

Enhanced Sarcolemmal FAT/CD36 Content and Triacylglycerol Storage in Cardiac Myocytes From Obese Zucker Rats

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In obesity, the development of cardiomyopathy is associated with the accumulation of myocardial triacylglycerols (TAGs), possibly stemming from elevation of myocardial long-chain fatty acid (LCFA) uptake. Because LCFA uptake is regulated by insulin and contractions, we examined in cardiac myocytes from lean and obese Zucker rats the effects of insulin and the contraction-mimetic agent oligomycin on the initial rate of LCFA uptake, subcellular distribution of FAT/CD36, and LCFA metabolism. In cardiac myocytes from obese Zucker rats, under basal conditions, FAT/CD36 was relocated to the sarcolemma at the expense of intracellular stores. In addition, the LCFA uptake rate, LCFA esterification rate into TAGs, and the intracellular unesterified LCFA concentration each were significantly increased. All these metabolic processes were normalized by the FAT/CD36 inhibitor sulfo-*N*-succinimidyleate, indicating its antidiabetic potential. In cardiac myocytes isolated from lean rats, *in vitro* administration of insulin induced the translocation of FAT/CD36 to the sarcolemma and stimulated initial rates of LCFA uptake and TAG esterification. In contrast, in myocytes from obese rats, insulin failed to alter the subcellular localization of FAT/CD36 and the rates of LCFA uptake and TAG esterification. In cardiac myocytes from lean and obese animals, oligomycin stimulated the initial rates of LCFA uptake and oxidation, although oligomycin only induced the translocation of FAT/CD36 to the sarcolemma in lean rats. The present results indicate that in cardiac myocytes from obese Zucker rats, a permanent relocation of FAT/CD36 to the sarcolemma is responsible for myocardial TAG accumulation. Furthermore, *in vitro* these cardiac myocytes, although sensitive to contraction-like stimulation, were completely insensitive to insulin, as the basal

conditions in hyperinsulinemic, obese animals resemble the insulin-stimulated condition in lean littermates. *Diabetes* 53:1655–1663, 2004

Cardiovascular diseases are the most serious complications of obesity (1). A common feature of obesity is insulin resistance, which is characterized by a diminished ability of insulin-sensitive tissues, such as heart, to take up and metabolize glucose in response to insulin (1–3). There is a strong relation between the development of insulin resistance and the accumulation of intracellular triacylglycerols (TAGs) in skeletal muscle from rodents and humans (4–6). Several studies have demonstrated that in an insulin-resistant state, TAGs accumulate not only in skeletal muscle but also in the heart (2,7). This cardiac accumulation of TAGs could be due to an increased myocardial long-chain fatty acid (LCFA) uptake. Rodent models in which myocardial LCFA uptake is elevated, such as mice overexpressing peroxisome proliferator-activated receptor- α or acyl-CoA synthetase, indeed showed markedly increased cardiac TAG levels (8,9). Interestingly, it has been shown in hearts from obese Zucker rats that lipid accumulation is associated with impaired cardiac function (2,7).

Evidence is accumulating that cellular LCFA uptake is a rate-governing step in LCFA utilization (10). Using heart giant membrane vesicles, a model used to investigate cardiac LCFA uptake dissected from LCFA metabolism (11) and cardiac myocytes, in which LCFA uptake is closely linked to metabolism (12), we previously showed that ~50% of cardiac LCFA uptake is mediated by the 88-kDa putative LCFA transport protein, fatty acid translocase (FAT)/CD36. Under physiological conditions, both insulin (13–15) and cellular contractions (16) are able to elevate LCFA uptake into cardiac myocytes. We recently demonstrated that insulin induces translocation of FAT/CD36 from an intracellular pool toward the plasma membrane, leading to a 1.5-fold increase of LCFA uptake by cardiac myocytes both *in vivo* and *in vitro* (13–15). Moreover, in electrically stimulated cardiac myocytes, FAT/CD36 is translocated toward the plasma membrane, and there is an accompanying 1.6-fold increase in LCFA uptake (16). Despite the fact that both insulin and cellular contractions induce FAT/CD36-mediated LCFA uptake to the same magnitude, their effects are additive and different

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AMPK, AMP kinase; FAT, fatty acid translocase; HPTCL, high-performance thin-layer chromatography; LCFA, long-chain fatty acid; LDM, low-density microsome; PI, phosphatidylinositol; PL, phospholipid; SL fraction, sarcolemmal fraction; SSO, sulfo-*N*-succinimidyleate; TAG, triacylglycerol.

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signaling pathways are involved. Notably, insulin activates phosphatidylinositol (PI) 3-kinase (15), whereas cellular contractions activate AMP kinase (AMPK) (17,18).

Upon their transport into the cytoplasm of the cardiac myocyte, LCFAs are directed toward different target sites: mitochondrial β -oxidation, esterification into TAG and phospholipids (PLs), and signal transduction pathways (19,20). Insulin predominantly directs intracellular LCFAs toward esterification (21), whereas during cellular contractions LCFAs are efficiently used for energy production via mitochondrial β -oxidation (22). Recently, we unmasked a pivotal role of FAT/CD36 in the altered cardiac LCFA uptake in obese Zucker rats (23). It appears that in giant membrane vesicles isolated from the heart of obese Zucker rats, LCFA uptake was elevated, whereas neither the total abundance of FAT/CD36 mRNA nor the total amount of protein was different in the heart of obese rats compared with that of lean Zucker rats. Notably, an increased amount of FAT/CD36 was detectable at the sarcolemma in the obese rat heart. These findings indicate that in cardiac myocytes from obese Zucker rats, a portion of the intracellular FAT/CD36 pool is permanently relocated to the sarcolemma (23). However, it is not known whether this permanent relocation is due to an impaired FAT/CD36 translocation from intracellular pools toward the sarcolemma in response to insulin or due to cellular contractions. Moreover, it remains unclear whether the regulation of LCFA uptake and metabolism by insulin and cellular contractions is impaired in cardiac myocytes from obese Zucker rats.

