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ORIGINAL ARTICLE

Metformin Suppresses Lipid Accumulation in Skeletal Muscle by Promoting Fatty Acid Oxidation

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SUMMARY

Background: **Obesity is a major risk factor for metabolic syndrome, including insulin resistance (IR), type 2 diabetes mellitus (T2DM), and cardiovascular disease; ectopic fat deposition plays a key role in the development of these conditions. In insulin-resistant and/or T2DM patients, lipid accumulation is increased in skeletal muscle; the intramuscular accumulation of fatty acid metabolites is recognized to play a critical role in metabolic syndrome. Besides improving insulin sensitivity, the anti-diabetic drug metformin can reduce lipid accumulation in skeletal muscle; however, its mechanism of action remains unclear.**
Methods: ***ob/ob*** **mice and C2C12 cells were used to explore the effects of metformin on the morphological and physiological changes of lipid droplets. To clarify the mechanism by which metformin regulates fatty acid metabolism, a cDNA microarray and quantitative real-time PCR were used to examine the effects of metformin on the transcriptome of C2C12 cells treated with 200 µmol/L oleic acid.**
Results: **Metformin could retard body weight gain, improve insulin sensitivity and reduce intramyocellular lipid accumulation in** ***ob/ob*** **mice. In C2C12 cells, metformin inhibited lipid accumulation, stimulated fatty acid oxidation, and decreased triglyceride synthesis. Twenty-seven differentially expressed genes, including 12 upregulated and 15 downregulated genes, were involved in fatty acid metabolism. Interestingly, several genes involved in acyl-CoA synthesis and fatty acid oxidation were also upregulated, such as *Ppard*, *Acsbg1*, *Ascl3*, and *Mlycd*. However, several genes related to lipolysis were downregulated, such as *Ces1d* and *Cel.* Moreover, several important genes related to lipid metabolism were also downregulated, such as *Fabp4*, *Adipoq*, and *Apoc2*.**
Conclusions: **Metformin retards body weight gain, improves insulin sensitivity, and suppresses lipid accumulation in skeletal muscle by promoting fatty acid oxidation.**
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KEY WORDS

metformin, fatty acid oxidation, skeletal muscle, lipid droplets, obesity

ABBREVIATIONS

Acsbg1 - acyl-CoA synthetase bubblegum family member 1
Adipoq - adiponectin, C1Q and collagen domain-containing
AMPK - AMP-dependent protein kinase
Apoc2 - apolipoprotein C-II
Ascl3 - acyl-CoA synthetase long-chain family member 3
Cel - carboxyl ester lipase
Ces1d - carboxylesterase 1D
CPT1 - carnitine-palmitoyl transferase 1
DAG - diacylglycerol
DPM - disintegrations per minute
ER - endoplasmic reticulum
Fabp4 - fatty acid binding protein 4
FFA - free fatty acid
Gapdh - glyceraldehyde-3-phosphate dehydrogenase
GO - gene ontology
IR - insulin resistance

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Irs4 - insulin receptor substrate 4
MCD - malonyl-CoA decarboxylase
Met - metformin
Mlycd - malonyl-CoA decarboxylase
OGTT - oral glucose tolerance test
Phip - pleckstrin homology domain interacting protein
Ppard - peroxisome proliferator activator receptor delta
qRT-PCR - quantitative real-time PCR
ROS - reactive oxygen species
SEM - standard error of measurement
T2DM - type 2 diabetes mellitus
TLC - thin-layer chromatography
WT - wild-type

INTRODUCTION

The incidence of obesity has dramatically increased throughout the world in recent decades [1-3]; this problem is increasingly extending to developing countries. The numbers of obese individuals are expected to increase most rapidly in developing regions of the world and, depending on future trends, these estimates may range from increases of 71 - 263% [4]. Obesity predisposes individuals to the development of metabolic syndrome including insulin resistance (IR), type 2 diabetes mellitus (T2DM), and cardiovascular disease; ectopic fat deposition plays a key role in these processes [5,6]. Other than adipose tissue, tissues such as the liver, skeletal muscle, and heart normally only contain a small amount of fat. Previous studies demonstrated that ectopic fat is associated with insulin resistance and T2DM, as it interferes with cellular function and, hence, organ function [7,8]. Skeletal muscle is relevant to the regulation of energy homeostasis in the entire body. As the main site of fatty acid and glucose oxidation, skeletal muscle has a high potential to adjust fuel selection, depending on energy supply and demand. In insulin-resistant and/or T2DM patients, increased lipid accumulation occurs in skeletal muscle as a result of increased free fatty acid (FFA) availability, impaired fatty acid oxidation, and the toxic effects of metabolites of fatty acid oxidation on insulin signaling [9-11]. The intramuscular accumulation of fatty acid metabolites is a critical factor which is increasingly recognized to play an important role in metabolic syndrome.

The biguanide metformin is the most commonly used drug for the initial management of T2DM, and its application is also indicated to be beneficial for obesity and metabolic syndrome. Although metformin has been used for over 50 years, its mechanism of action remains unclear and some of the available data are contradictory [12-15]. However, it is accepted that metformin does not alter insulin secretion; instead it exerts a number of effects such as improving insulin sensitivity in pre-adipocytes, hepatocytes, and skeletal muscle, and reduces the plasma lipid concentration [16-18]. Recently, it was discovered that metformin could activate AMP-dependent protein kinase (AMPK), a central regulator of energy metabolism [19]. AMPK is considered to be a key controller of energy metabolism in skeletal muscle. Furthermore, a commonly proposed mechanism indicates that metformin inhibits mitochondrial respiration at complex I [20]. This interruption of energy production would decrease ATP production and, hence, elevate the concentration of AMP.

Although metformin can stimulate fatty acid oxidation, it is difficult to rationalize an inhibition of mitochondrial respiration due to stimulation of fatty acid oxidation. Furthermore, it is also possible that other metabolic processes in skeletal muscle are affected by metformin. In this context, we aimed to determine whether metformin could reduce lipid accumulation in skeletal muscle tissue. We also examined the effects of metformin on the transcriptome of cultured C2C12 cells, to further clarify the mechanism by which metformin regulates fatty acid metabolism.

MATERIALS AND METHODS

Experimental animals and procedures

C57BL/6 and *ob/ob* mice were generated by mating the heterozygous leptin-deficient mice (*ob/ob*) on a C57BL/6 background. The mice were housed with free access to water and food, and maintained at a constant temperature under a 12 hour light/12 hour dark cycle. All experiments were conducted in accordance with protocols approved by the Fourth Military Medical University Animal Care and Use Committee. Male *ob/ob* mice were divided into four groups with 6 animals per group. The mice were treated with metformin (500 μg/kg body weight/day) by oral gavage for 4 weeks. The animals were fed every day over the four weeks, and the food intake per cage, as well as the body weight of the individual animals, was monitored every week.

Oral glucose tolerance test

Mice were subjected to an oral glucose tolerance test after 4 weeks of metformin treatment.

