DGAT1 overexpression in muscle by in vivo DNA electroporation increases intramyocellular lipid content

Berber D. Roorda,[*,,††] Matthijs K. C. Hesselink,[†,**,††] Gert Schaart,[†,††] Esther Moonen-Kornips,[*,†,††] Pilar Martínez-Martínez,[§] Mario Losen,[§] Marc H. De Baets,[§] Ronald P. Mensink,[*,**,††] and Patrick Schrauwen[1,*,**,††]**

Departments of Human Biology,* Movement Sciences,[†] and Neuro Psychology,[§] Maastricht University, 6200 MD Maastricht, The Netherlands; Wageningen Center for Food Sciences,** Nutrition and Toxicology Research Institute Maastricht,[††] 6200 MD Maastricht, The Netherlands

Abstract In adipose tissue, the microsomal enzyme 1,2-acyl CoA:diacylglyceroltransferase-1 (DGAT1) plays an important role in triglyceride storage. Because DGAT1 is expressed in skeletal muscle as well, we aimed to directly test the effect of DGAT1 on muscular triglyceride storage by overexpressing DGAT1 using in vivo DNA electroporation. A pcDNA3.1-DGAT1 construct in saline was injected in the left tibialis anterior muscle of rats, followed by the application of eight transcutaneous pulses, using the contralateral leg as sham-electroporated control. Electroporation of the DGAT1 construct led to significant overexpression of the DGAT1 protein. The functionality of DGAT1 overexpression is underscored by the pronounced diet-responsive increase in intramyocellular lipid (IMCL) storage. In chow-fed rats, DGAT1-positive myocytes showed significantly higher IMCL content compared with the control leg, which was almost devoid of IMCL (1.99 ± 1.13% vs. 0.017 ± 0.014% of total area fraction; $P < 0.05$). High-fat feeding increased IMCL levels in both DGAT1-positive and control myocytes, resulting in very high IMCL levels in DGAT1-overexpressing myocytes (4.96 ± 1.47% vs. 0.80 ± 0.14%; $P < 0.05$). **Our findings indicate that DGAT1 contributes to the storage of IMCL and that in vivo DNA electroporation is a promising tool to examine the functional consequences of altered gene expression in mature skeletal muscle.**—Roorda, B. D., M. K. C. Hesselink, G. Schaart, E. Moonen-Kornips, P. Martínez-Martínez, M. Losen, M. H. De Baets, R. P. Mensink, and P. Schrauwen. **DGAT1 overexpression in muscle by in vivo DNA electroporation increases intramyocellular lipid content. *J. Lipid Res.* 2005.** 46: **230–236.**

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In addition to white adipose tissue, fatty acids can be stored in nonadipose tissues, such as heart, liver, and skeletal muscle. In skeletal muscle, fatty acids are stored as lipid droplets mostly in the vicinity of the mitochondria, where they may serve as a rapid available energy store (1). In highly trained subjects, the level of these intramyocellular lipids (IMCLs) is increased (2), and endurance training in previously untrained subjects increases IMCL levels after only 2 weeks (3). These findings are compatible with a role for IMCL as a substrate to fuel muscle, especially during prolonged exercise. However, increased IMCL can also be the consequence of increased fatty acid availability, such as after intravenous lipid infusion (4, 5), on a high-fat diet (6, 7), and in type 2 diabetes mellitus and/or obesity (8).

Despite these findings, little is known about the regulation of triglyceride storage in muscle. In adipose tissue, the microsomal enzyme 1,2-acyl CoA:diacylglyceroltransferase (DGAT) may play an important role in triglyceride storage. DGAT catalyzes the final step in triacylglycerol synthesis by using diacylglycerol and fatty acyl CoAs as substrates. Two isoforms of DGAT have been discovered, DGAT1 and DGAT2, which are members of different families of genes. DGAT1 is widely expressed in all tissues, with high expression in white adipose tissue, skeletal muscle, and intestine (9), whereas the expression of DGAT2 is restricted to liver and adipose tissue (10). To test DGAT1 functionality, Chen et al. (11) generated transgenic mice with white adipose tissue-specific overexpression. These mice had increased adipocyte size, increased adipose mass, and increased susceptibility to high-fat diet-induced obesity compared with wild-type mice. The increased adiposity was limited to adipose tissue and did not affect the liver and skeletal muscle, indicating that, at least in white adipose tissue, DGAT1 has a direct effect on triglyceride storage (11). On the other hand, transgenic DGAT1-deficient mice have normal plasma triglyceride levels but abundantly store triglycerides in their adipose tissue, probably attributable to the remnant activity of DGAT2 (12). Interestingly, DGAT1-deficient mice have 30–40% lower triglyc-