We hypothesized that in obesity, the regulation of myocardial FAT/CD36-mediated LCFA uptake and utilization by the hormone insulin and by cellular contractions is altered. Isolated, quiescent cardiac myocytes were used to investigate the direct regulation of myocardial LCFA uptake and utilization under well-controlled conditions in which vascular factors were eliminated. The effect of cellular contractions on myocardial LCFA utilization was studied by means of oligomycin, a compound that inhibits F_0/F_1 ATPase activity at relatively low concentrations. Oligomycin, at an optimal concentration (30 $\mu\text{mol/l}$), increased the AMP kinase activity in cardiac myocytes by elevating the intracellular AMP/ATP ratio to approximately the same extent as that observed with cellular contractions (18). To investigate the role of FAT/CD36 in myocardial LCFA uptake and utilization in lean and obese Zucker rats, cardiac myocytes were treated with sulfo-*N*-succinimidyl oleate (SSO), which inactivates FAT/CD36 by covalently binding to its LCFA binding site (24,25).

RESEARCH DESIGN AND METHODS

The study animals, 11-week-old female lean and obese Zucker rats, were obtained from the Harlan Laboratory, kept on a 12-h light/dark cycle, and fed a standard laboratory diet and water ad libitum. Before any surgical interference, the rats, in the fed state, were anesthetized with an intraperitoneal injection of sodium pentobarbital. In blood plasma collected from anesthetized lean and obese Zucker rats, glucose (hexokinase method) and TAGs (glycerol kinase-lipase method) were analyzed on a COBAS BIO analyzer; insulin was analyzed using a radioimmunoassay kit (23). The Experimental Animal Committee of Maas-

tricht University gave approval for all experiments involving animals.

Treatment of isolated cardiac myocytes. Cardiac myocytes from lean and obese Zucker rats were isolated using a Langendorff perfusion system and a modified Krebs-Henseleit bicarbonate medium (12,26). After being isolated, myocytes were washed and treated with 0.5% DMSO or 0.4 mmol/l SSO in DMSO for 30 min at room temperature. SSO is routinely synthesized in our laboratory according to the method of Anjaneyulu and Staros (27). After being washed, the DMSO- and SSO-treated cardiac myocytes were incubated for 15 min at 37°C with continuous shaking. Thereafter, the DMSO-treated myocytes were incubated for 15 min under continuous shaking at 37°C, either not treated (basal) or treated with 10 nmol/l insulin or 30 $\mu\text{mol/l}$ oligomycin. Subsequently, these cardiac myocytes were used for further investigations, but only when >80% of the cells had a rod-shaped appearance, which was determined by a trypan blue staining.

[1- ^{14}C]palmitate uptake, oxidation, and incorporation into intracellular lipid pools. At the start of the incubation, a [1- ^{14}C]palmitate/BSA complex (molar ratio 0.3) was added to non-treated (basal) and SSO-, insulin-, and oligomycin-treated cardiac myocytes so that the final concentration of [1- ^{14}C]palmitate was 100 $\mu\text{mol/l}$. This palmitate concentration, which was below the K_m of 435 $\mu\text{mol/l}$ (corresponding to a "free" palmitate concentration of 6 nmol/l) (28) allowed us to study unidirectional LCFA uptake in the absence of saturation of transporters. Initial rates of [1- ^{14}C]palmitate uptake (3-min incubation period) were determined as previously described (12). In other experiments, rates of [1- ^{14}C]palmitate oxidation (measured as the production of $^{14}\text{CO}_2$ after 20 min of incubation) (29) and esterification (measured as incorporation of the ^{14}C -label into intracellular PLs, TAGs, and unesterified LCFAs after 20 min of incubation) (30) were determined.

Subcellular fractionation of cardiac myocytes and determination of FAT/CD36. Non-treated (basal) and insulin- and oligomycin-treated cardiac myocyte suspensions from lean and obese Zucker rats were diluted with distilled H_2O , and NaN_3 was added to a final concentration of 5 mmol/l to stop ATP-dependent vesicular trafficking events (31). Immediately thereafter, cardiac myocyte suspensions were homogenized and frozen in liquid nitrogen. Subsequently, fractionation was carried out according to a modified procedure of Fuller et al. (32), as previously described (22). To determine the FAT/CD36 protein content in the total heart homogenates and the sarcolemmal (SL) and low-density microsomal (LDM) fractions, SDS-PAGE and Western blotting were performed. The SL fraction was enriched with ouabain-sensitive *p*-nitrophenyl-phosphatase, whereas in the LDM fraction, no activity of *p*-nitrophenyl-phosphatase or Ca^{2+} -ATPase could be detected, indicating that this fraction was devoid from sarcolemma or sarcoplasmic reticulum. Furthermore, caveolin-3 was found to be much more abundant in the SL fraction than in the LDM fraction (data not shown). A monoclonal antibody (MO25) directed against human CD36 was used to detect FAT/CD36. The immunoblot intensities were analyzed by densitometry using the computer program Quantity One (Bio-Rad).

Cardiac triacylglycerol content. Total TAG content was determined in heart homogenates from lean and obese Zucker rats. Lipids were extracted and separated by high-performance thin-layer chromatography (HPTLC), as previously described (33). Quantification was performed by scanning plates and integrating the density areas using Quantity One software (Bio-Rad).

Statistical analysis. Data are presented as means \pm SE. Differences between lean and obese animals were tested by ANOVA and the nonparametric Mann-Whitney *U* test. The paired sample *t* test was used to define difference between treatments within a group. $P < 0.05$ indicates statistical significance.