The mice were fasted for 12 hours. After collection of baseline (t = 0) blood samples by snipping the tail, the mice received an oral gavage (1 mg/g body weight) of *D*-glucose dissolved in sterilized 0.9% sodium chloride, and additional blood samples were taken 15, 30, 60, and 120 minutes after the administration of glucose. The blood glucose levels were measured using an ACCU-CHEK glucose analyzer (Roche Diagnostics, Basel, Switzerland).

Cell culture

The C2C12 myoblast cell line derived from mouse skeletal muscle was purchased from the ATCC Global Bioresource Center (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and

100 μg/mL streptomycin at 37ºC in a humidified atmosphere with 5% (v/v) CO_2. The cells were incubated in control media or media supplemented with 100, 250, or 500 μmol/L metformin (Sigma-Aldrich, St. Louis, MO, USA). Both the control media (without metformin) and media supplemented with metformin contained 10% FBS and 200 μmol/L oleic acid (Sigma-Aldrich). After 12 hours of incubation, cell samples were collected for the following experiments.

Oil red O staining

The parallel control and metformin-treated C2C12 cell cultures were incubated as described above. At the end of incubation, the cells were washed three times with ice-cold PBS, fixed in 4% paraformaldehyde for 30 minutes, washed three times with PBS, and stained with 0.5% (w/v) Oil Red O solution in 60% (v/v) isopropanol for 30 minutes. The stain was removed, cells were washed briefly with 60% isopropanol and subsequently with PBS, and counter-stained with hematoxylin prior to microscopy.

Lipid extraction and triglyceride analysis

For the tissue samples, approximately 100 mg skeletal muscle was homogenized in 1 mL ice-cold PBS. For the cultured cells, the cell monolayers in 60 mm dishes were washed with ice-cold PBS and scraped into 1 mL PBS.

The total lipid contents of the tissues or cells were extracted using the Folch method, as previously described [21]. The entire lower phase containing lipids was dried down under nitrogen, resuspended in isopropanol and assayed using a triglyceride kit (InTec Products, Inc., Xiamen, China). The proteins were dried, dissolved in 2 mol/L KOH solution, and then measured by the Bradford method using a commercial kit (Bio-Rad, Hercules, CA, USA).

Fatty acid oxidation and uptake

C2C12 cells were cultured in 6-well plates, and the experiments were performed when the cells reached 70 - 80% confluence. The cells were treated with or without metformin in the presence of 1 μCi/mL [9,10-^{3}H] oleate (Amersham Pharmacia Biotech, Milan, Italy). After 12 hours, the media were collected and extracted using the Folch method as previously described [22]. The amount of tritium incorporated into H_2O, which represents the rate of fatty acid oxidation, was measured using a liquid scintillation counter. To monitor the rate of triglyceride synthesis, the amount of tritium incorporated into triglyceride was determined. Cellular lipids were extracted from the cells using the Folch method, separated by thin-layer chromatography (TLC), the triglyceride spots were scratched off the TLC plates, dissolved in 500 μL of methanol/water (1:2 v/v), and the radioactivity was counted. The results were expressed as disintegrations per minute (DPM) and normalized to the protein concentration.

RNA isolation and quantification

Total RNA was extracted from C2C12 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA was quantified using the Nanodrop ND-2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA), and RNA integrity was assessed using the Agilent 2100 Bioanalyzer and 2100 Expert software (Agilent Technologies, Santa Clara, CA, USA) and standard denaturing agarose gel electrophoresis. Only RNA with an RNA integrity number (RIN) $\geq$ 9.0 was processed for labeling. Each total RNA extract constituted a single test sample, and an equimolar pool of all RNAs was used as the reference sample. Three independent experiments were performed for each array hybridization experiment.

Agilent hybridization and analysis

Total RNA from C2C12 cells treated with or without (control group) metformin was amplified and transcribed into fluorescent cRNA in accordance with Agilent's Quick Amp Labeling protocol (version 5.7, Agilent Technologies). The labeled cRNAs were hybridized onto the Whole Mouse Genome Oligo Microarray (4 x 44K, Agilent Technologies). After washing the slides, the arrays were scanned using the Agilent Scanner G2505B. Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies). After quantile normalization of the raw data, genes which had flags in at least 1 of the 2 samples were chosen for further data analysis. Differentially expressed genes were identified through Fold Change filtering between the two samples. Pathway analysis and gene ontology (GO) analysis were used to determine the roles the differentially expressed genes play in biological pathways or GO terms. We also used GenMAPP software to detect the differentially expressed genes associated with metabolic pathways [23].

Quantitative real-time PCR

Quantitative real-time PCR was performed to detect and quantify the expression of selected genes which were more than 2-fold differentially expressed between the metformin treated groups and control groups, according to the microarray profiling results.

Total RNA was reverse transcribed to cDNA using Super-Script II (Takara, Dalian, China), and qRT-PCR was performed on the ABI Step One Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using gene-specific primers (Table 1) and SYBR Green PCR Master Mix (Takara) according to the manufacturer's recommendation. All primers were synthesized by AuGCT (Bejing, China). Each analysis had three to six replicates. Relative gene expression was normalized to the control gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) using the $\Delta\Delta^{CT}$ method.

Statistical analysis

All data are presented as mean ± SEM. The statistical significance of the differences between groups were assessed by one-way ANOVA and Student's *t*-tests using SPSS 13.0 software (SPSS, Chicago, IL, USA). The criterion for significance was set at $p < 0.05$.

RESULTS

Metformin retards body weight gain, improves insulin sensitivity, and reduces intramyocellular lipid accumulation in *ob/ob* mice

Excess body weight gain and increased lipid accumulation in the organs, apart from adipose tissue, are considered to be the major risk factors for the development of insulin resistance and T2DM. When ectopic fat accumulates, the mechanisms leading to the disruption of organ function in these organs are quite similar at the cellular level [8]. It has been noted that fat can be dispersed or accumulated intracellularly. Intracellular lipid accumulation may impair cellular function via induction of endoplasmic reticulum (ER) stress, reactive oxygen species (ROS) accumulation or other processes associated with decreased insulin sensitivity [24]. Although skeletal muscle insulin resistance is a common metabolic disorder in obese individuals, and a key contributor to the etiology of T2DM [25], the molecular signals linking impaired lipid metabolism to insulin sensitivity within skeletal muscle have not yet been completely defined. In this study, the effect of metformin on body weight gain was assessed in *ob/ob* mice. Male *ob/ob* mice were gavaged with metformin (500 μg/kg/day) for 4 weeks, and then the final body weight and blood glucose levels were monitored. Administration of metformin for 4 weeks reduced body weight gain in *ob/ob* mice (Figure 1A). Furthermore, the glucose level curves during the OGTT showed that glucose intolerance in *ob/ob* mice was also reversed by 4 weeks administration of metformin (Figure 1B). To clarify the effects of metformin on the intramuscular lipid content, Oil Red O staining and lipid quantification were performed. Oil Red O staining showed that metformin decreased both the number and size of the intracellular lipid droplets in the skeletal muscle of *ob/ob* mice (Figure 1C). Similarly, lipid quantification indicated that the levels of triglycerides in skeletal muscle decreased by approximately 2-fold in *ob/ob* mice treated with 500 μg/kg/day metformin for 4 weeks compared with the control group (Figure 1D). These results suggested that metformin could retard body weight gain, improve insulin sensitivity, and reduce intramyocellular lipid accumulation in obese mice.

