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Proteomic Analysis of Mitochondrial Proteins of Basal and Lipolytically (Isoproterenol and TNF-α)-Stimulated Adipocytes

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ABSTRACT

The regulation of adipocyte lipolysis is increasingly believed to influence insulin resistance, in a process that may be associated with mitochondrial dysfunction. However, the molecular basis of the relationship between mitochondrial protein expression, lipolytic responsiveness, and insulin resistance remains unknown. A set of proteins that shows altered abundances in the mitochondria of untreated and treated 3T3-L1 adipocytes with TNF-α or isoproterenol was identified. These include the proteins associated with energy production, including fatty acid oxidation, TCA cycle, and oxidative phosphorylation. Proteins associated with oxidative stress dissipation were down-regulated in lipolytically stimulated adipocytes. Lipolytic stimulation with isoproterenol and TNF-α, which is also a potent proinflammatory cytokine, showed some noticeable differences in mitochondrial protein expression. For example, isoproterenol markedly enhanced the expression of prohibitin which is involved in the integrity of mitochondria but TNF-α did not. These results provide valuable information on mitochondrial dysfunction associated with oxidative stress induced by lipolytic stimulation. J. Cell. Biochem. 106: 257–266, 2009.

KEY WORDS: ADIPOCYTE; MITOCHONDRIA; PROTEOMICS; LIPOLYSIS

Obesity develops only if energy intake, in the form of feeding, chronically exceeds total body expenditure. Energy expenditure includes physical activity, basal metabolism, and adaptive thermogenesis. Mitochondria play a critical role in the process of energy expenditure, as they are the cellular furnaces where fuels (derived from fatty acids and glucose) are oxidized and energy is either stored in the energy phosphate bonds of ATP or is released as heat [Spiegelman and Flier, 2001]. Mitochondrial dysfunction can lead to diseases characterized by lipid metabolism disorders and pathological triglyceride accumulation in several cell types [Klopstock et al., 1997; Kakuda, 2000; Munoz-Malaga et al., 2000]. In patients with insulin resistance and type 2 diabetes, mitochondrial metabolism and ATP synthesis are reduced in concert with a reduction of key factors regulating mitochondrial biogenesis [Petersen et al., 2004; Bogacka et al., 2005]. Moreover, recent studies report that adipose conversion is accompanied by increases in the mitochondrial mass and by alterations in the mitochondrial composition [Wilson-Fritch et al., 2003].

Lipolytic stimulation of adipocytes induces the hydrolysis of triacylglycerol to fatty acids and consequent mitochondrial stimulation. The hydrolyzed fatty acids are transported into mitochondria and oxidized to acetyl-CoA, which can enter TCA cycle, where the acetate is further oxidized to CO_2 and H_2O, yielding NADH, $FADH_2$, and ATP. Therefore, mitochondrial stimulation induced by adequate lipolytic stimulus such as aerobic physical exercise can improve obesity and related disease. However, the chronic and excessive elevation of fatty acid concentration by too much lipolytic stimulus, dysfunctions in lipid storage mechanism or chronic high fat diet and its oxidation induces the generation of excessive reactive oxygen species (ROS). As ROS are produced mainly in mitochondria, it appears that mitochondria are a primary target for the destructive action of ROS. This mitochondrial damage triggered by excessive ROS plays an important role in the development of insulin resistance and type 2 diabetes [Moore et al., 2004; Fridlyand and Philipson, 2006]. However, there remains an incomplete mechanistic understanding of how the processes

Additional Supporting Information may be found in the online version of this article.

