Thiazolidinediones and Fatty Acids Convert Myogenic Cells into Adipose-like Cells*

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Lydia Teboul, Danielle Gaillard, Laurence Staccini, Hidekuni Inadera, Ez-Zoubir Amri, and Paul A. Grimaldi‡
From the Centre de Biochimie, UMR-134 CNRS, Université de Nice-Sophia Antipolis, Faculté des Sciences, 06108 Nice Cedex 2, France

Fatty acids and thiazolidinediones act as potent activators of the adipose differentiation program in established preadipose cell lines. In this report, the effects of these agents on the differentiation pathway of myoblasts have been investigated. Exposure of C2C12N myoblasts (a subclone of the C2C12 cell line) to thiazolidinediones or fatty acids prevents the expression of myogenin, α-actin, and creatine kinase, thus abolishing the formation of multinucleated myotubes. These treatments lead in parallel to the expression of a typical adipose differentiation program including acquisition of adipocyte morphology and activation of adipose-related genes. A similar transition toward the adipose differentiation pathway also occurs in mouse muscle satellite cells maintained in primary culture. Thiazolidinediones exert their adipogenic effects only in non-terminally differentiated myoblasts; myotubes are insensitive to the compounds. Continuous exposure to inducers after growth arrest is not required to maintain the adipose phenotype, but proliferation of adipose-like C2C12N cells leads to a complete reversion toward undifferentiated cells able to undergo either myogenic or adipogenic differentiation depending on the composition of culture medium. These results indicate that adipogenic inducers, such as thiazolidinediones or fatty acids, specifically convert the differentiation pathway of myoblasts into that of adipoblasts.

Formation of muscle, bone, and adipose tissue are multistep processes that include the determination of a common progenitor mesodermal cell toward a specific differentiation pathway followed by the expression of various terminal differentiation phenotypes. The multipotentiality of progenitor mesodermal cells has been illustrated in vitro by the spontaneous and concomitant differentiation of embryonal carcinoma cells (1) or of clonal cell populations derived from fetal rat calvaria (2) into myotubes, adipocytes, and chondrocytes. Multiple differentiated phenotypes can also be chemically induced by 5-aza-cytidine treatment of fibroblasts, such as C3H10T1/2 or Swiss 3T3 cells (3). Several lines of evidence indicate that exogenous regulatory factors play a crucial role in the determination of common progenitor cell into specific differentiation lineages. Glucocorticoids exert positive effects on the differentiation of the RCJ 3.1 clonal line from fetal rat calvaria into myotubes, adipocytes, and chondrocytes (2), whereas transforming growth factor-β exerts negative effects on all these differentiation processes (4). Other factors, such as bone morphogenetic protein-2 (5), triggers specific determination of pluripotent fibroblasts (6) or L6 and C2C12 myoblasts (7, 8) toward the osteoblast lineage.

To our knowledge, factors that are able to promote transition from the myoblast lineage to that of the adipoblast have not been yet discovered. Muscle and adipose differentiation involve complex processes that lead to the induction of several differentiation-linked genes specifically expressed either in muscle cells, i.e. MyoD, myogenin, α-actin, or muscle creatine kinase (MCK), or in adipose cells, i.e. adipocyte lipid binding protein (ALBP) or hormone-sensitive lipase (9, 10). Other genes, such as glycerol-3-phosphate dehydrogenase (GPDH), lipoprotein lipase, insulin-responsive glucose transporter-4 (Glut-4), or fatty acid transporter, are expressed in both tissues but at higher levels in adipose tissue. Factors controlling muscle and adipose differentiation processes are clearly different. Differentiation of myoblasts in cell culture is initiated by peptide growth factor withdrawal (11, 12) while adipose differentiation is controlled by addition of various hormones and nutrients (10). We have shown that long chain fatty acids act in preadipose cells as adipogenic agents (13–16). These effects of fatty acids are mediated by activation of a nuclear receptor called fatty acid-activated receptor (FAAR) expressed in a variety of tissues including adipose tissue and muscle (17). FAAR is activated by fatty acids (17) and thiazolidinediones (18), a new class of antidiabetic agents, which have also been described as exerting potent adipogenic effects in preadipose cell lines (19, 20). The mode of action of thiazolidinediones as antidiabetic agents is not yet understood, but it has been shown that their administration to diabetic animals improves insulin sensitivity of muscle and adipose tissue (21, 22).