Metformin inhibits lipid accumulation in C2C12 cells

We found that metformin could inhibit lipid accumulation in the skeletal muscle of *ob/ob* mice. Therefore, we used C2C12 cells to clarify the direct effects of metformin on skeletal muscle cells. The lipid droplet contents were determined in cultured C2C12 cells treated with 100, 250, and 500 μmol/L metformin for 12 hours while supplemented with 200 μmol/L oleic acid to induce triglyceride synthesis.

Oil Red O staining demonstrated that both the number and size of lipid droplets decreased in C2C12 cells after treatment with metformin (Figure 2A). The number of lipid droplets in control C2C12 cells was 66.0 ± 4.74 per cell, which decreased to 53.6 ± 6.35, 50.8 ± 4.15, and 49.8 ± 6.72 per cell in C2C12 cells treated with 100, 250, and 500 μmol/L metformin, respectively (Figure 2B). Triglyceride quantification revealed that the triglyceride content decreased by about 12.6%, 17.4%, and 19.3% in C2C12 cells treated with 100, 250, and 500 μmol/L metformin, respectively, compared with the control group (Figure 2C). These results suggested that metformin could directly reduce lipid accumulation in skeletal muscle cells.

Metformin stimulates fatty acid oxidation and decreases triglyceride synthesis

In skeletal muscle cells, the majority of FFAs are used for generating energy by β-oxidation, or re-esterified to triglycerides and stored in lipid droplets. Metformin is known to act by inhibiting the substrates linked to mitochondrial complex I, which is required during the process of β-oxidation. However, it is also well accepted that metformin activates AMPK and promotes catabolism, including fatty acid oxidation. Therefore, we determined the process of fatty acid metabolism in metformin-treated C2C12 cells. In this experiment, we used [^{3}H]oleic acid as a substrate, to evaluate the role of metformin in the regulation of fatty acid oxidation and triglyceride synthesis. Interestingly, the rates of oleic acid oxidization in C2C12 cells treated with 100, 250, and 500 μmol/L metformin were 17.5%, 27.0%, and 29.8% higher, respectively, than the control group at 12 hours (Figure 3A). Moreover, the rates of radioactive oleate incorporation into triglycerides decreased by 11.3%, 17.1%, and 19.9% in C2C12 cells treated with 100, 250, and 500 μmol/L metformin, respectively (Figure 3B). These results indicated that metformin could promote fatty acid β-oxidation and reduce triglyceride synthesis, which resulted in decreased lipid accumulation in skeletal muscle cells.

Metformin upregulates the mRNA expression levels of fatty acid oxidation-related genes

As previously mentioned, metformin inhibits lipid accumulation in skeletal muscle cells by promoting fatty acid oxidation and reducing triglyceride synthesis. To determine the molecular pathway involved in lipid metabolism in metformin-treated skeletal muscle cells, the transcriptome of metformin-treated C2C12 cells was analyzed using an oligonucleotide microarray. Using the selection criteria of an absolute fold change value > 2.00 and $p < 0.01$, more than one thousand genes were differentially expressed in cultured C2C12 cells treated with 250 μmol/L metformin, compared to con

Table 1. Quantitative real-time PCR primers.

Gene Name	Primer Sequences (5' - 3')
Ppard	S: TCCATCGTCAACAAAGACGGG
	A: ACTTGGGCTCAATGATGTCAC
Acsbg1	S: GGTGGAGCCCACATCACACA
	A: CGGATGAAGCCAGACTGAGC
Acsl3	S: CTCCAACCTGGTCTGAGTTCC
	A: CTGCAATATCTGAGGGCAGTG
Mlycd	S: TGTTCTGATGGGCCAGGCTTACTT
	A: TAGAGCTTTCTGAAGGCACAGGCT
Ces1d	S: AGAGCCCTGGAGCTTCGTG
	A: GAGCACATAGGCGGGTAGGAG
Fabp4	S: TCACCTGGAAGACAGCTCCT
	A: TGCCTGCCACTTTCCTTGT
Apoc2	S: TGGGGTCTCGGTTCTTCCTG
	A: GCCTGCGTAAGTGCTCATGG
Adipoq	S: TGTTCCTCTTAATCCTGCCCA
	A: CCAACCTGCACAAGTTCCCTT
Cel	S: CTTGGGTTTCCTTAGCAC
	A: CCACTCTGACTGATGGCTCT
Gapdh	S: ACCCCTTCATTGACCTCAACTACATGG
	A: ATTTGATGTTAGTGGGGTCTCGCTCCT

trol cells.

When GO tools were applied to analyze the differentially expressed genes, the main GOs were related to the cell membrane and integral to membrane with respect to the cellular component. Amongst the most differentially regulated biological processes were those involved in signaling transduction (including signal transducer activity, molecular transducer activity, receptor activity, transmembrane signaling receptor activity, and signaling receptor activity), and protein binding (such as zinc ion binding, actin binding, cytoskeletal protein binding, and transition metal ion binding; Suppl. Table 1A and B).

Amongst the differentially expressed genes, several genes in the insulin signaling pathway were upregulated, such as insulin receptor substrate 4 (*Irs4*) and pleckstrin homology domain interacting protein *(Phip*; Suppl. Table 2). In this work, we were primarily concerned with fatty acid metabolism. We found that 27 of the differentially expressed genes in metformin-treated C2C12 cells were involved in fatty acid metabolism, including 12 upregulated and 15 downregulated genes (Table 2). Interestingly, several other upregulated genes were involved in the process of acyl-CoA synthesis and fatty acid oxidation, such as peroxisome proliferator activator receptor delta (*Ppard*), acyl-CoA synthetase bubblegum family member 1 (*Acsbg1*), acyl-CoA synthetase long-chain family member 3 (*Ascl3*), and malonyl-CoA decarboxylase (*Mlycd*). However, several genes related to lipolysis were downregulated in metformin-treated C2C12 cells, such as carboxylesterase 1D (*Ces1d*) and carboxyl ester lipase (*Cel*). Moreover, several important genes related to lipid metabolism were also down regulated in metformin-treated C2C12 cells, such as fatty acid binding protein 4 (*Fabp4*), adiponectin, C1Q and collagen domain-containing (*Adipoq*), and apolipoprotein C-II (*Apoc2*).

To confirm the results of the microarray, the mRNA levels of several differentially expressed genes were determined by quantitative real-time PCR in both C2C12 cells treated with 250 μmol/L metformin and skeletal muscle tissues from *ob/ob* mice gavaged with 500 μg/kg/day metformin. The mRNA levels of *Ppard*, *Acsbg1*, *Ascl3*, and *Mlycd* were upregulated, and the mRNA levels of *Ces1d*, *Fabp4*, and *Cel* were downregulated in both C2C12 cells and skeletal muscle tissues of *ob/ob* mice treated with metformin. Compared to the control group, the mRNA levels of *Ppard*, *Acsbg1*, *Ascl3*, and *Mlycd* were 2.615 ± 0.370, 2.218 ± 0.156, 2.435 ± 0.521, and 2.138 ± 0.382-fold higher, respectively, in C2C12 cells treated with metformin, and 2.035 ± 0.528, 1.875 ± 0.539, 1.924 ± 0.580, and 1.672 ± 0.435-fold

Table 2. Differentially expressed genes related to fatty acid metabolism in C2C12 cells treated with metformin.