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connected with mitochondrial alteration by chronic lipolytic stimulation and/or elevated free fatty acid concentration can lead to insulin resistance at the cellular level in adipocytes. The 3T3-L1 cell line is widely used as a model of adipogenic differentiation and insulin action. Cells of this line undergo lipolysis upon TNF-α orisoproterenol stimulation. Isoproterenol, a β-adrenoreceptor agonist, is a strong activator of lipolysis by activating adenylyl cyclases, elevating intracellular cAMP content and activating cAMP-dependent protein kinase A and eventually leads to insulin resistance by inhibiting insulin signaling [Londos et al., 1999; Fasshauer et al., 2001]. TNF-α is also a potent stimulus of lipolysis in adipocytes by activating mitogen-activated protein kinase kinase (MEK) and extracellular signal-related kinase (ERK), suppressing cAMP-specific phosphodiesterase, elevating intracellular cAMP and activating cAMP-dependent protein kinase A and causes insulin resistance [Hoffmann et al., 1999; Zhang et al., 2002; Green et al., 2004]. However, in our experiment, isoproterenol stimulated the fatty acid oxidation with increased rate of lipolysis but TNF-α did not stimulate the fatty acid oxidation (Fig. 1), suggesting that isoproterenol and TNF-α might affect fatty acid oxidation in 3T3-L1 adipocytes by different mechanism. Unlike isoproterenol, TNF-α, is also a well-known proinflammatory cytokine and known to increase oxidative stress and to induce damages in mitochondrial functions and consequently to inhibit energy production. Moreover, increased levels of TNF-α, are found in both obese people and type II diabetes patients [Schulze-Osthoff et al., 1992; Wlodek and Gonzales, 2003]. So, with expectation to find some clues of the different mechanism and relation of mitochondrial dysfunction with chronic elevated free fatty acid concentration or excessive oxidative stress, we have examined the mitochondrial protein expression, in either isoproterenol or TNF-α, treated adipocytes. Using a separation approach consisting of subcellular fractionation, 2D-PAGE and LC-MS/MS, we have identified 70 up- and down-regulated mitochondrial proteins in adipocytes after lipolytic stimulation with isoproterenol or TNF-α. The identification of 70 up- and down-regulated proteins and their assignments to specific cellular processes provides a depiction of the manner in which adipocyte adapts to lipolytic stimulation. The increase in mitochondrial capacity coupled with a selective increase in the levels of enzymes involved in fatty acid metabolism and the decrease in expression of enzymes involved in oxidative stress dissipation were identified.

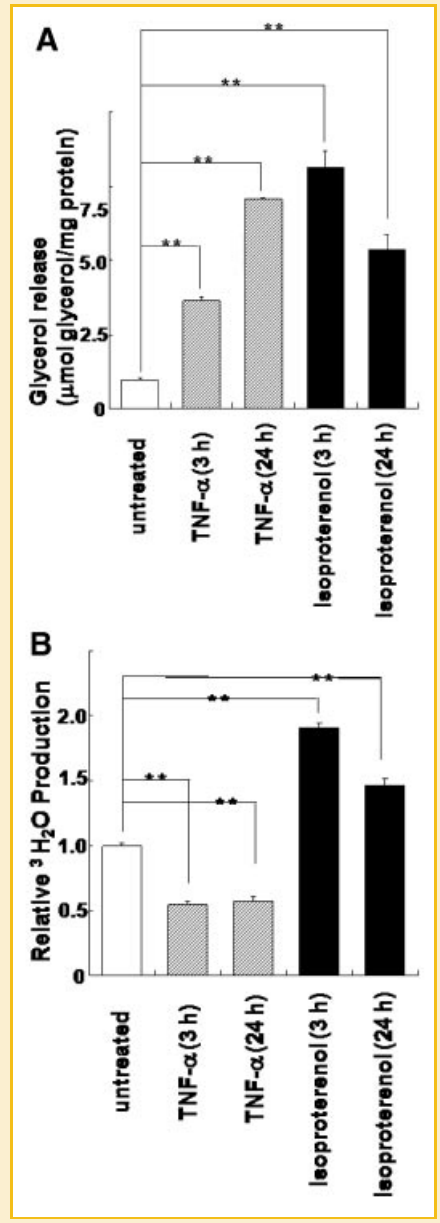

Fig. 1. Effect of either isoproterenol or TNF-α on lipolysis and fatty acid oxidation. Fully differentiated adipocytes were treated without or with either 10 μM isoproterenol or 10 ng/ml TNF-α for 3 and 24 h. A: Media were then collected and assayed for glycerol. Data shown are means ± SD and were obtained from two independent experiments carried out in duplicate ($^{**}P < 0.01$ compared with untreated cells). B: The cells were changed to serum-free medium containing ^{3}H-palmitic acid. ^3H_2O production was assayed 7 h after incubation. Data shown are means ± SD and were obtained from two independent experiments carried out in duplicate ($^{**}P < 0.01$ compared with untreated cells).

MATERIALS AND METHODS

REAGENTS

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) and calf serum were purchased from GIBCO-BRL of Life Technologies (Gaithersburg, MD). (-)-Isoproterenol, TNF-α, dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate (ABC), trifluoroacetic acid (TFA), TCEP (tris(2-carboxyethyl) phosphine), protease inhibitor cocktail, insulin, 3-isobutyl-1-methylxanthine (IBMX), and dexamethasone were purchased from Sigma-Aldrich. ReadyPrep™ Rehydration buffer, acrylamide/bis (30%) solution and Coomassie Brilliant Blue (CBB) G-250 were purchased from BioRad (Hercules, CA). Immobilized pH gradient (IPG) Drystrip, IPG buffer, and dry-strip cover fluid were purchased from Amersham Biosciences (Piscataway, NJ), which is now part of GE Healthcare. Trypsin was from Promega (Madison, WI).