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[1] To whom correspondence should be addressed.
e-mail: p.schrauwen@hb.unimaas.nl

eride levels in skeletal muscle, suggesting that DGAT1 plays a key role in triglyceride storage in muscle. However, DGAT1-deficient mice are also characterized by increased energy expenditure, leaving open the possibility that the reduced IMCL levels in DGAT1-deficient mice are the result of increased use of these IMCLs.

Therefore, to directly test the role of DGAT1 in triglyceride storage in skeletal muscle, we overexpressed DGAT1 in skeletal muscle of rats in vivo. To this end, we used the unique in vivo DNA electroporation technique to achieve overexpression of the DGAT1 protein. In vivo DNA electroporation has been shown to be very successful for the transfection of genes, especially in skeletal muscle (13). A major advantage of this methodology is that DGAT1 can be overexpressed locally, in one specific skeletal muscle, allowing the use of the corresponding muscle in the contralateral leg as an internal control. Therefore, many potential confounding factors, such as substrate endocrine milieu or dietary intake, are identical in overexpression vs. control muscle. In addition, using in vivo DNA electroporation techniques, the protein of interest is expressed within a couple of days. This is an advantage over traditional lifelong overexpression of the protein of interest in transgenic mice, which often results in compensatory changes in other proteins, thereby affecting the overall phenotype of the transgenic animal. To test the functionality of DGAT1 overexpression, lipid accumulation was examined in DGAT1-transfected muscle of adult rats fed either a chow or a high-fat diet.

MATERIALS AND METHODS

Animals

Eight male Wistar rats, 12 weeks of age, weighting 293.4 ± 10.2 g at the onset of the study, were housed individually in environmentally controlled conditions (21–22°C) with a 12 h/12 h light/dark-cycle (light from 7:00 AM to 7:00 PM). During the experiments, rats had free access to tap water and food. All experiments were approved by the Institutional Animal Care and Use Committee and complied with the principles of laboratory animal care.

Injection of the construct and electroporation

An in vivo DNA electroporation technique was used to obtain specific overexpression of DGAT1 in the left tibialis anterior (TA) muscle of the rat, whereas the right TA muscle served as a sham-electroporated internal control. DNA electroporation was performed under halothane anesthesia. Left TA muscles were injected with 200 μg (2 μg/μl) of pcDNA3.1-DGAT1 construct containing a FLAG-tagged epitope (kindly provided by Dr. R. Farese, Jr., J. David Gladstone Institutes) in 0.9% sterile NaCl. Right TA muscles were injected with 100 μl of 0.9% sterile NaCl as a control. Five minutes after injection, eight transcutaneous electric pulses were applied by two stainless steel plate electrodes placed at each side of the leg to obtain transient permeabilization of the cell membrane, facilitating the passage of the injected construct. Impedance was minimized by shaving the leg and the application of a conductive gel. Four square wave pulses followed by four pulses of the opposite polarity were generated by an ECM 820 electroporator (BTX, San Diego, CA). The ratio of applied voltage to electrode distance was 20 V/mm (13).

Diets

Immediately after electroporation, rats were randomly divided into two groups. One group (n = 5) received a regular chow low-fat diet (7% energy from fat) for 15 days, whereas the other group (n = 3) was placed on a high-fat diet (46% energy from fat). Both diets were purchased from Hope Farms (Woerden, The Netherlands) and provided ad libitum.