In this study, we investigated the effects of fatty acids and thiazolidinediones, on the differentiation pathway of C2C12N myogenic cells and satellite cells from newborn mouse muscle. We report that these compounds prevent myotube formation and induce the expression of a new phenotype resembling that of adipose cells.

EXPERIMENTAL PROCEDURES

Materials—Culture media were obtained from Life Technologies, Inc. (Cergy-Pontoise, France). Bovine serum and other chemical products were purchased from Sigma Chimie (Saint-Quentin, France). Radioactive materials, the random priming kit, Hybond membranes, and Hyperfilm MP were from Amersham (Les Ullis, France). Thiazo-
of post-confluent cells to 5 days from seeding today until 5 days post-confluence (Fig. 1). Multinucleated myotubes when maintained in standard medium expressed high levels of MCK activity (2250 ± 225 milliunits/mg of protein) and low levels of GPDH activity (24 ± 4 milliunits/mg of protein). Exposure to 5 μM BRL 49653, 10 μM CS045, or 100 μM pioglitazone led to a strong inhibition of MCK activity and to induction of GPDH activity. Treatment with 100 μM γ-linolenic acid or 5,8,11,14-eicosatetraynoic acid also effectively inhibited MCK expression and induced GPDH expression. Palmitate appeared to be less effective than polyunsaturated fatty acids in this process.

RESULTS

Effects of BRL 49653 on C2C12N Cell Differentiation—C2C12N cells displayed an almost complete morphology of multinucleated myotubes when maintained in standard medium without additions (A) or in the presence of 5 μM BRL 49653 added at confluence (B) or 2 days before confluence (C). Bars, 0.1 mm.

FIG. 1. Effects of BRL 49653 on C2C12N morphological differentiation. Cells were maintained to day 5 post-confluence in standard medium without additions (A) or in the presence of 5 μM BRL 49653 added at confluence (B) or 2 days before confluence (C). Bars, 0.1 mm.

FIG. 2. Effects of various thiazolidinediones and fatty acids on muscle and adipose-like differentiation of C2C12N cells. MCK and GPDH activities were determined in 5 day post-confluent cells maintained in standard medium (a) or exposed from day −2 to day +5 relative to confluence to 5 μM BRL 49653 (b), 10 μM CS 045 (c), 10 μM pioglitazone (d), 100 μM palmitate (e), 100 μM γ-linolenate (f), or 100 μM 5,8,11,14-eicosatetraynoic acid (g). Values obtained with cells maintained in standard medium are taken as 1 and represent the mean ± S.D. from three separate experiments.

FIG. 3. Effects of BRL 49653 on the expression of myogenic or adipose differentiation markers in C2C12N cells. Cells were maintained in standard medium until day 5 post-confluence and exposed (lane 2) or not (lane 1) from confluence to 5 μM BRL 49653. 20 μg of total RNA was analyzed by Northern blot as described under "Experimental Procedures." Similar results have been obtained in three separate experiments.

FIG. 4. Effects of BRL 49653 on satellite cell differentiation. A and B, satellite cells were isolated from muscle of newborn mice as described under "Experimental Procedures" and maintained in standard medium in the absence (A) or presence (B) of 5 μM BRL 49653 from seeding to 5 days post-confluence. Bars, 0.1 mm. C, RNA (20 μg/lane) from 5 days post-confluent untreated cells (lane 1) or cells exposed to 5 μM BRL 49653 (lane 2) was analyzed as described in the legend to Fig. 3. Similar results have been obtained in three separate experiments.