RESULTS

Characteristics of lean and obese Zucker rats. The body and heart weight of 11-week-old obese Zucker rats were significantly higher than those of their age-matched lean littermates (Table 1). The obese Zucker rats, compared with their lean controls, had significantly elevated TAG plasma concentrations. In addition, the obese rats were euglycemic and markedly hyperinsulinemic, demonstrating the presence of systemic insulin resistance (Table 1). **Initial rate of myocardial LCFA uptake.** The initial rates of basal myocardial LCFA uptake were 1.4-fold higher ($P < 0.05$) in obese Zucker rats compared with their lean littermates (Fig. 1). The inhibitor SSO reduced initial basal myocardial LCFA uptake rates in both lean (-34% ; $P < 0.05$) and obese (-46% ; $P < 0.05$) rats (Fig. 1).

TABLE 1
Characteristics of lean and obese Zucker rats

	Lean	Obese
Body weight (g)	184 ± 18	333 ± 35*
Heart weight (g)	0.98 ± 0.08	1.30 ± 0.12*
Glucose _{plasma} (mmol/l)	11.5 ± 1.3	13.5 ± 2.3
Insulin _{plasma} (nmol/l)	0.44 ± 0.29	3.14 ± 0.65*
Triacylglycerols _{plasma} (mmol/l)	0.36 ± 0.07	2.06 ± 1.43*

Data are means ± SE for 8–10 rats. * $P < 0.05$ vs. lean Zucker rats.

Notably, in SSO-treated cardiac myocytes, initial rates of LCFA uptake were not significantly different in lean and obese rats (Fig. 1).

Insulin stimulated the initial rates of LCFA uptake into cardiac myocytes from lean rats by up to 1.3-fold ($P < 0.05$). In contrast, in myocytes isolated from obese rats, in vitro treatment with insulin failed to stimulate LCFA uptake (Fig. 1). The contraction-mimetic agent oligomycin increased the initial rates of myocardial LCFA uptake by 1.7-fold ($P < 0.05$) in lean and 1.8-fold ($P < 0.05$) in obese rats (Fig. 1). In the oligomycin-stimulated cardiac myocytes, initial LCFA uptake rates were greater ($P < 0.05$) in obese than in lean animals (Fig. 1).

Sarcolemmal and intracellular FAT/CD36 protein content. The total protein content of FAT/CD36 in the heart of obese Zucker rats was not significantly different from that in lean rats (Fig. 2A). However, under basal conditions, the sarcolemmal FAT/CD36 protein content was elevated by 74% ($P < 0.05$) in myocytes from obese rats compared with myocytes from lean rats. Concomitantly, the intracellular FAT/CD36 protein content was reduced by 50% ($P < 0.05$) in obese Zucker rats (Fig. 2B).

In isolated cardiac myocytes from lean rats, in vitro

administration of insulin and oligomycin increased the total sarcolemmal protein amount of FAT/CD36 by 73 and 86% ($P < 0.05$), respectively. This coincided with a decrease in intracellular FAT/CD36 protein content of -46 and -51% ($P < 0.05$), respectively (Fig. 2B). In contrast, in isolated myocytes from obese rats, in vitro administration of both insulin and oligomycin failed to increase sarcolemmal FAT/CD36 beyond the increase observed in the basal state (Fig. 2B). Concurrently, in isolated myocytes from obese rats, insulin and oligomycin failed to decrease the intracellular depot of FAT/CD36 beyond the reduction observed in the basal state (Fig. 2B).

Myocardial LCFA oxidation rate. Under basal conditions, myocardial LCFA oxidation rates were not significantly different in obese Zucker rats compared with in lean control rats (Fig. 3). SSO reduced the myocardial LCFA oxidation to a similar extent in lean (76%; $P < 0.05$) and in obese (86%; $P < 0.05$) rats (Fig. 3). In vitro addition of insulin had no effect on the rates of LCFA oxidation in isolated cardiac myocytes obtained from lean and obese rats (Fig. 3). In contrast to the effects of insulin, oligomycin increased the LCFA oxidation rate in isolated cardiac myocytes from lean (2.6-fold; $P < 0.05$) and obese (3.7-fold; $P < 0.05$) rats. The effect of oligomycin was greater in myocytes from obese rats compared with those from lean rats ($P < 0.05$, Fig. 3).

LCFA esterification rate into myocardial triacylglycerols and phospholipids and the myocardial unesterified LCFA pool. The total TAG pool was threefold higher ($P < 0.05$) in heart homogenates of obese Zucker rats compared with lean rats (Fig. 4). Under basal conditions, the rate of incorporation of extracellular LCFAs into intracellular TAGs in myocytes from obese rats was 2.3-

