced adaptive remodeling. Muscle remodeling in response to muscular activity differs according to the type of activity involved. Three weeks of chronic slow frequency electrical stimulation of the motor nerve of rabbit tibialis anterior muscles led to a twofold increase in capillary number and oxidative fiber switching (7). Similar increases in capillary density and oxidative fiber number were also reported after several weeks of long-term endurance training and voluntary running in rodent models (1, 7, 12, 25). Clearly, the muscle remodeling induced by PPAR\(\beta\) agonist administration is very fast compared with the period of time required for remodeling in response to muscular activity.

Additionally, the effects of PPAR\(\beta\) agonist on myogenic and angiogenic markers also recapitulated those observed during muscle adaptation to physical exercise. Several studies revealed that, in trained animals, upregulation of myogenic and angiogenic markers occurred within hours or days, returned to control values rapidly, and preceded muscle remodeling (30, 48, 51).

An intriguing observation is the GW0742-promoted reduction in cross-section area of both SDH-positive and SDH-negative fibers in the tibialis anterior. This phenotype was not observed during endurance exercise-induced muscle remodeling (1) and is more suggestive of the initiation of an
PPARβ agonist promotes exercise-like muscle remodeling

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<th>Table 1. PPARβ overexpression or pharmacological activation increases myofiber and capillary numbers</th>
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Values are means ± SD; n = 5 mice of each genotype. PPARβ, peroxisome proliferator-activated receptor-β; SDH, succinate dehydrogenase. *P < 0.01, †P < 0.005 vs. control animals. ‡P < 0.001 vs. nontreated animals.

Atrophy program. Interestingly, it has been recently reported (11) that GW0742 administration upregulated the expression of two muscle specific E3 ligases, atrogin-1/MAFbx and MuRF-1, that play important roles in ubiquitin-proteasome-dependent muscle proteolysis. Further investigations are needed to evaluate the implications of such an effect of PPARβ activation. However, it should be noted that fasting induces muscle atrophy and also promotes PPARβ upregulation in mouse skeletal muscle (27), suggesting a potential role of the nuclear receptor in this physiological process.

PPARβ is expressed in several cell types present in adult skeletal muscle, including myofibers, myoblasts (27), and endothelial cells (39, 44), and it is likely that these cell types are contributing to the muscle remodeling promoted by PPARβ agonist treatment. Two hypotheses can be proposed to explain the formation of new fibers, which requires myoblast recruitment. PPARβ activation in myoblasts could initiate terminal differentiation by promoting a transient upregulation of myogenic genes and formation of the new fibers. Alternatively or concomitantly, PPARβ activation in myofibers could promote the production of signals that, in turn, trigger myoblast terminal differentiation. The existence of signals produced by myofibers to activate differentiation and/or fusion of myoblasts, such as specific interleukins or growth factors, has been documented (15, 45). The fiber hyperplasia produced by administration of the PPARβ agonist was very similar to that observed with HSA-Cre-mediated overexpression of PPARβ (Table 1). This could argue in favor of a prominent role of the functional fibers in myoblast recruitment, since in the transgenic model, PPARβ overexpression occurred specifically in functional myofibers and not in myoblasts (31, 34).

In contrast, the amplitude of capillarization was significantly higher in GW0742-treated animals than in the muscles of PPARβ overexpressing mice (Table 1). This suggests that activation of the PPARβ pathway specifically in functional fibers induced a limited angiogenic response, while the angiogenic response in agonist-treated mice could involve other cell types, such as endothelial cells. Involvement of both myofibers and endothelial cells in the production of VEGF-A and in the remodeling of skeletal muscle capillary network has been shown. Several studies have demonstrated that VEGF-A mRNA and protein are upregulated in myofibers during training-induced muscle remodeling (5, 6, 8) and that endothelial cells from skeletal muscle are also able to produce VEGF-A (13).

Noteworthy, a proangiogenic action of various PPARβ agonists implicating upregulation of VEGF-A and VEGF receptor was recently described in murine aortic and human umbilical endothelial cells (39, 44). The mechanisms of PPARβ-mediated activation of VEGF-A gene expression remain unclear. The presence of a PPAR-responsive element in the VEGF-A gene promoter has been reported and implicated in the repression of the gene by PPARγ agonists in adenocarcinoma cells (38). However, Fauconnet et al. (17) showed that the PPARβ agonist-mediated activation of VEGF-A gene expression in bladder cancer cells involves an indirect mechanism requiring the synthesis of an intermediary regulatory protein through the MAPK pathway.

Our data demonstrating a complete blunting of PPARβ agonist-mediated muscle remodeling by coadministration of CsA (Fig. 6) strongly suggest an indirect action through the calcineurin pathway rather than a direct transactivation of myogenic and angiogenic markers. It has been established that calcineurin, a Ca2+/calmodulin-regulated phosphatase, plays an important function in myofiber type specification and angiogenesis by dephosphorylation and nuclear translocation of the transcription factors of the NFAT family. Activation of the calmodulin/calcineurin/NFAT signaling pathway leads to increased transcription of genes expressed in oxidative fibers and results in enhanced mitochondrial biogenesis (2, 10, 32). Other findings (22, 35, 40) have implicated the calcineurin/NFAT signaling pathway in endothelial cell proliferation and angiogenesis.

In summary, our results confirmed the role of PPARβ in adaptive responses of skeletal muscle and demonstrate for the first time that pharmacological activation of the nuclear receptor results in a very fast enhancement of oxidative capability of the tissue by increasing both oxidative fiber number and capillary density. As already proposed for exercise-induced muscle remodeling, these actions of PPARβ on muscle morphology implicate an activation of the calcineurin pathway. How PPARβ pharmacological activation is affecting the calcineurin signaling remains to be investigated.

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