Effects of BRL 49653 on C2C12N Cell Differentiation—C2C12N cells displayed an almost complete morphology of multinucleated myotubes when maintained in standard medium from seeding to day 5 post-confluence (Fig. 1A). Exposure of post-confluent cells to 5 μM BRL 49653 (30) led to a net decrease in the number of multinucleated myotubes with a parallel appearance of small cells containing lipid droplets (Fig. 1B). A homogenous monolayer of lipid-containing cells was observed when the treatment was started 2 days before confluence (Fig. 1C). This phenomenon was next investigated biochemically by the determination of MCK activity, as a muscle differentiation marker, and GPDH activity, as an adipocyte differentiation marker, in C2C12N cells exposed from day −2 to +5 relative to confluence to thiazolidinediones, namely BRL 49653, CS 045 (20), and pioglitazone (19), or various long chain fatty acids (Fig. 2). C2C12N cells maintained in standard medium expressed high levels of MCK activity (2250 ± 225 milliunits/mg of protein) and low levels of GPDH activity (24 ± 4 milliunits/mg of protein). Exposure to 5 μM BRL 49653, 10 μM CS 045, or 10 μM pioglitazone led to a strong inhibition of MCK activity and to induction of GPDH activity. Treatment with 100 μM γ-linolenic acid or 5,8,11,14-eicosatetraynoic acid also effectively inhibited MCK expression and induced GPDH expression. Palmitate appeared to be less effective than polyunsaturated fatty acids in this process.

Northern blot analyses were performed to investigate the effects of BRL 49653 on the expression of RNA markers characteristic of either muscle or adipose differentiation in C2C12N cells (Fig. 3). Cells maintained in standard medium were clearly differentiated into myotubes since high levels of muscle-specific form of α-actin mRNA and myogenin mRNA were expressed 5 days after confluence. By contrast, these cells did not express detectable levels of ALBP, GPDH, and hormone-sensitive lipase mRNAs, and they showed only weak signals for Glut-4, lipoprotein lipase, and fatty acid transporter mRNAs. Exposure to BRL 49653 strongly reduced the expression of myogenin and α-actin mRNAs and led to the emergence of adipose markers including ALBP, GPDH, and hormone-sensitive lipase mRNAs. Significant increases in Glut-4, lipoprotein lipase, and fatty acid transporter mRNA expression were also

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Effects of BRL 49653 on satellite cell differentiation. A and B, satellite cells were isolated from muscle of newborn mice as described under "Experimental Procedures" and maintained in standard medium in the absence (A) or presence (B) of 5 μM BRL 49653 from seeding to 5 days post-confluence. Bars, 0.1 mm. C, RNA (20 μg/lane) from 5 days post-confluent untreated cells (lane 1) or cells exposed to 5 μM BRL 49653 (lane 2) was analyzed as described in the legend to Fig. 3. Similar results have been obtained in three separate experiments.
Cells cultured in standard medium were exposed from day −2 to day +5 to increasing concentrations of BRL 49653 (filled symbols) or CS 045 (open symbols). A, RNA was analyzed as described under "Experimental Procedures." Results are expressed by taking the maximal value obtained for each probe as 100. Symbols are: □. myogenin mRNA; □, □, ALBP mRNA; B. MCK □, □ and GPDH (□, □) enzymatic activities were determined in the same cells as in A. Results are presented as in Fig. 2 and represent the mean ± S.D. from three separate experiments.

FIG. 5. Dose-response effects of BRL 49653 and CS 045 on myogenic and adipose marker expression in C2C12N cells. Cells cultured in standard medium were maintained in standard medium supplemented or not with 5 μM BRL 49653 or CS 045. After plating until day 5 post-confluence, satellite differentiation process of satellite cells from thigh muscles of 1-day-old mice. After plating until day 5 post-confluence, satellite cells were maintained in standard medium supplemented or not with 5 μM BRL 49653. At that time, cells maintained in standard medium differentiated into myotubes (Fig. 4A), whereas cells exposed to BRL 49653 presented an adipose-like morphology (Fig. 4B). Northern blot analyses confirmed these morphological observations, since control cells expressed high levels of myogenin and α-actin mRNAs and did not express ALBP and fatty acid transporter mRNAs, whereas BRL 49653-treated cells strongly expressed the adipose markers ALBP and fatty acid transporter and only weakly expressed the muscle markers (Fig. 4C). Similar results were obtained for satellite cells exposed to 10 μM CS 045 or 100 μM linoleic acid instead of BRL 49653 (not shown). Taken together, these observations strongly suggest that thiazolidinediones and fatty acids inhibit myogenic differentiation and induce the expression of a typical adipose differentiation program in C2C12N cells and in primary cultures of muscle cells.