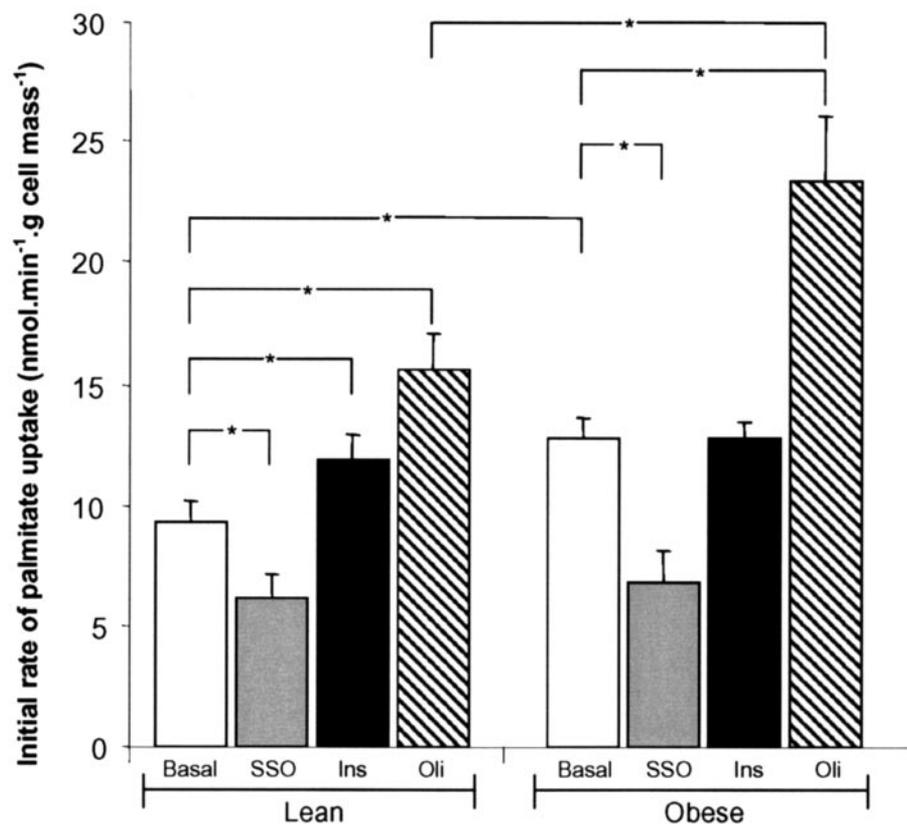


FIG. 1. Influence of SSO, insulin (Ins), and oligomycin (Oli) on the initial palmitate uptake rate in cardiac myocytes from lean and obese Zucker rats. [¹⁴C]palmitate uptake in 3 min was determined in cardiac myocytes from lean and obese Zucker rats either not treated (basal) or treated with 0.4 mmol/l SSO, 10 mmol/l insulin, or 30 μmol/l oligomycin and expressed as nanomoles per minute per gram of cell mass. Data are means ± SE, $n = 8$. * $P < 0.05$.

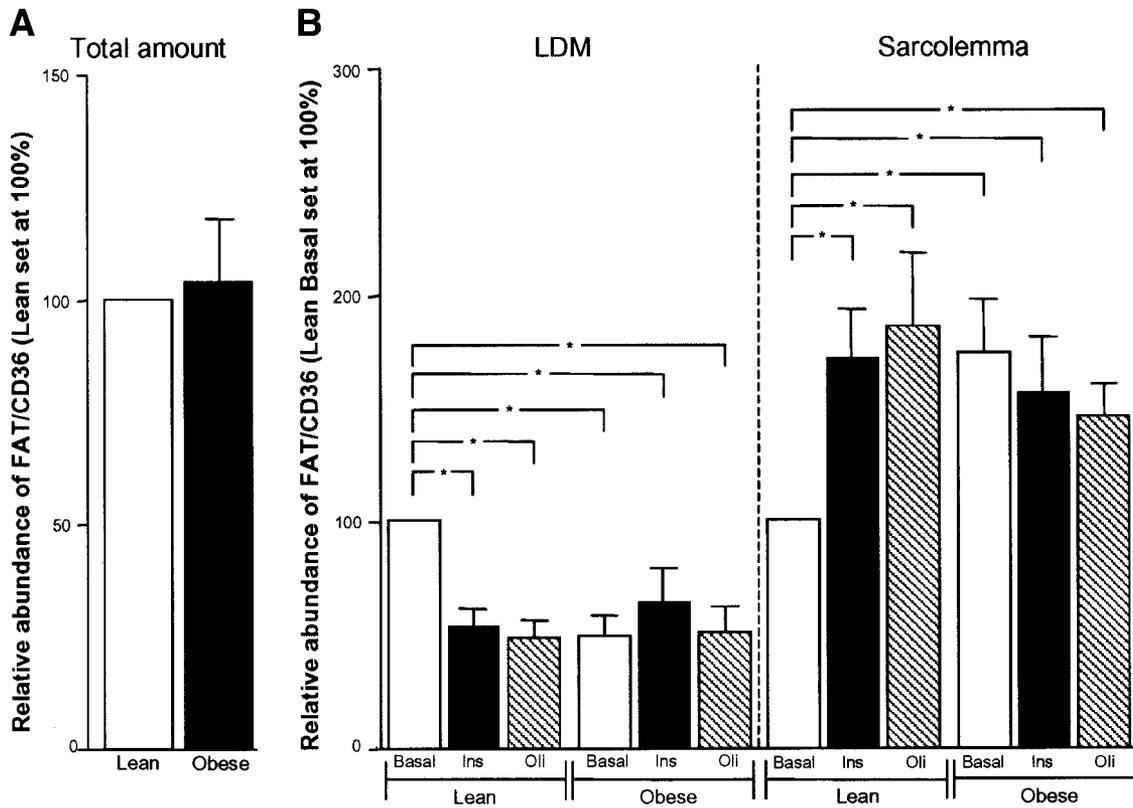


FIG. 2. Quantitation of Western blots of the LCFA transporter FAT/CD36 in total heart homogenates ($n = 6$; A) and LDM and SL compartment of cardiac myocytes from lean and obese Zucker rats ($n = 8$; B), under basal conditions or treated with 10 nmol/l insulin (Ins) or 30 μ mol/l oligomycin (Oli). The FAT/CD36 protein content was expressed as the percent of FAT/CD36 content in nontreated cardiac myocytes from lean Zucker rats in the corresponding fractions. Data are means \pm SE. * $P < 0.05$ vs. basal values in lean Zucker rats.