CELL CULTURE

Mouse 3T3-L1 preadipocytes, purchased from the American Type Culture Collection, were maintained in DMEM containing 10% calf

serum. For the differentiation process, the medium was replaced with DMEM containing 10% FBS, 10 μg/ml of insulin, 0.5 mM IBMX, and 1 μM dexamethasone at 2 days postconfluence. After 2 days, the medium was changed to DMEM containing 10 μg/ml of insulin and 10% FBS, and the cells were then refed with 10% FBS every 2 days.

INCUBATION OF 3T3-L1 ADIPOCYTES IN LIPOLYTICALLY STIMULATION CONDITIONS

Fully differentiated adipocytes were incubated with in low glucose DMEM medium (GIBCO-BRL) containing 2% (w/v) fatty acid-free BSA for 24 h. After 24 h, the cells were treated either with 10 μM isoproterenol or with 10 ng/ml TNF-α for 3 and 24 h.

LIPOLYSIS

3T3-L1 adipocytes were incubated in a low glucose DMEM medium (GIBCO-BRL) containing 2% (w/v) fatty acid-free BSA without or with either 10 μM isoproterenol or 10 ng/ml TNF-α for 3 and 24 h. Glycerol content of the incubation medium was determined using a colorimetric assay (GPO-Trinder, Sigma-Aldrich). Protein content was determined using a BCA protein assay (Pierce, Rockford, IL).

FATTY ACID OXIDATION

3T3-L1 adipocytes placed in a 12-well plate were washed and incubated in low glucose DMEM medium (GIBCO-BRL) containing 2% (w/v) fatty acid-free BSA, 0.3 mM L-carnitine, and ^{3}H-palmitic acid (3 μCi/well). Excess ^{3}H-palmitic acid in the medium was removed by trichloroacetic acid precipitation twice. The supernatant was extracted with chloroform/methanol (2:1) twice and then counted for $^{3}H_2O$ production.

ISOLATION OF MITOCHONDRIA

Mitochondria were isolated from 3T3-L1 adipocytes as described by Wilson-Fritch et al. [2003]. Untreated and treated 3T3-L1 cells (two 150-mm dishes) with either 10 ng/ml of TNF-α for 24 h or with 10 μM isoproterenol for 3 h were washed twice with ice-cold phosphate-buffered saline and twice with isolation buffer (250 mM sucrose, 0.5 mM EDTA, 5 mM HEPES pH 7.4). Cells were homogenized by 10 passages through a 27-gauge needle and centrifuged at 500*g* for 10 min. The postnuclear supernatant was removed and centrifuged at 18,000*g* for 25 min. The pellet was resuspended in 20% sucrose-10 mM Tris-0.1 mM EDTA and centrifuged at 18,000*g* for 30 min. The pellet was resuspended in 60% sucrose-10 mM Tris-0.05 mM EDTA. The suspension was overlaid with a 53% sucrose layer (sucrose, 10 mM Tris, 0.05 mM EDTA) and a 44% sucrose layer (sucrose, 10 mM Tris, 0.05 mM EDTA). The sucrose step gradient was centrifuged at 141,000*g* for 2 h. The purified mitochondria settled at the 44–53 interface. The mitochondrial layer was removed, diluted in isolation buffer, and centrifuged at 18,000*g* for 30 min. The mitochondrial pellet was suspended in ReadyPrep™ Rehydration buffer, and the protein concentration was determined using a Bradford assay (BioRad).

TWO-DIMENSIONAL GEL ELECTROPHORESIS

First-dimension isoelectric focusing (IEF) was performed using 18-cm, pH 4–7 or pH 6–9, IPG strips (Amersham Biosciences) in three steps under step-n-hold mode. Six hundred micrograms of protein were used and focused for 28,000 V h (pH 4–7) or 45,000 V h (pH 6–9). After the IEF process, proteins were reduced and alkylated by soaking the IPG strips in the equilibration solution (6 M urea, 2% SDS, 30% glycerol, 50 mM Tirs–HCl, pH 8.8) containing 130 mM DTT for 10 min at room temperature. This was then followed by soaking them in an equilibration solution containing 135 mM IAA for 10 min. The equilibrated IPG strips were sealed on top of 12% SDS–PAGE gel, using 0.5% agarose. The 2-D SDS–PAGE was carried out using a Tris–glycine–SDS buffer system (25 mM Tris, 192 mM glycine, and 0.1% SDS) at 5 W/gel for 1 h and then 15 W/gel until the dye front reached the bottom edge of the gel. Protein spots were visualized by staining with CBB G-250. Mitochondrial proteins were obtained from untreated and isoproterenol or TNF-α-treated cells three separate times, and two gels were run from each of the samples. Thus, six gels from each sample were used for comparative analyze.