Potency of Thiazolidinediones on C2C12N Adipose Differentiation—C2C12N cells maintained in standard medium were exposed to increasing concentrations of BRL 49653 or CS 045 from day −2 to day +5 relative to confluence. The potency of the compounds to change the differentiation pathway of the cells was evaluated by determining the amounts of myogenin and ALBP mRNAs (Fig. 5A), and of MCK and GPDH enzymatic activities (Fig. 5B). Morphological analysis revealed that, increasing the concentration of inducer lead, in a dose-dependent manner, to a decrease in the rate of myotube formation with a concomitant increase in the appearance of lipid-containing cells in the whole cell population (not shown). Biochemical parameters reflected this dual process, since inhibition of muscle markers, i.e. myogenin mRNA and MCK activity, and induction of adipose markers, i.e. ALBP mRNA and GPDH activity, were also dose-dependent. Both compounds exerted their effects at very low concentrations, BRL 49653 appearing more active than CS 045 with half-maximal effective concentrations at about 100 and 300 nM, respectively.

Time-dependence of BRL 49653 Effects on Adipose C2C12N Differentiation—To determine the temporal action of thiazolidinediones on inhibition of myotube formation and induction of adipose differentiation, C2C12N cells maintained in standard medium were exposed for various periods of time to a maximally effective concentration of BRL 49653. Five days after confluence, the extent of muscle and adipose differentiation was estimated by determining MCK and GPDH enzymatic activities (Fig. 6). The maximal adipogenic effect of the compound, observed in cells treated from day −2 to day +5 relative to confluence, resulted in a complete absence of MCK activity and a 24-fold induction of GPDH activity. However, chronic treatment by BRL 49653 was not required, since cells treated from day −2 to day 0 or from day 0 to day +3 expressed high GPDH activities and low MCK activities. By contrast, BRL 49653 appeared to be ineffective on myotubes as illustrated by the lack of effect on MCK and GPDH activities in cells maintained until day 3 after confluence in standard medium and then exposed to the compound for 2 days (Fig. 6) or longer (not shown).

To investigate whether or not the adipose phenotype is inherited, C2C12N cells exposed to 5 μM BRL 49653 from day −2 to day +3 relative to confluence were replated in standard medium at a 20-fold dilution in order to promote cell proliferation. These cells were already differentiated into adipocytes since expressing a high level of GPDH activity (Fig. 7). After attachment, cells began to proliferate actively to reach conflu-
ence after 5 days. At that time, cells presented the same morphology as cells which had never been exposed to the compound and expressed low GPDH activity (35 milliunits/mg of protein). In addition, these dedifferentiated cells were found to have recovered the ability to undergo either new myogenic differentiation when maintained after confluence in standard medium, illustrated by the induction of MCK and the lack of expression of GPDH, or new adipose differentiation when exposed to BRL 49653, illustrated by the induction of GPDH and the lack of expression of MCK (Fig. 7). These observations demonstrate that induction of the adipose phenotype by BRL 49653 does not require the continuous exposure of nonproliferative confluent cells. Rather, cell proliferation leads to complete reversion of this differentiated phenotype.