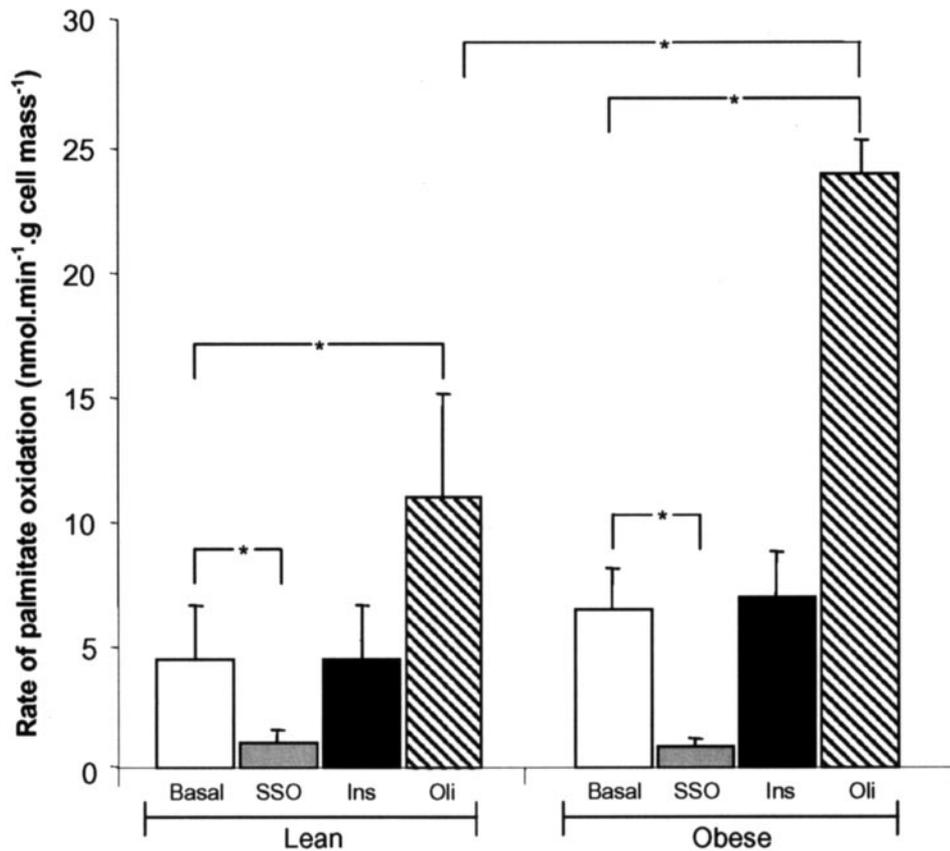


FIG. 3. Influence of SSO, insulin, and oligomycin on the palmitate oxidation rate in cardiac myocytes from lean and obese Zucker rats. ¹⁴CO₂ production (palmitate oxidation) in 20 min was determined in cardiac myocytes from lean and obese Zucker rats either not treated (basal) or treated with 0.4 mmol/l SSO, 10 nmol/l insulin (Ins), or 30 μ mol/l oligomycin (Oli) and expressed as nanomoles per minute per gram of cell mass. Data are means \pm SE, $n = 8$. * $P < 0.05$.

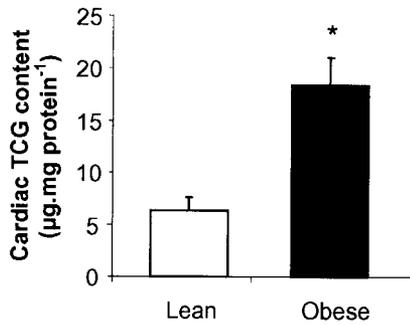


FIG. 4. Cardiac TAG content in lean and obese Zucker rats, measured using HPTLC in heart homogenates and expressed as micrograms per milligram of protein. Data are means \pm SE, $n = 6$. * $P < 0.05$ vs. lean Zucker rats.

fold higher ($P < 0.05$) than in myocytes from lean rats (Fig. 5A). SSO reduced the incorporation of LCFA into intracellular TAG in myocytes from lean rats and significantly ($P < 0.05$) reduced it in myocytes from obese rats (Fig. 5A).

In vitro insulin administration stimulated the incorporation of LCFAs into intracellular TAGs by 2.3-fold ($P < 0.05$) in cardiac myocytes isolated from lean rats. In contrast, in myocytes from obese rats, this hormone had no additional effect, over basal conditions, on the incor-

poration of LCFAs into the TAG pool (Fig. 5A). Oligomycin had no significant effect on LCFA incorporation into intracellular TAGs in myocytes from lean and obese rats (Fig. 5A).

In nontreated cardiac myocytes, the rate of LCFA incorporation into PLs was not different in obese Zucker rats compared with lean rats (Fig. 5B). In myocytes from lean rats, SSO significantly reduced the rate of incorporation into the intracellular PL pool by 42% ($P < 0.05$) compared with the basal incorporation rate; however, in myocytes from obese rats, SSO had no significant effect on PL esterification. Insulin and oligomycin had no effect on the incorporation rate of LCFA into PL in myocytes from either lean or obese rats (Fig. 5B).

The rate of deposition of extracellular LCFA into the intracellular, unesterified LCFA pool in cardiac myocytes from obese Zucker rats was increased by more than twofold ($P < 0.05$) under basal conditions compared with myocytes from lean rats (Fig. 6). In myocytes from obese rats, SSO reduced the LCFA deposition into the intracellular LCFA pool to the same absolute level as in myocytes from lean rats under basal conditions (Fig. 6). In cardiac myocytes from lean rats, SSO did not alter LCFA deposition into the LCFA pool (Fig. 6). Insulin and oligomycin did not alter this parameter in cardiac myocytes from either lean or obese Zucker rats (Fig. 6).