Tissue sampling and staining

Fifteen days after DNA electroporation, tissue dissection was performed under ketamine (Nimatek; 1.0 ml/kg) and xylazine (Sedamun; 0.5 ml/kg) anesthesia by subcutaneous injection. Left and right TA muscles were dissected carefully, and any visible fat and blood were removed. The mid belly was embedded in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and rapidly frozen in liquid nitrogen-cooled isopentane and stored at −80°C until further analysis. Immediately after tissue sampling, rats were killed by cervical dislocation.

Fresh serial sections (5 μm) were thaw mounted on uncoated precleaned (96% ethanol) glass slides. Air-dried cryosections from the electroporated and control TA muscles were fixed in 3.7% formaldehyde (Merck, Darmstadt, Germany) in deionized water for 1 h at 4°C. Formaldehyde-fixed sections were rinsed three times in deionized water for 30s to remove excess formaldehyde. The sections were then treated with 0.5% Triton X-100 (Merck) in PBS for 5 min, washed with three exchanges of PBS for 5 min, and processed for combined oil red O (ORO) and immunofluorescence staining according to Koopman, Schaart, and Hesselink (14). In brief, sections were incubated with an antibody against the basement membrane protein laminin (Sigma-Aldrich, St. Louis, MO; 1:40 dilution) in PBS for 30 min at room temperature to define the myocellular border. Sections for the determination of fiber types were coincubated with A4.951-myosin heavy chain 1 antibody [Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA; 1:50 dilution]. Incubation was followed by washing with one exchange of PBS and two exchanges of TBS for 5 min each. Next, sections were incubated with FITC-conjugated anti-FLAG antibody (Sigma-Aldrich; 1:100 dilution) for visualization of overexpressed DGAT1 or with Alexafluor 488-labeled goat anti-mouse IgG 1488 (Molecular Probes, Leiden, The Netherlands; 1:200 dilution) and Alexafluor 488-labeled goat anti-rat IgG1 Alexa 350 (Molecular Probes; 1:130 dilution) in TBS for 30 min at room temperature. After washing with one exchange of TBS and two exchanges of PBS for 5 min each, glass slides were immersed in the ORO working solution for 30 min for the detection of lipid droplets. The working solution consists of a 36% triethylphosphate solution containing 12 ml of ORO stock solution and 8 ml of deionized water. The working solution was filtered through Whatman paper number 42 (Whatman, Maidstone, UK) to remove crystallized ORO. The stock solution was made by adding 500 mg of ORO (Fluka Chemie, Buchs, Switzerland) to 60 ml of triethylphosphate (Fluka Chemie) and 40 ml of deionized water. After ORO staining, slides were rinsed three times with deionized water for 30s followed by 10 min of washing with running tap water. Stained sections were embedded in Mowiol.

Image capturing, processing, and analysis

All sections were examined using a Nikon E800 fluorescence microscope (Uvikon, Bunnik, The Netherlands) coupled to a Basler A101C progressive scan color charge-coupled device camera. Multiple random images were captured for every single color (red, green, and blue) from all sections and processed and analyzed using Lucia GF 4.80 software (Nikon, Düsseldorf, Germany). Special care was taken to use the same camera settings (gain and exposure time) while grabbing all images.

All images were analyzed for the number of myocytes overexpressing DGAT1 and for the lipid droplet over myocyte area fraction. To this end, a semiautomated macro was written that allowed *1*) autodetection of the cellular membrane (identified in the blue channel); *2*) measurement of the area covered by the measured myocytes; *3*) measurement of the area covered by lipid droplets (identified in the red channel); and 4) measurement of the intensity of the fluorescent stain yielded by the anti-FLAG FITC [identified in the green channel and represented at an 8 bit level; 0–255 arbitrary units (AU)], representing overexpressed DGAT1. The area fraction was computed by dividing the area covered by lipid droplets in micrometers squared by the area covered by the measured myocytes in micrometers squared.

Measuring background intensity of the anti-FLAG FITC in the sham-electroporated control leg revealed that in every single cell the intensity of the green signal did not exceed 40 AU. Therefore, we defined myocytes exhibiting a green intensity of >40 AU as DGAT1-positive myocytes. The efficiency of transfection was computed by dividing the number of myocytes with green intensity >40 AU over the total number of myocytes examined in the DGAT1-electroporated muscle. In total, 1,105 myocytes were examined for DGAT1 expression and lipid droplet content in the electroporated leg. In the contralateral leg, devoid of exogenous DGAT1 protein and therefore exhibiting low intermyocellular variation in lipid droplet content, 405 myocytes were examined.