RefSeq	Gene Symbol	Gene description	FC	p-value
Upreguated				
NM_023530	Pla2g12b	phospholipase A2, group XIIB	+3.357	0.0028
NM_011145	Ppard	peroxisome proliferator activator receptor delta	+3.090	0.0033
NM_018887	Cyp39a1	cytochrome P450, family 39, subfamily a, polypeptide 1	+2.819	0.005
NM_010953	Oc90	otoconin 90	+2.741	0.0033
NM_001082484	Snx27	sorting nexin family member 27	+2.703	0.0094
NM_053178	Acsbg1	acyl-CoA synthetase bubblegum family member 1	+2.693	0.0071
NM_133220	Sgk3	serum/glucocorticoid regulated kinase 3	+2.578	0.0093
NM_028817	Acsl3	acyl-CoA synthetase long-chain family member 3	+2.562	0.0022
NM_019966	Mlycd	malonyl-CoA decarboxylase	+2.368	0.0085
NM_031395	Sytl3	synaptotagmin-like 3	+2.183	0.0047
NM_172285	Plcg2	phospholipase C, gamma 2	+2.097	0.0076
NM_010011	Cyp4a10	cytochrome P450, family 4, subfamily a, polypeptide 10	+2.079	0.008
Downregulated				
NM_053200	Ces1d	carboxylesterase 1D	-10.413	0.003
NM_024406	Fabp4	fatty acid binding protein 4	-7.412	0.0084
NM_009695	Apoc2	apolipoprotein C-II	-6.588	8.24551E-05
NM_009605	Adipoq	adiponectin, C1Q and collagen domain containing	-6.148	0.006
NM_026334	Lipf	lipase, gastric	-4.139	0.0045
NM_013486	Cd2	CD2 antigen	-3.735	0.0027
NM_010475	Hsd17b1	hydroxysteroid (17-beta) dehydrogenase 1	-3.710	0.0095
NM_011169	Prlr	prolactin receptor	-3.671	0.0009
NM_001441	Faah	fatty acid amide hydrolase	-3.586	0.0042
NM_009117	Saa1	serum amyloid A 1	-2.671	0.0007
NM_015731	Atp9a	ATPase, class II, type 9A (Atp9a)	-2.610	0.0016
NM_010069	Doc2a	double C2, alpha	-2.584	0.0054
NM_009885	Cel	carboxyl ester lipase	-2.421	3.85255E-05
NM_028035	Snx10	sorting nexin 10 (Snx10)	-2.412	0.0075
NM_013492	Clu	clusterin	-2.380	0.0064

Microarray analysis was performed on six samples of cultured C2C12 cells from three independent experiments; three samples were treated with 250 µmol/L metformin, three without metformin, all six had been treated with 200 µmol/L oleic acid to induce triglyceride synthesis. The table lists the 27 differentially regulated gene transcripts related to fatty acid metabolism, selected on the basis of their fold change (FC) and ordered by the magnitude of the absolute FC. The first column lists the NCBI Reference Sequence (RefSeq) accession number of the transcripts, the second the gene symbol, the third the description of the gene, the fourth the FC (with a positive symbol for upregulated genes and negative symbol for downregulated genes), and the fifth the significance of differences as estimated by the Students *t*-test.

higher, respectively, in the skeletal muscle tissues of *ob/ob* mice treated with metformin. In contrast, the mRNA levels of *Ces1d*, *Fabp4*, and *Cel* were 0.349 ± 0.09, 0.386 ± 0.072, and 0.588 ± 0.155 in C2C12 cells treated with metformin, and 0.472 ± 0.02, 0.536 ±0.085, and 0.71 ± 0.153, respectively, in the skeletal muscle tissues of *ob/ob* mice treated with metformin, compared to the levels in the control groups.

These data were consistent with the results of the microarray. However, *Adipoq* and *Apoc2* mRNA were only decreased in metformin-treated C2C12 cells (0.492 ± 0.14 and 0.416 ± 0.14 compared to the control group, respectively), but not in the skeletal muscle tissues of *ob/ob* mice treated with metformin, as shown in Figure 4.

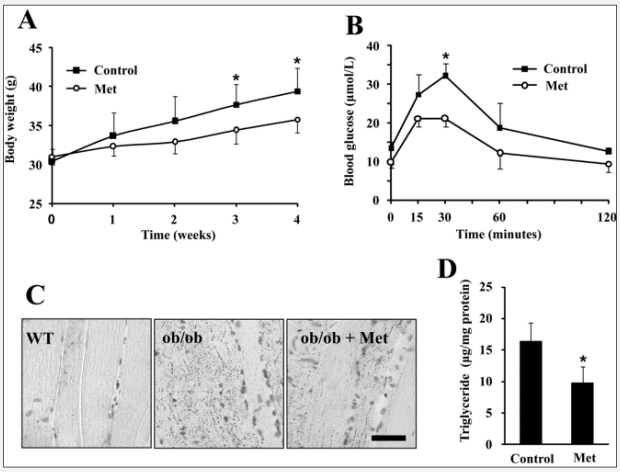

Figure 1. Effect of metformin on lipid metabolism in *ob/ob* mice.

A: Body weight changes of *ob/ob* mice treated with or without metformin (500 µg/kg body weight/day) for 4 weeks.
B: Oral glucose tolerance test after 4 weeks treatment with or without metformin. Mice were fasted for 12 hours and glucose (1 mg/g body weight) was administered. Plasma glucose levels were monitored over time, as described in the Materials and Methods.
C: Oil Red O staining of skeletal muscle tissue from *ob/ob* mice treated with or without metformin. C57BL/6 mice were used as controls. Bar = 20 µm.
D: Quantification of triglycerides in the skeletal muscle tissue of *ob/ob* mice treated with or without metformin for 4 weeks. Triglyceride content was determined using the Folch method. Data are expressed as the mean ± SEM, n = 6, * - $p < 0.05$, Student's *t*-test.

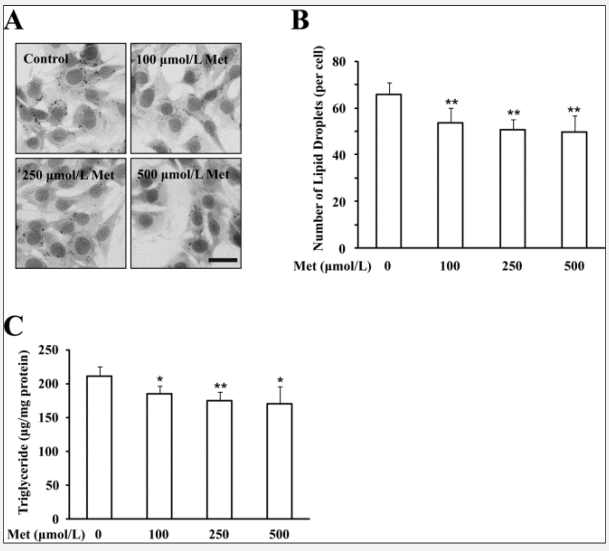

Figure 2. Effect of metformin on lipid content in C2C12 cells.