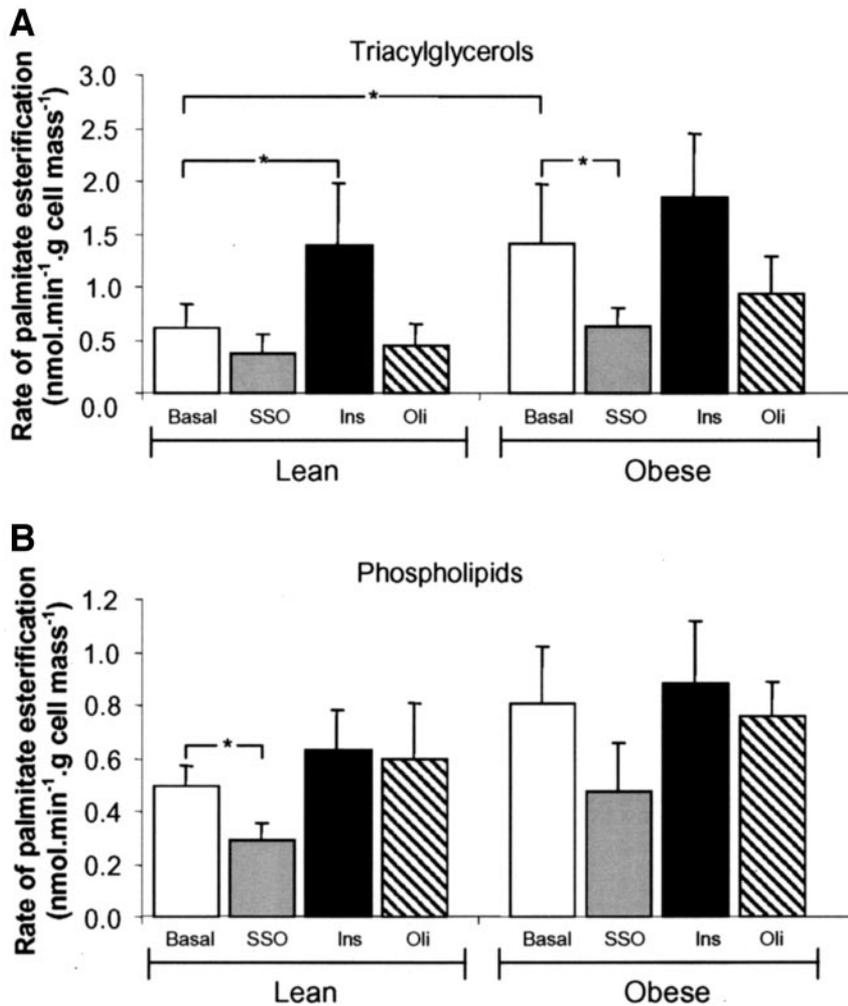


FIG. 5. Influence of SSO, insulin, and oligomycin on [¹⁴C]palmitate esterification into TAGs and PLs in cardiac myocytes from lean and obese Zucker rats. Incorporation of [¹⁴C]palmitate into intracellular TAGs (A) and PLs (B) was determined after 20 min in cardiac myocytes either not treated (basal) or treated with 0.4 mmol/l SSO, 10 nmol/l insulin (Ins), or 30 µmol/l oligomycin (Oli) and expressed as nanomoles per minute per gram of cell mass. Data are means \pm SE, $n = 8$. * $P < 0.05$.

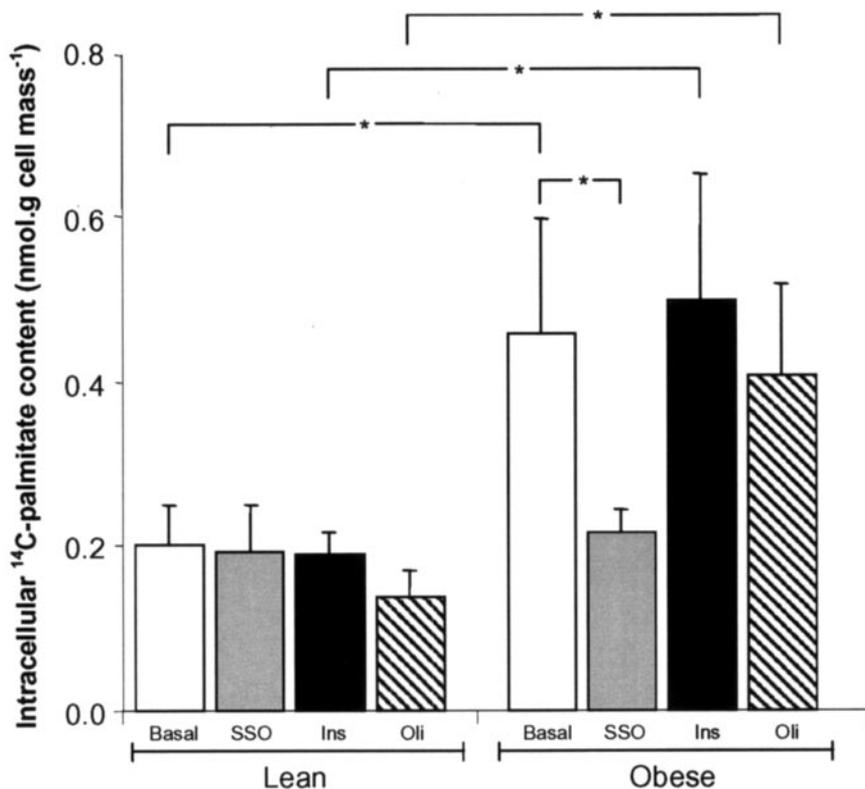


FIG. 6. Influence of SSO, insulin, and oligomycin on the intracellular [¹⁴C]palmitate content in cardiac myocytes from lean and obese Zucker rats. The intracellular content of [¹⁴C]palmitate was determined after 20 min in cardiac myocytes either not treated (basal) or treated with 0.4 mmol/l SSO, 10 nmol/l insulin (Ins), or 30 μ mol/l oligomycin (Oli) and expressed as nanomoles per minute per gram of cell mass. Data are means \pm SE, $n = 8$. * $P < 0.05$.