RESULTS

Impact of in vivo DNA electroporation on muscle structure

The effect of in vivo DNA electroporation on muscle structure was examined using routine light microscopy. Muscles were screened for the well-known markers of muscle damage, such as centrally located nuclei, membrane disruption, and macrophage infiltration. Fifteen days after electroporation, light microscopic examination revealed no signs of muscle damage (data not shown). These findings indicate that electroporation was well tolerated by the animals and that 15 days after electroporation muscle structure was normal.

In vivo DNA electroporation of DGAT1 in muscle

Electroporation of the pcDNA3.1-DGAT1 construct into the TA muscle of rats in vivo led to a significant increase in green fluorescence intensity compared with the sham-electroporated contralateral control (67.7 ± 30.5 AU vs. 31.3 ± 2.23 AU; $P < 0.001$). Thus, DGAT1-positive myocytes could easily be distinguished from DGAT1-negative myocytes by the detection of green fluorescence from the FLAG-tagged antibody. The efficiency of transfection in the DGAT1-electroporated muscle equaled 29 ± 12%.

In skeletal muscle, different muscle fiber types can be distinguished by marked differences in oxidative capacity. Type I muscle fibers have the highest oxidative capacity, whereas type II muscle fibers are highly glycolytic. In healthy control muscles, the majority of IMCLs are confined to the oxidative type I muscle fibers. To test if an effect of DGAT1 overexpression on IMCL content could be biased by a putative fiber type selectivity in DGAT1 overexpression, we stained serial sections with the FLAG-tagged antibody (green), a laminin antibody that identifies the sarcolemma and thus indicates individual muscle fibers (blue) and ORO (red) (**Fig. 1A**), or the FLAG-tagged antibody (green) combined with laminin antibody (blue) and a myosin heavy chain 1 antibody, which can be used to identify type I muscle fibers (red) (Fig. 1B). From Fig. 1A, B it can be deduced that overexpression of DGAT1 did not occur in a fiber type-selective manner.

Effect of DGAT1 overexpression on IMCL accumulation

In chow-fed rats, hardly any lipid droplets could be detected in the sham-electroporated control muscle (**Fig. 2A**, **B**). DGAT1 electroporation resulted in increased accumulation of lipid droplets in DGAT1-positive myocytes (Fig. 2C, D, **Fig. 3**). Quantification showed a significantly increased area fraction (percent of the total cell surface covered by lipid droplets) in DGAT1-positive myocytes compared with control myocytes (DGAT1-positive, 1.99 ± 1.13% vs. control, 0.017 ± 0.014%; $P < 0.05$; **Fig. 4**).