A: Oil Red O staining of C2C12 cells treated with different concentrations of metformin; 200 µmol/L OA (oleic acid) was administered with or without metformin to induce triglyceride synthesis. Bar = 20 µm.
B: Number of lipid droplets per cell in C2C12 cells treated with different concentrations of metformin; 200 µmol/L OA was administered with or without metformin to induce triglyceride synthesis.
C: Triglyceride content of C2C12 cells treated with different concentrations of metformin; 200 µmol/L OA was administered with or without metformin to induce triglyceride synthesis. Each bar represents the mean ± SEM (n = 3 - 6); * - $p < 0.05$, ** - $p < 0.01$, Student's *t*-test.

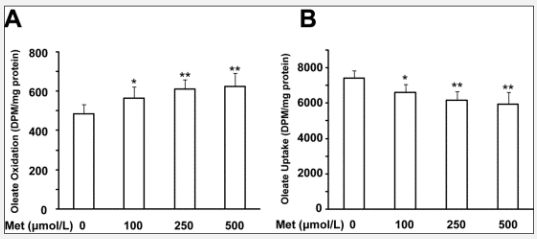

Figure 3. Effects of metformin on oleate uptake and oxidation in C2C12 cells.

A: Fatty acid β-oxidation increased in response to metformin administration. The rates of oleic acid oxidization in C2C12 cells treated with 100, 250, or 500 μmol/L metformin were 17.5%, 27.0%, and 29.8% higher, respectively, than the control group at 12 hours.
B: Triglyceride synthesis decreased during administration of metformin. The rates of radioactive oleate incorporation into triglycerides decreased by 11.3%, 17.1%, and 19.9% in C2C12 cells treated with 100, 250, or 500 μmol/L metformin, respectively; * - $p < 0.05$, ** - $p < 0.01$, Student's *t*-test.

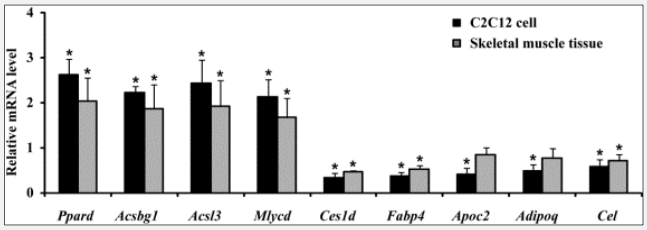

Figure 4. Quantitative real-time PCR analysis of nine selected differently expressed genes related to lipid metabolism.

Nine of the 27 differentially expressed genes related to fatty acid metabolism in C2C12 cells treated with metformin were selected. *Ppard, Acsbg1, Acsl3*, and *Mlycd* were upregulated and *Ces1d, Fabp4, Apoc2, Adipoq*, and *Cel* were downregulated in the microarray. The quantitative real-time PCR results correlated with the fold changes of these genes observed in the microarray. The mRNA levels of the target genes were normalized to *Gapdh* and the relative mRNA expression levels were calculated using the $\Delta\Delta^{CT}$ method. Each bar represents the mean ± SEM of 3 - 6 samples, * - $p < 0.05$, Student's *t*-test.

DISCUSSION

Skeletal muscle accounts for about 40% of the total body mass, > 30% of energy expenditure in human and other mammalian species, and is the predominant tissue responsible for lipid disposal under various physiological conditions [26]. Recently, defects in fatty acid oxidation in skeletal muscle have been considered to be the primary cause of insulin resistance [27]. Incomplete fatty acid oxidation leading to intramyocellular lipid accumulation may contribute to the development of insulin resistance [27-29]. In skeletal muscle, free fatty acids (FFAs) are mainly taken up by protein-mediated membrane transport along with passive diffusion, and then converted to fatty acyl-CoA before being taken up by the mitochondria via carnitine-palmitoyl transferase 1 (CPT1) [30]. Inside the mitochondria, β-oxidation and further degradation takes place via the Kreb's cycle. When there is an oversupply of FFAs and/or an impairment in mitochondrial β-oxidation, acyl-CoA accumulates and is broken down to intermediates such as diacylglycerol (DAG) and ceramide, which could damage the insulin signaling pathway [31] and affect glucose transport across the cell membrane.

As an oral antidiabetic drug in the biguanide class, metformin is the most commonly used drug for the treatment of type 2 diabetes, especially in overweight and obese individuals [32]. It is surprising that the mechanism of action of metformin remains largely unclear. Recently, metformin was shown to act via the inhibition of substrates linked to mitochondrial complex I, which is required for fatty acid β-oxidation. However, paradoxically, metformin is also known to promote catabolism, particularly fatty acid oxidation, by activation of AMPK.

In our study, we found that metformin could reduce the body weight gain and blood glucose level in *ob/ob* mice, which is consistent with previously published results [33,34]. Moreover, lipid accumulation was attenuated in the skeletal muscle tissues of *ob/ob* mice treated with metformin for 4 weeks. Using C2C12 cells, we found that metformin could directly inhibit lipid accumulation by reducing the size and number of intracellular lipid droplets. Furthermore, the results indicated that metformin could promote fatty acid oxidation and inhibit triglyceride synthesis in C2C12 cells. These results suggest that metformin can promote the oxidation of fatty acids and reduce lipid accumulation in skeletal muscle cells *in vivo* and *in vitro*.

To further clarify the molecular mechanism of metformin, the transcriptome of C2C12 cells treated with 250 μmol/L metformin was analyzed using an oligonucleotide microarray. Analysis of the oligonucleotide microarray revealed that metformin affects more than one thousand genes in the transcriptome of cultured C2C12 cells. Similarly, we found that several genes related to the insulin signaling pathway were upregulated, such as *Irs4* and *Phip*. More interestingly, 27 of the genes which were highly regulated by metformin in cultured C2C12 cells are involved in fatty acid metabolism, which provides insight into the molecular mechanism of metformin on fatty acid metabolism.

Of the highly differentially expressed genes, a number of genes related to acyl-CoA synthesis and fatty acid oxidation were found to be upregulated, such as *Ppard*, *Acsbg1*, *Ascl3* and *Mlycd*. As members of the nuclear receptor superfamily, the peroxisome proliferator-activated receptors (PPARs) can be activated by both dietary fatty acids and their metabolic derivatives *in vivo*, and thus serve as lipid sensors that, when activated, can markedly affect metabolism [35-37]. PPARδ, encoded by *Ppard*, is abundantly expressed in mammals and is a powerful regulator of fatty acid catabolism and energy homeostasis, with a broad role in lipid catabolism [35]. During energy storage and metabolism, fatty acids are activated by acyl-CoA synthetases, such as Acsbg1 and Ascl3, which form an activating thioester linkage between the fatty acid and CoA. In this work, we found that both *Acsbg1* and *Ascl3* were upregulated in the skeletal muscle cells of mice treated with metformin. *Mlycd,* another gene related to fatty acid oxidation, was also upregulated in the skeletal muscle cells of mice treated with metformin. *Mlycd* encodes malonyl-CoA decarboxylase (MCD), which catalyzes the conversion of malonyl-CoA to acetyl-CoA and carbon dioxide. Hepatic expression of MCD in rats can lower circulating FFA levels, leading to lower muscle β-OH-butyrate levels and improved insulin sensitivity [38].