DISCUSSION

In the present study, we investigated the regulation of the initial rate of LCFA uptake, the subcellular localization of the LCFA transporter FAT/CD36, and the rates of LCFA oxidation and esterification into intracellular lipids in cardiac myocytes isolated from lean and obese Zucker rats. The following novel observations were made: 1) in cardiac myocytes isolated from obese Zucker rats, there was increased LCFA uptake due to a permanent translocation of FAT/CD36 to the sarcolemma; 2) in vitro, the stimulatory effect of insulin on LCFA uptake was lost in cardiac myocytes from obese rats, whereas the stimulatory effect of oligomycin was maintained; 3) insulin as well as oligomycin lost the ability in vitro to induce FAT/CD36 translocation in cardiac myocytes from obese rats; 4) the rate of LCFA esterification into intracellular TAGs was markedly elevated in cardiac myocytes from obese rats in vitro, which likely accounted for the increased cardiac TAG content, as rates of LCFA oxidation did not differ and in vitro insulin failed to stimulate TAG esterification further; and 5) the FAT/CD36 inhibitor SSO was able to reduce the elevated incorporation of LCFAs into both the intracellular unesterified LCFAs and the TAG pools in cardiac myocytes from obese rats to normal physiological levels, as was seen in cardiac myocytes from lean littermates.

Alterations in myocardial LCFA uptake by obese Zucker rats. The 11-week-old female obese Zucker rats were hyperinsulinemic and euglycemic, indicating that they were insulin resistant. The rats were also hyperlipidemic, which is characterized by elevated TAG plasma levels (Table 1). In a previous study with these obese Zucker rats using heart giant membrane vesicles (23), we demonstrated that obesity was associated with a marked elevation of the transsarcolemmal LCFA transport rate. In

the present study we were able to confirm in isolated cardiac myocytes the previously observed elevated uptake of LCFAs by giant membrane vesicles obtained from hearts of obese Zucker rats. The same previous study (23) also revealed that membranes from giant sarcolemmal vesicles displayed a higher content of FAT/CD36, whereas total heart tissue amounts of this protein were unaltered, suggesting that FAT/CD36 is permanently relocated to the sarcolemma in myocytes from obese rats. In the present study, the subcellular distribution of FAT/CD36 in cardiac myocytes was examined by subcellular fractionation (Fig. 2A and B). The findings demonstrated that the total protein amount of FAT/CD36 in the obese heart is not altered, whereas its abundance in the sarcolemmal fraction is increased, coinciding with a decline in the intracellular pool of this transporter. Thus, these subcellular fractionation studies provide definitive evidence that a permanent relocation of FAT/CD36 from an intracellular compartment to the sarcolemma is responsible for the elevated myocardial LCFA uptake in obese Zucker rats. In the present study we also demonstrated that in myocytes isolated from obese rats and treated with SSO, which specifically inhibits FAT/CD36-mediated LCFA uptake, LCFA uptake was markedly reduced to a level not significantly different from that in SSO-treated cardiac myocytes from lean rats. This observation provides the causal link between chronically elevated sarcolemmal FAT/CD36 protein levels and enhanced myocardial LCFA uptake. Taken together, these findings show that in obesity, FAT/CD36 is permanently relocated to the sarcolemma and increases the myocardial LCFA uptake rate. However, the mechanisms that maintain FAT/CD36 at the sarcolemma are incompletely understood.

Previously we established that there are two signaling pathways linked to the translocation of FAT/CD36 from an

intracellular compartment to the sarcolemma: insulin-induced PI 3-kinase-dependent signaling (13–15) and contraction-induced AMPK-dependent signaling (18). Accordingly, in the present study, we found that *in vitro* insulin and oligomycin were effective in inducing FAT/CD36 translocation and stimulating LCFA uptake in cardiac myocytes from lean rats. In contrast, neither stimulus was able to further translocate FAT/CD36 in cardiac myocytes from obese Zucker rats. We speculate that FAT/CD36-mediated LCFA uptake by cardiac myocytes from obese rats is already maximally stimulated due to physiologically high plasma insulin levels *in vivo*. Thus, the permanent relocation of FAT/CD36 seen in the myocytes from obese rats would then be a normal response to increased concentrations of circulating insulin. Importantly, the loss of insulin's ability to additionally induce FAT/CD36 translocation in cardiac myocytes from obese rats *in vitro* was paralleled by a loss in the ability of insulin to further stimulate LCFA uptake.

Oligomycin retained its ability to stimulate myocardial LCFA uptake in obese Zucker rats despite the loss of its ability to induce FAT/CD36 translocation. Apparently, oligomycin stimulates myocardial LCFA uptake in obese rats by a different mechanism than it does for FAT/CD36 translocation. A possible explanation could be that oligomycin increases the intrinsic activity of FAT/CD36 by a signaling event that is not at all or only partly operative in cardiac myocytes from lean rats. We have evidence that supports the fact that the intrinsic activity of FAT/CD36 in hearts of lean rats can be changed (22). Based on the presence of putative phosphorylation motifs in the primary sequence of FAT/CD36, it is feasible that an increase in intrinsic activity is due to phosphorylation. We speculate that certain members of the protein kinase C family, which are known to be differentially expressed and activated in the heart of obese Zucker rats (34–36), are responsible for an increased FAT/CD36 activation at the sarcolemma.