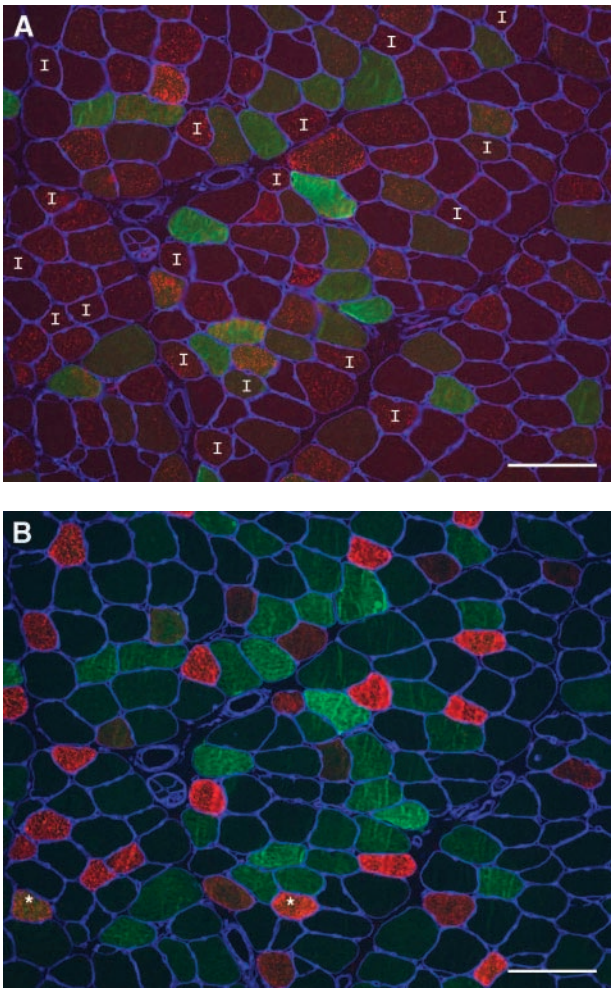

Fig. 1. 1,2-Acyl CoA:diacylglyceroltransferase-1 (DGAT1) electroporation and fiber types. Serial sections of the DGAT1-electroporated muscle. A: The green fluorescent signal indicates the presence of DGAT1 protein containing a FLAG-tagged epitope; lipid droplets are stained using oil red O (in red). Myocytes are marked with I for type I fibers. B: Type I fibers are stained in red, DGAT1 in green. Myocytes marked with asterisks are DGAT1-positive type I fibers. Bars = 100 μm.