Several genes encoding enzymes associated with hydrolysis, such as *Ces1d* and *Cel*, were also downregulated in response to metformin. Hydrolysis is required for further metabolism of the neutral lipids stored in lipid droplets. Therefore, the downregulation of these enzymes may be a response to the reduced lipid accumulation observed in the skeletal muscle cells treated with metformin. Furthermore, the gene *Fabp4* encodes an adipocyte fatty acid-binding protein which plays an important role in intracellular fatty acid transport. Downregulation of *Fabp4* indicates that FFA levels may decrease in metformin-treated skeletal muscle cells, due to enhanced fatty acid oxidation.

In conclusion, the results of this study clearly demonstrate that metformin inhibits the accumulation of lipids in skeletal muscle by promoting fatty acid oxidation. Microarray and quantitative real-time PCR indicated that metformin leads to upregulation of genes associated with fatty acid oxidation. These results provide an explanation of how metformin favors lipid metabolism in skeletal muscle, which offers further insight into the mechanism of action of metformin in the treatment of T2DM and obesity.

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Declaration of Interest:

No conflicts of interest are involved in this manuscript.

References:

1. Merle P, Trépo C, Zoulim F. Current Management Strategies for Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. Nature 2001;414:782-7.
2. Ogden CL, Carroll MD, Curtin LR, et al. Prevalence of overweight and obesity in the United States, 1999-2004. JAMA 2006; 295:1549-55.
3. Gregg EW, Cheng YJ, Narayan KM, et al. The relative contributions of different levels of overweight and obesity to the increased prevalence of diabetes in the United States: 1976-2004. Prev Med 2007;45:348-52.
4. Kelly T, Yang W, Chen CS, et al. Global burden of obesity in 2005 and projections to 2030. Int J Obes (Lond) 2008;32:1431-7.
5. Mokdad AH, Ford ES, Bowman BA, et al. Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. JAMA 2003;289:76-9.
6. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006;444: 840-6.
7. Lettner A, Roden M. Ectopic fat and insulin resistance. Curr Diab Rep 2008;8:185-91.
8. Szendroedi J, Roden M. Ectopic lipids and organ function. Curr Opin Lipidol 2009;20:50-6.
9. Anderwald C, Bernroider E, Krssak M, et al. Effects of insulin treatment in type 2 diabetic patients on intracellular lipid content in liver and skeletal muscle. Diabetes 2002;51:3025-32.

10. Cascio G, Schiera G, Di Liegro I. Dietary fatty acids in metabolic syndrome, diabetes and cardiovascular diseases. Curr Diabetes Rev 2012;8:2-17.

11. Roden M, Price TB, Perseghin G, et al. Mechanism of free fatty acid-induced insulin resistance in humans. J Clin Invest 1996;97: 2859-65.

12. Maida A, Lamont BJ, Cao X, et al. Metformin regulates the incretin receptor axis via a pathway dependent on peroxisome proliferator-activated receptor-alpha in mice. Diabetologia 2011;54:339-49.

13. Turban S, Stretton C, Drouin O, et al. Defining the contribution of AMP-activated protein kinase (AMPK) and protein kinase C (PKC) in regulation of glucose uptake by metformin in skeletal muscle cells. J Biol Chem 2012;287:20088-99.

14. Zhang Y, Wang Y, Bao C, et al. Metformin interacts with AMPK through binding to gamma subunit. Mol Cell Biochem 2012;368: 69-76.

15. Miller RA, Birnbaum MJ. An energetic tale of AMPK-independent effects of metformin. J Clin Invest 2010;120:2267-70.

16. Alexandre KB, Smit AM, Gray IP, et al. Metformin inhibits intracellular lipid accumulation in the murine pre-adipocyte cell line, 3T3-L1. Diabetes Obes Metab 2008;10:688-90.

17. Collier CA, Bruce CR, Smith AC, et al. Metformin counters the insulin-induced suppression of fatty acid oxidation and stimulation of triacylglycerol storage in rodent skeletal muscle. Am J Physiol Endocrinol Metab 2006;291:E182-9.

18. Zang M, Zuccollo A, Hou X, et al. AMP-activated protein kinase is required for the lipid-lowering effect of metformin in insulin-resistant human HepG2 cells. J Biol Chem 2004;279:47898-905.

19. Hardie DG. AMPK: a key regulator of energy balance in the single cell and the whole organism. Int J Obes (Lond) 2008;32 Suppl 4:S7-12.

20. Gonzalez-Barroso MM, Anedda A, Gallardo-Vara E, et al. Fatty acids revert the inhibition of respiration caused by the antidiabetic drug metformin to facilitate their mitochondrial beta-oxidation. Biochim Biophys Acta 2012;1817:1768-75.

21. Ye J, Li JZ, Liu Y, et al. Cideb, an ER- and lipid droplet-associated protein, mediates VLDL lipidation and maturation by interacting with apolipoprotein B. Cell Metab 2009;9:177-90.

22. Li JZ, Ye J, Xue B, et al. Cideb regulates diet-induced obesity, liver steatosis, and insulin sensitivity by controlling lipogenesis and fatty acid oxidation. Diabetes 2007;56:2523-32.

23. Salomonis N, Hanspers K, Zambon AC, et al. GenMAPP 2: new features and resources for pathway analysis. BMC Bioinformatics 2007;8:217.

24. Sozio MS, Liangpunsakul S, Crabb D. The role of lipid metabolism in the pathogenesis of alcoholic and nonalcoholic hepatic steatosis. Semin Liver Dis 2011;30:378-90.

25. Lillioja S, Mott DM, Spraul M, et al. Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus. Prospective studies of Pima Indians. N Engl J Med 1993;329:1988-92.

26. Smith AG, Muscat GE. Skeletal muscle and nuclear hormone receptors: implications for cardiovascular and metabolic disease. Int J Biochem Cell Biol 2005;37:2047-63.

27. Mohlig M, Isken F, Ristow M. Impaired mitochondrial activity and insulin-resistant offspring of patients with type 2 diabetes. N Engl J Med 2004;350:2419-21.

28. Chow L, From A, Seaquist E. Skeletal muscle insulin resistance: the interplay of local lipid excess and mitochondrial dysfunction. Metabolism 2010;59:70-85.

29. Holloszy JO. Skeletal muscle "mitochondrial deficiency" does not mediate insulin resistance. Am J Clin Nutr 2009;89:463S-6S.

30. Glatz JF, Bonen A, Luiken JJ. Exercise and insulin increase muscle fatty acid uptake by recruiting putative fatty acid transporters to the sarcolemma. Curr Opin Clin Nutr Metab Care 2002;5:365-70.

31. Morino K, Petersen KF, Shulman GI. Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction. Diabetes 2006;55 Suppl 2:S9-S15.