Alterations in myocardial LCFA metabolism by obese Zucker rats. The increased FAT/CD36-mediated LCFA uptake by cardiac myocytes from obese Zucker rats will provide the intracellular metabolic machinery with a greater supply of LCFA. Therefore, it was of interest to monitor the possible differences in the metabolic fate of LCFAs taken up by myocytes from lean versus obese rats. When considering the main metabolic pathways that are involved in LCFA processing, it appeared that under basal conditions, the rate of esterification of LCFAs into intracellular TAGs is significantly elevated in myocytes from obese rats, whereas the rates of LCFA oxidation and esterification into PLs were not altered. We expect that the enhanced LCFA esterification rate into myocardial TAGs is causally related to the elevated intracellular TAG content found in hearts of obese Zucker rats. Another important metabolic alteration in myocytes from obese rats is that the intracellular concentration of unesterified LCFAs was significantly increased by more than twofold. Previously, we established that LCFAs taken up by cardiac myocytes are rapidly equilibrated (<30 s) with the total intracellular unesterified LCFA pool (12). Therefore, we can reasonably assume that a twofold increase in deposition of radiolabeled product in the myocardial unesterified LCFA pool,

measured after 20 min, reflected a twofold increase in the LCFA pool size. Because intracellular unesterified LCFAs are highly disruptive to cellular membranes and to a great number of enzymes, the mammalian cell generally attempts to maintain a very low level of unesterified LCFAs (19). As a result, the rise in intracellular unesterified LCFA is a sensitive indicator of a mismatch between LCFA uptake and subsequent LCFA metabolism (37). It is likely that esterification of LCFAs into TAGs provides an escape route to prevent a further rise in unesterified LCFAs. An intracellular TAG accumulation and an increased amount of intracellular unesterified LCFAs are considered to be critical steps in the development of cellular insulin resistance. These excess lipid depots will also result in an increased intracellular long-chain acyl-CoA pool, leading to cardiac lipotoxicity (4,7,38). Moreover, the excess amount of myocardial LCFAs may enter the lipotoxic pathways of *de novo* synthesis of ceramide, which appears to play a central role in lipoapoptosis. Ceramide increases the expression of inducible NO synthetase, leading to an increased production of NO and reactive oxygen species causing lipoapoptosis (39). Our findings strengthen the relation between TAG storage and LCFA uptake and implicate FAT/CD36 in this process.

Insulin had no effect on the rates of LCFA oxidation and esterification into PLs but stimulated the rate of esterification into TAGs in cardiac myocytes from lean rats. These observations are in line with our earlier observations (15). However, in cardiac myocytes from obese rats, the *in vitro* administration of insulin had no additional metabolic effects. The loss of insulin's ability to stimulate LCFA esterification is likely related to elevated basal levels of the LCFA esterification rate into intracellular TAGs. Analogous to the changes seen in the initial LCFA uptake rate, we speculate that insulin signaling is already maximally activated under basal conditions due to the high levels of insulin *in vivo*, so that the addition of insulin *in vitro* does not result in further activation of the LCFA esterification rate into TAGs. This would indicate that the excess amount of LCFAs taken up under basal conditions is preferentially channeled into storage.

When examining the metabolic effects of contraction signaling, oligomycin strongly stimulated LCFA oxidation and had no effect on the esterification of LCFAs into TAGs and PLs in cardiac myocytes from lean rats. It was found that these metabolic actions of oligomycin are preserved in cardiac myocytes from obese Zucker rats and that, strikingly, LCFA oxidation is even more sensitive to oligomycin in cardiac myocytes from obese compared with lean rats. This hypersensitivity of LCFA oxidation to oligomycin might be caused by a more potent activation of AMPK in cardiac myocytes from obese Zucker rats. In this respect, it should be noted that in skeletal muscle from both obese Zucker rats and type 2 diabetic patients, AMPK activity is still highly inducible (40,41).

A total blockade of FAT/CD36-mediated LCFA uptake by SSO significantly reduced the rate of LCFA utilization by two major LCFA metabolic pathways, LCFA oxidation and esterification into intracellular TAGs, in cardiac myocytes from both lean and obese Zucker rats. This finding is in agreement with the notion that the inhibitory effects of SSO on LCFA metabolism are secondary to its inhibitory

effect on the LCFA uptake process (12,16,25). Hence, SSO treatment proved to be an effective tool in normalizing LCFA metabolism in myocytes from obese rats. Specifically, the normalization by SSO of the elevated esterification rate into TAGs in myocytes from obese rats suggests that increased sarcolemmal FAT/CD36 is responsible for TAG accumulation in the obese rat heart. Interestingly, and of potential therapeutic significance, is the observation that SSO is able to reduce the intracellular level of unesterified LCFAs, whereas the stimulatory effect of the AMPK-activating agent oligomycin on LCFA oxidation failed to do so. This indicates that SSO is able to not only decrease LCFA esterification into TAGs, but also to restore the balance between LCFA uptake and metabolism in obese cardiac myocytes. Therefore, SSO is possibly more suited as antidiabetic agent than currently used drugs, such as 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), which acts via activation of AMPK (40,42,43).

The results of the present study indicate that in cardiac myocytes from obese Zucker rats, a permanent translocation of FAT/CD36 to the sarcolemma results in enhanced extracellular LCFA uptake and channeling into TAGs, which is expected to lead to an accumulation of myocardial TAGs. Furthermore, these cardiac myocytes, although sensitive to contraction-like stimulation, are completely insensitive to insulin when administered in vitro, as basal conditions in hyperinsulinemic, obese animals resemble the insulin-stimulated condition in lean littermates. Nonetheless, the action of insulin in these lean littermates will not lead to cardiac lipotoxicity, as opposed to the case with obese rats, because in vivo cardiac myocytes will be exposed to insulin only during a short postprandial insulin peak. Finally, the studies with SSO suggest that blocking FAT/CD36 would be an effective means to limit LCFA uptake and to prevent myocardial TAG accumulation, which most likely will result in an improved function of the heart in obesity.

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