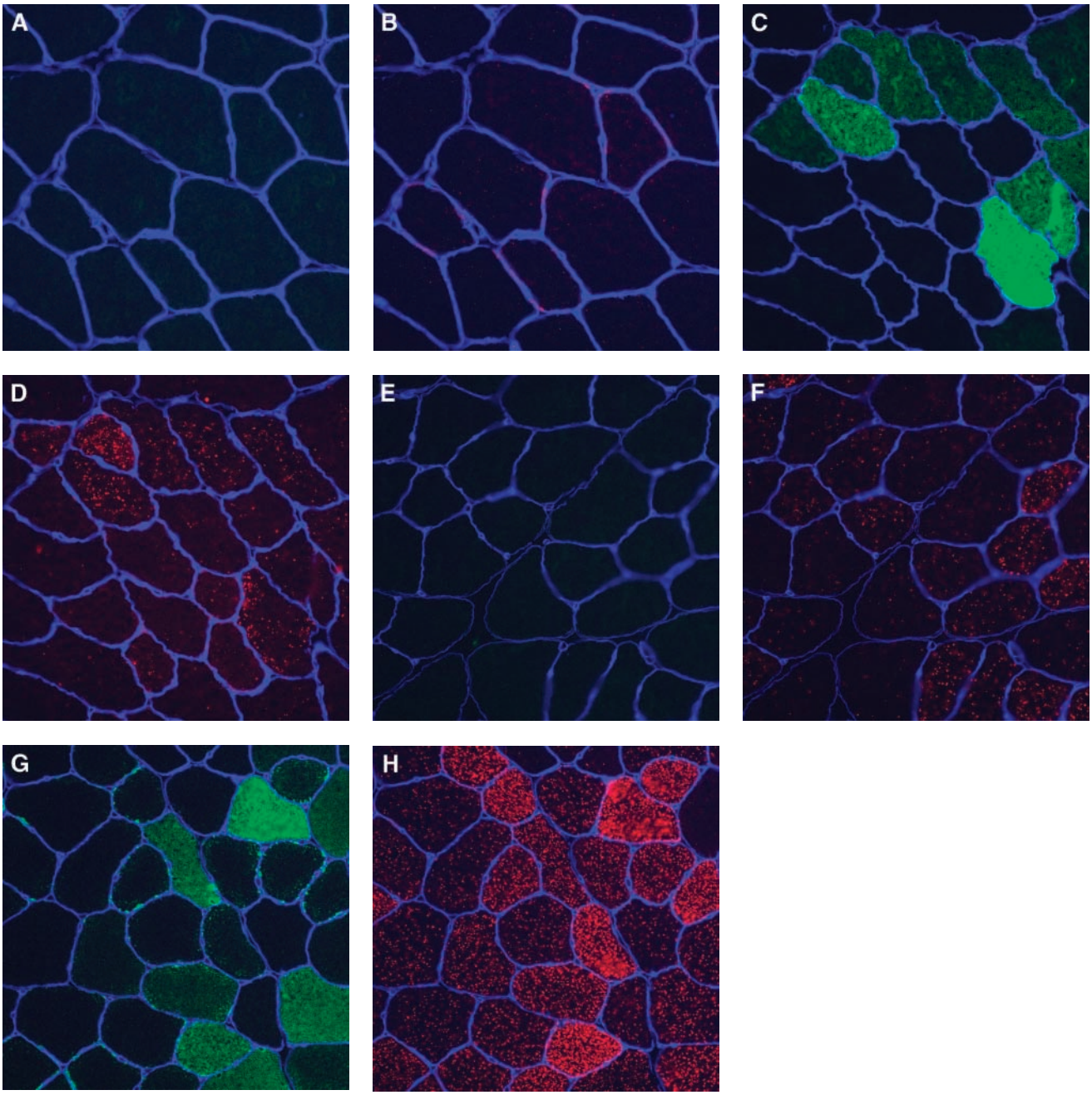

Fig. 2. DGAT1 staining and lipid accumulation under different conditions. Staining of DGAT1-positive myocytes (in green) and lipid accumulation stained with ORO (in red) in control muscle (A, B) and DGAT1-electroporated muscle (C, D) of rats fed a chow diet and in control muscle (E, F) and DGAT1-electroporated muscle (G, H) of rats fed a high-fat diet.

Effect of nutritional abundance of fatty acids on lipid accumulation

A high-fat diet for 15 days increased the accumulation of lipid droplets in control muscle compared with a chow diet (Fig. 2E, F). Quantification of the area fraction covered by lipid droplets showed a significant increase compared with a chow diet (high fat, 0.80 ± 0.14% vs. chow, 0.017 ± 0.014%; $P < 0.005$; Fig. 4). However, the area fraction covered by lipids on a high-fat diet was still 2.5-fold less compared with the effect of DGAT1 overexpression on a chow diet. Moreover, DGAT1 overexpression further increased the high-fat diet-induced lipid accumulation by ~6-fold compared with control myocytes (Fig. 2G, H), thereby reaching the highest levels of IMCL accumulation (DGAT1-positive, 4.96 ± 1.47% vs. control, 0.80 ± 0.14%; $P < 0.05$; Fig. 4).

DISCUSSION

DGAT1 is known to increase triglyceride storage in white adipose tissue, but its effect in skeletal muscle is unknown. Therefore, the aim of the present study was to test the role of DGAT1 in IMCL storage in skeletal muscle in vivo. To this end, we used in vivo DNA electroporation to

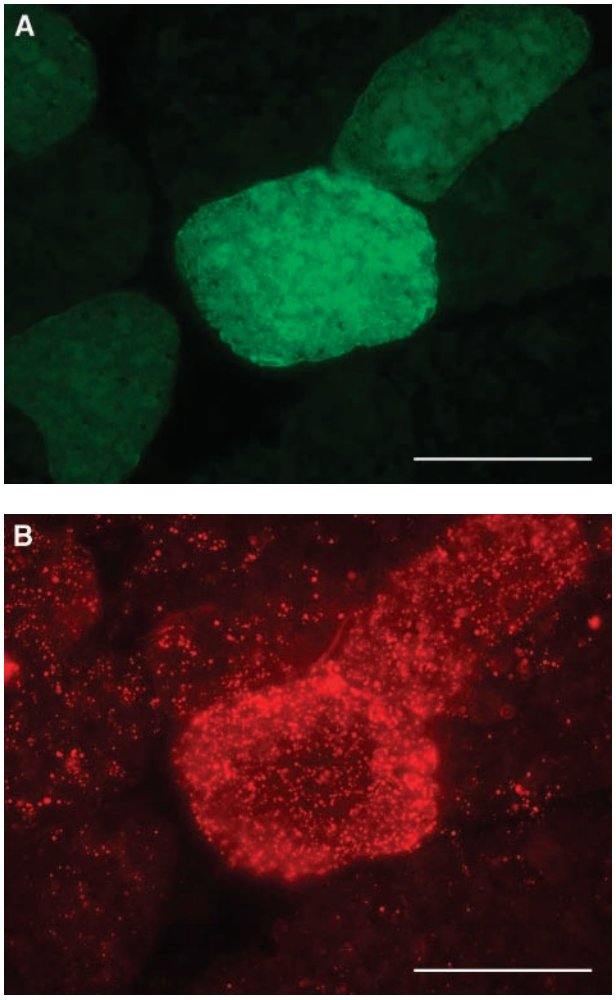

Fig. 3. DGAT1 protein overexpression and lipid accumulation. Double staining of DGAT1-positive myocytes (in green; A) and corresponding increase of lipid accumulation stained with ORO (in red; B). Bars = 100 μm.