32. Nathan DM, Buse JB, Davidson MB, et al. Medical management of hyperglycaemia in type 2 diabetes mellitus: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement from the American Diabetes Association and the European Association for the Study of Diabetes. Diabetologia 2009; 52:17-30.

33. Lin HZ, Yang SQ, Chuckaree C, et al. Metformin reverses fatty liver disease in obese, leptin-deficient mice. Nat Med 2000;6: 998-1003.

34. Cohen SE, Tseng YH, Michael MD, et al. Effects of insulin-sensitising agents in mice with hepatic insulin resistance. Diabetologia 2004;47:407-11.

35. Evans RM, Barish GD, Wang YX. PPARs and the complex journey to obesity. Nat Med 2004;10:355-61.

36. Ravnskjaer K, Frigerio F, Boergesen M, et al. PPARdelta is a fatty acid sensor that enhances mitochondrial oxidation in insulin-secreting cells and protects against fatty acid-induced dysfunction. J Lipid Res 2009;51:1370-9.

37. Schuler M, Ali F, Chambon C, et al. PGC1alpha expression is controlled in skeletal muscles by PPARbeta, whose ablation results in fiber-type switching, obesity, and type 2 diabetes. Cell Metab 2006;4:407-14.

38. An J, Muoio DM, Shiota M, et al. Hepatic expression of malonyl-CoA decarboxylase reverses muscle, liver and whole-animal insulin resistance. Nat Med 2004;10:268-74.

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Supplemental Table 1A. Gene ontology annotation of upregulated genes in cultured C2C12 cells treated with metformin.

Gene ontologies of upregulated genes			
GO Term	**GO term description**	**Number of genes**	**p-value**
Cellular component			
GO:0016020	membrane	220	0.0008
GO:0044425	membrane part	179	0.0032
GO:0016021	integral to membrane	154	0.0097
GO:0071944	cell periphery	106	0.0071
GO:0005886	plasma membrane	105	0.0048
GO:0030054	cell junction	26	0.0091
GO:0005819	spindle	9	0.0047
GO:0001726	ruffle	8	0.0010
GO:0072686	mitotic spindle	3	0.0013
Molecular function			
GO:0004872	receptor activity	79	0.0099
GO:0004871	signal transducer activity	77	0.0007
GO:0060089	molecular transducer activity	77	0.0007
GO:0038023	signaling receptor activity	65	0.0087
GO:0004888	transmembrane signaling receptor activity	64	0.0062
GO:0046914	transition metal ion binding	51	0.0092
GO:0008270	zinc ion binding	47	0.0019
GO:0008092	cytoskeletal protein binding	23	0.0049
GO:0003779	actin binding	16	0.0024
Biological process			
GO:0065007	biological regulation	237	0.0008
GO:0050789	regulation of biological process	227	0.0014
GO:0050794	regulation of cellular process	221	0.0002
GO:0032501	multicellular organismal process	153	0.0038
GO:0007154	cell communication	138	0.0050
GO:0023052	signaling	137	0.0033
GO:0007165	signal transduction	133	0.0009
GO:0071840	cellular component organization or biogenesis	100	0.0063
GO:0016043	cellular component organization	97	0.0060
GO:0003008	system process	71	0.0104
GO:0007186	G-protein coupled receptor signaling pathway	60	0.0095
GO:0035556	intracellular signal transduction	52	0.0050
GO:0044093	positive regulation of molecular function	35	0.0102
GO:0007010	cytoskeleton organization	26	0.0061
GO:0006140	regulation of nucleotide metabolic process	19	0.0053
GO:0051249	regulation of lymphocyte activation	12	0.0085
GO:0010639	negative regulation of organelle organization	9	0.0057
GO:0048660	regulation of smooth muscle cell proliferation	7	0.0044
GO:0048659	smooth muscle cell proliferation	7	0.0053
GO:0030282	bone mineralization	6	0.0071

GO:0007189	activation of adenylate cyclase activity by G-protein signaling pathway	5	0.0020
GO:0010578	regulation of adenylate cyclase activity involved in G-protein signaling pathway	5	0.0020
GO:0010579	positive regulation of adenylate cyclase activity by G-protein signaling pathway	5	0.0020
GO:0032024	positive regulation of insulin secretion	5	0.0028
GO:0007190	activation of adenylate cyclase activity	5	0.0059
GO:0090277	positive regulation of peptide hormone secretion	5	0.0064
GO:0045762	positive regulation of adenylate cyclase activity	5	0.0069
GO:0002793	positive regulation of peptide secretion	5	0.0075
GO:0031281	positive regulation of cyclase activity	5	0.0081
GO:0051349	positive regulation of lyase activity	5	0.0094
GO:0042398	cellular modified amino acid biosynthetic process	4	0.0035
GO:0042522	regulation of tyrosine phosphorylation of Stat5 protein	3	0.0062
GO:0042506	tyrosine phosphorylation of Stat5 protein	3	0.0099
GO:0070373	negative regulation of ERK1 and ERK2 cascade	3	0.0099

List of relevant GO annotations from the set of upregulated transcripts with an absolute FC > 2.0 and p < 0.01 in cultured C2C12 cells treated with metformin. Only GOs with more than one gene observed are shown. The first column lists the GO term identifier, the second the GO term description, the third the number of genes annotated with this GO term, and the fourth the significance testing value of that term.

Supplemental Table 1B. Gene ontology annotation of downregulated genes in cultured C2C12 cells treated with metformin.

Gene ontologies of downregulated genes			
GO Term	GO term description	Number of genes	p-value
Cellular component			
GO:0016020	membrane	226	0.0010
GO:0044425	membrane part	192	0.0003
GO:0031224	intrinsic to membrane	169	0.0019
GO:0016021	integral to membrane	165	0.0022
GO:0005576	extracellular region	76	8.35E-07
GO:0044459	plasma membrane part	53	0.0053
GO:0044421	extracellular region part	52	3.90E-07
GO:0005615	extracellular space	41	1.85E-06
GO:0005624	membrane fraction	32	0.0088
GO:0009986	cell surface	25	0.0011
GO:0031012	extracellular matrix	17	0.0078
GO:0009897	external side of plasma membrane	15	0.0009
GO:0045121	membrane raft	11	0.0100
GO:0030016	myofibril	10	0.0011
GO:0043292	contractile fiber	10	0.0022
GO:0016459	myosin complex	6	0.0030
GO:0032982	myosin filament	4	0.0005
GO:0034364	high-density lipoprotein particle	4	0.0020