Expression of Nuclear Adipose-regulatory Factors in C2C12N Cells—To investigate the mechanisms underlying the adipogenic effect of BRL 49653, expression of nuclear proteins known to play crucial roles in the control of adipose differentiation was examined. Fig. 8 presents the time course for induction of mRNAs encoding these proteins in C2C12N cells exposed or not to 5 μM BRL 49653 at confluence. α-Actin and ALBP mRNAs were used as indicators of muscle and adipose differentiation, respectively (Fig. 8A). In cells maintained in standard medium, α-actin mRNA emerged at day 1 post-confluence and accumulated thereafter to reach a maximal expression at day 5 post-confluence, whereas treatment with BRL 49653 almost completely prevented this accumulation. In contrast, ALBP mRNA expression was very low in untreated cells, and increased in cells exposed to the drug to attain maximal levels at day 5. As shown in Fig. 8B, FAAR mRNA was detectable 2 days before confluence and reached 75% of its maximal expression at confluence. Exposure of the cells to BRL 49653 led to a moderate, but significant increase in FAAR mRNA expression. C/EBPα and PPARγ mRNAs were below the limit of detection in growing cells and remained undetectable during the myogenic differentiation of cells kept in standard medium. Emergence of both mRNAs occurred in parallel to that of ALBP mRNA in cells exposed to BRL 49653, reaching plateau values at day 5 after confluence.

**DISCUSSION**

The present study demonstrated that thiazolidinediones and fatty acids prevent the myogenic differentiation of myoblasts from a donor cell line or from primary muscle cell cultures, and also promote their differentiation into adipose-like cells. Exposure to thiazolidinediones or to fatty acids drives the developmental fate of C2C12N myoblasts in an adipogenic direction. At the gene level, these agents prevent the expression of muscle specific genes such as MCK, α-actin, and myogenin and lead to the expression of a typical adipose differentiation program. It is noteworthy that in C2C12N adipose-like cells the levels of expression of all the adipose-related genes investigated in this study are quite similar to those found in fully differentiated cells from adipose cell lines. For the most potent adipogenic agent, BRL 49653, this dual regulation takes place at low concentrations with a half-maximal effect of about 100 nM, indicating that C2C12N myoblasts are more sensitive to BRL 49653 than preadipose Ob1771 cells in which a stimulatory effect of the compound on ALBP gene expression is observed over a range of concentration at least one order of magnitude higher (18).

Thiazolidinediones and fatty acids are also able to promote a similar change in the cell fate of satellite cells from newborn mouse muscle (36, 37). When kept in standard medium from the time of seeding to day 5 post-confluence, about 50% of these cells differentiate into myotubes and express the muscle markers α-actin and myogenin, whereas chronic exposure to BRL 49653 leads to a nearly homogeneous monolayer of lipid-containing cells which express high levels of the adipose markers.
ALBP and fatty acid transporter, and only moderate levels of myogenin and α-actin mRNAs.

Several lines of evidence support the conclusion that thiazolidinediones exert an effect on non-terminally differentiated C2C12N cells. First, exposure of fully differentiated myotubes to the drug fails to reverse myotube formation and to induce adipose differentiation. Second, maximal adipogenic action of BRL 49653 is observed in cells treated before or just after confluence, i.e. before expression of the specific myotube markers. This is also evident morphologically as a complete differentiation into adipose-like cells is observed in cells exposed before confluence to the drug, whereas some myotubes still appear in cultures treated following confluence (Fig. 1, C versus B). It can also be concluded from our results that chronic treatment by the inducer is not required for adipose differentiation of C2C12N cells. Furthermore, the adipoblast commitment is not an inherited trait since the proliferation of adipose C2C12N cells in standard medium reverses the differentiated phenotype and leads to the appearance of cells capable of undergoing either myogenic or adipose differentiation depending on the presence or absence of BRL 49653 in the culture medium. These findings demonstrate that thiazolidinediones and fatty acids support the transition from myogenic lineage to that of adipogenic lineage and that this conversion event occurs at the end of the growing phase of committed myoblasts. Similar features emerge from a recent report describing the conversion pathway of C2C12 myoblasts to osteoblasts upon bone morphogenetic protein-2 treatment (8).

In conclusion, thiazolidinediones and fatty acids exert their adipogenic effects, as for fatty acids (17) is also activated by thiazolidinediones in C2C12N cells exposed to BRL 49653.

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In these cases, this phenomenon could be related to the increase of fatty acid disposal due to both an increase of fatty acid synthesis and decrease of mitochondrial fatty acid oxidation. Lipid accumulation has also been described in cardiac cells from diabetic rats (41) which are characterized by a high blood fatty acid concentration.

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