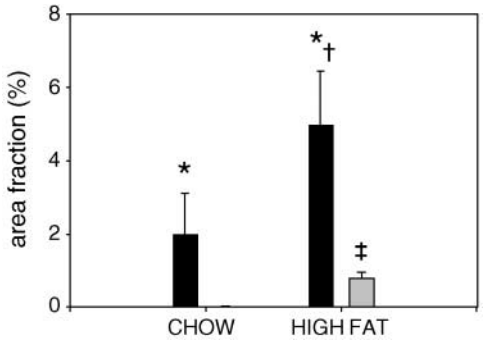

Fig. 4. Quantification of lipid accumulation. Area fraction (percent of the total cell surface covered by lipid droplets) of DGAT1-positive myocytes (black bars) and contralateral control myocytes (gray bars) of rats fed either a chow or a high-fat diet. * $P < 0.05$ compared with control; ‡ $P < 0.05$ compared with control myocytes of rats fed a chow diet; † $P < 0.005$ compared with DGAT1-positive myocytes of rats fed a chow diet. Error bars represent standard deviation.

achieve overexpression of the DGAT1 protein in living rats. We found that in vivo DNA electroporation increased the expression of the DGAT1 protein, which was restricted to a specific muscle of interest in adult, living rats, without affecting the muscle of the contralateral leg. We further showed that overexpression of DGAT1 resulted in a significant increase in IMCL content, indicating for the first time that overexpressing DGAT1 in skeletal muscle increases IMCL storage in vivo.

Using light microscopy, we found that in vivo DNA electroporation did not affect muscle structure, as measured 15 days after electroporation. Inevitably, in vivo DNA electroporation induces a certain degree of muscle damage, as electric fields induce the transient formation of hydrophilic pores in the cell membrane through which the DNA can enter the myocyte. However, with the voltages used in our study, electroporated muscles were completely recovered by 15 days after electroporation in young adult rats (data not shown).

It is well known, based on prior work with skeletal muscle, that muscle fiber types differ greatly in their lipid content (15–17). In humans, type I muscle fibers in lean individuals as well as in obese subjects and in subjects with type 2 diabetes mellitus have been found to contain significantly more lipid droplets than type II muscle fibers (18, 19). In addition, van Loon et al. (20) found a similar lipid distribution among fiber types in muscle biopsies of trained male cyclists. Therefore, DGAT1-specific overexpression in type I muscle fibers would confound the DGAT1-induced increase in lipid accumulation. For this reason, we checked whether DGAT1 transfection of myocytes was restricted to specific fiber types. As can be seen in Fig. 1, this was not the case, because DGAT1 overexpression was observed in all muscle fiber types and increased lipid accumulation was restricted to the DGAT1-overexpressing myocytes. These data indicate that the effect of DGAT1 overexpression on lipid accumulation was not dependent on fiber type.

An important role for DGAT1 in triglyceride synthesis in white adipose tissue had already been observed by Chen et al. (11), who generated transgenic mice that overexpressed DGAT1 by 2-fold in white adipose tissue. Reproductive fat pads of these mice contained ~1.75-fold more triglycerides (grams per fat pad), and DGAT1-overexpressing mice had increased total fat pad mass as a percentage of total body mass compared with wild-type mice when fed either a chow diet (~1.75-fold) or a high-fat diet (~1.20-fold) (11). Although the effects of DGAT1 overexpression seem less pronounced in white adipose tissue compared with the results of the present study, it should be noted that, in contrast to skeletal muscle, white adipose tissue contains huge amounts of triglycerides, suggesting that an ~2-fold increase in adipose tissue mass induced by DGAT1 indicates a major effect on triglyceride synthesis. However, instead of measuring relative amounts of triglycerides, our model allows us to determine the absolute changes in intramyocellular triglycerides by directly counting the surface area covered by lipid droplets.