GO:0030315	**T-tubule**	**4**	**0.0047**
GO:0032994	**protein-lipid complex**	**4**	**0.0083**
GO:0034358	**plasma lipoprotein particle**	**4**	**0.0083**
Molecular function			
GO:0005488	**binding**	**314**	**3.60E-07**
GO:0005515	**protein binding**	**179**	**2.20E-05**
GO:0003824	**catalytic activity**	**145**	**0.0056**
GO:0046872	**metal ion binding**	**110**	**0.0002**
GO:0043169	**cation binding**	**110**	**0.0004**
GO:0043167	**ion binding**	**110**	**0.0004**
GO:0004872	**receptor activity**	**82**	**0.0076**
GO:0016787	**hydrolase activity**	**69**	**0.0067**
GO:0046914	**transition metal ion binding**	**58**	**0.0007**
GO:0008270	**zinc ion binding**	**47**	**0.0034**
GO:0005102	**receptor binding**	**46**	**0.0001**
GO:0046983	**protein dimerization activity**	**31**	**0.0049**
GO:0008233	**peptidase activity**	**26**	**0.0022**
GO:0070011	**peptidase activity, acting on L-amino acid peptides**	**23**	**0.0091**
GO:0030246	**carbohydrate binding**	**22**	**0.0002**
GO:0004175	**endopeptidase activity**	**17**	**0.0059**
GO:0001664	**G-protein coupled receptor binding**	**16**	**2.04E-05**
GO:0046982	**protein heterodimerization activity**	**16**	**0.0052**
GO:0005125	**cytokine activity**	**13**	**0.0005**
GO:0048037	**cofactor binding**	**13**	**0.0094**
GO:0005126	**cytokine receptor binding**	**12**	**0.0023**
GO:0005506	**iron ion binding**	**12**	**0.0026**
GO:0004252	**serine-type endopeptidase activity**	**11**	**0.0010**
GO:0001871	**pattern binding**	**11**	**0.0021**
GO:0030247	**polysaccharide binding**	**11**	**0.0021**
GO:0008236	**serine-type peptidase activity**	**11**	**0.0091**
GO:0017171	**serine hydrolase activity**	**11**	**0.0100**
GO:0004497	**monooxygenase activity**	**10**	**0.0016**
GO:0005539	**glycosaminoglycan binding**	**10**	**0.0025**
GO:0001948	**glycoprotein binding**	**8**	**0.0002**
GO:0020037	**heme binding**	**8**	**0.0046**
GO:0008201	**heparin binding**	**8**	**0.0057**
GO:0046906	**tetrapyrrole binding**	**8**	**0.0066**
GO:0008009	**chemokine activity**	**7**	**1.21E-05**
GO:0042379	**chemokine receptor binding**	**7**	**0.0001**
GO:0015399	**primary active transmembrane transporter activity**	**7**	**0.0095**
GO:0005518	**collagen binding**	**6**	**0.0004**
GO:0032947	**protein complex scaffold**	**5**	**0.0013**
GO:0043178	**alcohol binding**	**5**	**0.0030**
GO:0004181	**metallocarboxypeptidase activity**	**4**	**0.0013**
GO:0008235	**metalloexopeptidase activity**	**4**	**0.0073**
GO:0004180	**carboxypeptidase activity**	**4**	**0.0090**

GO:0005344	oxygen transporter activity	3	0.0014
Biological process			
GO:0009987	cellular process	341	0.0008
GO:0008152	metabolic process	251	0.0009
GO:0065007	biological regulation	249	0.0001
GO:0050789	regulation of biological process	241	0.0001
GO:0050794	regulation of cellular process	226	0.0002
GO:0044238	primary metabolic process	208	0.0045
GO:0032501	multicellular organismal process	160	0.0011
GO:0051716	cellular response to stimulus	155	0.0012
GO:0007154	cell communication	147	0.0007
GO:0023052	signaling	145	0.0006
GO:0007165	signal transduction	138	0.0004
GO:0019222	regulation of metabolic process	125	0.0095
GO:0080090	regulation of primary metabolic process	111	0.0046
GO:0031323	regulation of cellular metabolic process	110	0.0084
GO:0019538	protein metabolic process	104	0.0020
GO:0048869	cellular developmental process	76	0.0074
GO:0043412	macromolecule modification	70	0.0054
GO:0006464	protein modification process	67	0.0074
GO:0065009	regulation of molecular function	64	0.0011
GO:0065008	regulation of biological quality	64	0.0033
GO:0051239	regulation of multicellular organismal process	60	0.0005
GO:0048583	regulation of response to stimulus	60	0.0055
GO:0035556	intracellular signal transduction	54	0.0031
GO:0050790	regulation of catalytic activity	52	0.0026
GO:0032879	regulation of localization	44	0.0085
GO:0051246	regulation of protein metabolic process	41	0.0071
GO:0042127	regulation of cell proliferation	39	0.0018
GO:0006468	protein phosphorylation	39	0.0094
GO:0048584	positive regulation of response to stimulus	37	0.0008
GO:0051049	regulation of transport	37	0.0029
GO:0022610	biological adhesion	31	0.0027
GO:0007243	intracellular protein kinase cascade	30	0.0010
GO:0034220	ion transmembrane transport	29	0.0007
GO:0023056	positive regulation of signaling	27	0.0068
GO:0044092	negative regulation of molecular function	26	0.0066
GO:0009967	positive regulation of signal transduction	25	0.0083
GO:0043086	negative regulation of catalytic activity	24	0.0011
GO:0055082	cellular chemical homeostasis	23	0.0053
GO:0010627	regulation of intracellular protein kinase cascade	22	0.0042
GO:0009894	regulation of catabolic process	21	0.0097
GO:0008285	negative regulation of cell proliferation	20	0.0029
GO:0045859	regulation of protein kinase activity	20	0.0069
GO:0034762	regulation of transmembrane transport	17	0.0034
GO:0043408	regulation of MAPK cascade	16	0.0024

GO:0003012	**muscle system process**	**15**	**1.62E-05**
GO:0006936	**muscle contraction**	**14**	**1.29E-05**
GO:0071900	**regulation of protein serine/threonine kinase activity**	**14**	**0.0071**
GO:0043405	**regulation of MAP kinase activity**	**12**	**0.0026**
GO:0044282	**small molecule catabolic process**	**12**	**0.0088**
GO:0032386	**regulation of intracellular transport**	**10**	**0.0028**
GO:0006941	**striated muscle contraction**	**9**	**6.09E-06**
GO:0016485	**protein processing**	**9**	**0.0005**
GO:0051604	**protein maturation**	**9**	**0.0009**
GO:0043491	**protein kinase B signaling cascade**	**8**	**0.0013**
GO:0045444	**fat cell differentiation**	**8**	**0.0039**
GO:0033157	**regulation of intracellular protein transport**	**8**	**0.0096**
GO:0051896	**regulation of protein kinase B signaling cascade**	**7**	**0.0011**
GO:0071901	**negative regulation of protein serine/threonine kinase activity**	**7**	**0.0044**
GO:0043407	**negative regulation of MAP kinase activity**	**6**	**0.0024**
GO:0022898	**regulation of transmembrane transporter activity**	**6**	**0.0057**
GO:0070613	**regulation of protein processing**	**5**	**0.0003**
GO:0050873	**brown fat cell differentiation**	**5**	**0.0004**
GO:0051897	**positive regulation of protein kinase B signaling cascade**	**5**	**0.0070**
GO:0003009	**skeletal muscle contraction**	**3**	**0.0046**
GO:0009071	**serine family amino acid catabolic process**	**3**	**0.0046**

List of relevant GO annotations from the set of downregulated transcripts with an absolute FC > 2.0 and p < 0.01 in cultured C2C12 cells treated with metformin. Only GOs with more than one gene observed are shown. The first column lists the GO term identifier, the second the GO term description, the third the number of genes annotated with this GO term, and the fourth the significance testing value of that term.