Using the electroporation technique, we found a significant overexpression of DGAT1 in almost 30% of the mus-

cle fibers. As expected, in myocytes of the contralateral internal control leg, hardly any lipid accumulation could be detected in chow-fed rats (~0.02% of total area fraction). However, overexpression of DGAT1 resulted in a significant increase in IMCL content, resulting in ~2% of the total cell surface covered by lipid droplets compared with the control myocytes of the contralateral muscle. This result is even more striking given that a chow diet contains little dietary fat. In human studies, area fractions of 3.6% in obese individuals with type 2 diabetes mellitus (8), 3.1% in type 2 diabetic subjects, and 2.7% in trained athletes (21) have been reported. Here, we show comparable area fractions of 1.99% and 4.96% in DGAT1-positive myocytes from chow-fed and high-fat-fed rats, respectively (Fig. 4). These data suggest that DGAT1 has an important role in triglyceride synthesis in skeletal muscle and that its overexpression can lead to the phenotype observed in type 2 diabetics and/or endurance-trained athletes (2, 8). In addition, our data show that the model of in vivo DGAT1 electroporation is an elegant tool to investigate within one organism the effect of high versus low levels of IMCLs on muscle metabolism while leaving whole body metabolism unaffected.

In contrast to a chow diet, in which hardly any lipids can be detected in normal (control) myocytes of rats, a high nutritional abundance of fatty acids attributable to either high-fat diets (6, 7, 22) or lipid infusions (4, 5) leads to a rapid accumulation of lipids inside muscle in both rats and humans within hours or days. Here, we found that in control muscle, high-fat feeding for 15 days (which was chosen to allow the muscle to recover from the electroporation procedure) led to a significant increase in the area fraction of lipid droplets compared with a chow diet. Interestingly, however, the IMCL content after high-fat feeding was less than 50% of the IMCL content after DGAT1 overexpression in chow-fed rats. This further illustrates the potent effect of DGAT1 overexpression on IMCL synthesis. On the high-fat diet, DGAT1 overexpression further enhanced the high-fat diet-induced lipid accumulation by 6-fold compared with the contralateral control muscle. Thus, our results show that overexpressing DGAT1 in skeletal muscle increases IMCL storage in vivo, indicating that DGAT1 plays an important role in the storage of triglycerides not only in white adipose tissue but also in skeletal muscle.

Several investigations have shown that IMCL levels are increased in type 2 diabetes (8, 23), in nondiabetic subjects with insulin resistance (24, 25), in obesity (8), and in endurance-trained subjects (21). Based on the present results, it could be speculated that increased DGAT1 expression contributes to the increased level of IMCL in these subjects. However, little is known about DGAT1 gene expression or protein content in diabetic patients or endurance-trained subjects. Ikeda et al. (26) found increased levels of DGAT1 mRNA in muscle after a 2 week swimming training in mice, which was suggested to enhance the replenishment of triglyceride in skeletal muscles after exercise. However, Schmitt et al. (27) found invariant levels of DGAT1 mRNA between TA muscles of endurance-trained and untrained male subjects. To the best of our knowledge, no data are available about DGAT1 levels in skeletal muscle of diabetic subjects or about the possible role of DGAT1 in IMCL storage in humans. It is tempting to speculate that DGAT1 content is increased in type 2 diabetic subjects, contributing to the increased IMCL storage in these subjects.

In conclusion, in vivo DNA electroporation is a promising tool for the examination of the functional consequences of altered gene expression in selected, mature skeletal muscle while whole body metabolism is not altered. In vivo electroporation of the DGAT1 construct in rat TA muscle accomplished a functional overexpression of the DGAT1 protein, leading to the accumulation of IMCL droplets in DGAT1-positive cells. From this study, it can be concluded that DGAT1 plays an important role not only in the storage of lipids in white adipose tissue but also in skeletal muscle. Future studies should focus on the role of DGAT1 in IMCL metabolism in human subjects.

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