IMAGE ANALYSIS

Densitometric comparative image analysis was performed using ImageMaster 2D Elite software (Amersham Biosciences). To correct for differences in the sample loading or staining intensity among gels, the "total quantity in valid spot" normalization method was used [Unlü et al., 1997]. Duplicate gels of a sample were grouped together using the "replicate groups" function, which allows the determination of the average quantities of protein spots. Statistical analysis using the Student's *t*-test function was then performed.

IN GEL TRYPTIC DIGESTION

Coomassie-stained protein spots were excised from the gel and destained with 45% acetonitrile in 100 mM ABC. The resulting gel slices were incubated with 10 mM TCEP, alkylated by an addition of 50 mM IAA, and then digested in situ with trypsin (100 ng per band in 50 mM ABC). The tryptic peptides were extracted using a Sonicator (BRANSON) in 0.2% TFA in 5% formic acid. The extracted peptides were concentrated using C18 zip-tips and eluted with 0.1% TFA in 30% acetonitrile followed by 0.1% TFA in 75% acetonitrile. The eluates were dried under a vacuum using a Speed Vac concentrator.

MASS SPECTROMETRY

HPLC/MS/MS analyses of the tryptic peptides were performed in a LCQ DECA XP plus ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA) coupled on-line with an in-house packed capillary column (150 μm × 50 cm, with precolumn flow splitting) Surveyor HPLC system (ThermoFinnigan) and an in-house built nanospray source. Experiments were performed in information-dependent analysis (IDA) mode. Precursor ions were selected for fragmentation using the following MS to MS/MS switch criteria: for ions greater than m/z 470.0 and smaller than 1470.0, charge state 1–3, intensity dependent manner, former target ions were excluded for 3 min, ion tolerance 50.0 mmu. CID experiment was used to fragment multiply charged ions using nitrogen as collision gas. For protein identification, MS/MS data were searched using BioWorks 3.0 software (ThermoFinnigan), a program for matching mass spectrometric information with sequence databases. Proteins were identified based on matching the MS/MS data with mass values

calculated for selected ion series of peptides. Typically, at least two sequence tags were used for the identification of each protein.

WESTERN BLOT ANALYSIS

3T3-L1 cells were lysed in RIPA buffer (PBS pH 7.4, containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail). Forty micrograms of proteins were resolved on 10% NuPAGE gels run in an MES buffer system (Invitrogen, Gaithersburg, MD) and transferred to nitrocellulose membranes according to the manufacturer's protocol. Immunoreactive proteins were revealed by enhanced chemiluminescence with ECL+ (Amersham Biosciences). The antibodies 3-hydroxyacyl-CoA dehydrogenase and aldehyde dehydrogenase 2 were purchased from GenWay Biotech, Inc. (San Diego, CA), that to adenylate kinase 2 from Abgent (San Diego, CA), that to peroxiredoxin 4 from Abcam (Cambridge, UK) and that to mitochondrial Hsp60 from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal antibody to isocitrate dehydrogenase 3 (NAD+) alpha was raised in rabbits and affinity-purified by LabFrontier (Seoul, Korea). Anti-Hsp60 antibody was used to assess equal loading of the protein. Blots were quantified using an ImageMaster (Amersham Biosciences).

STATISTICAL ANALYSIS

Experiments were performed at least three times. The levels of statistical significance were determined using an unpaired two-tailed Student's *t*-test. Results are presented as means ± SD. *P*-values of less than 0.05 were considered significant.

RESULTS

To study alterations in the protein content of mitochondria that occur during lipolytic stimulation, fully differentiated adipocytes were incubated with either TNF-α or isoproterenol. The duration and concentration of each reagent used were based on published in vitro studies to produce a maximum lipolytic response [Souza et al., 1998; Garcia-Vicente et al., 2005]. When the cells were incubated with isoproterenol, the rate of lipolysis and fatty acid oxidation was maximal at 3 h. On the other hand, the treatment of TNF-α gradually increased lipolysis until 24 h while fatty acid oxidation was already significantly decreased at 3 h (Fig. 1).

The spots showing markedly altered expression in 2-D gels for either isoproterenol or TNF-α-treated adipocytes compared untreated adipocytes were excised (Fig. 2 and Supplement Fig. 1),

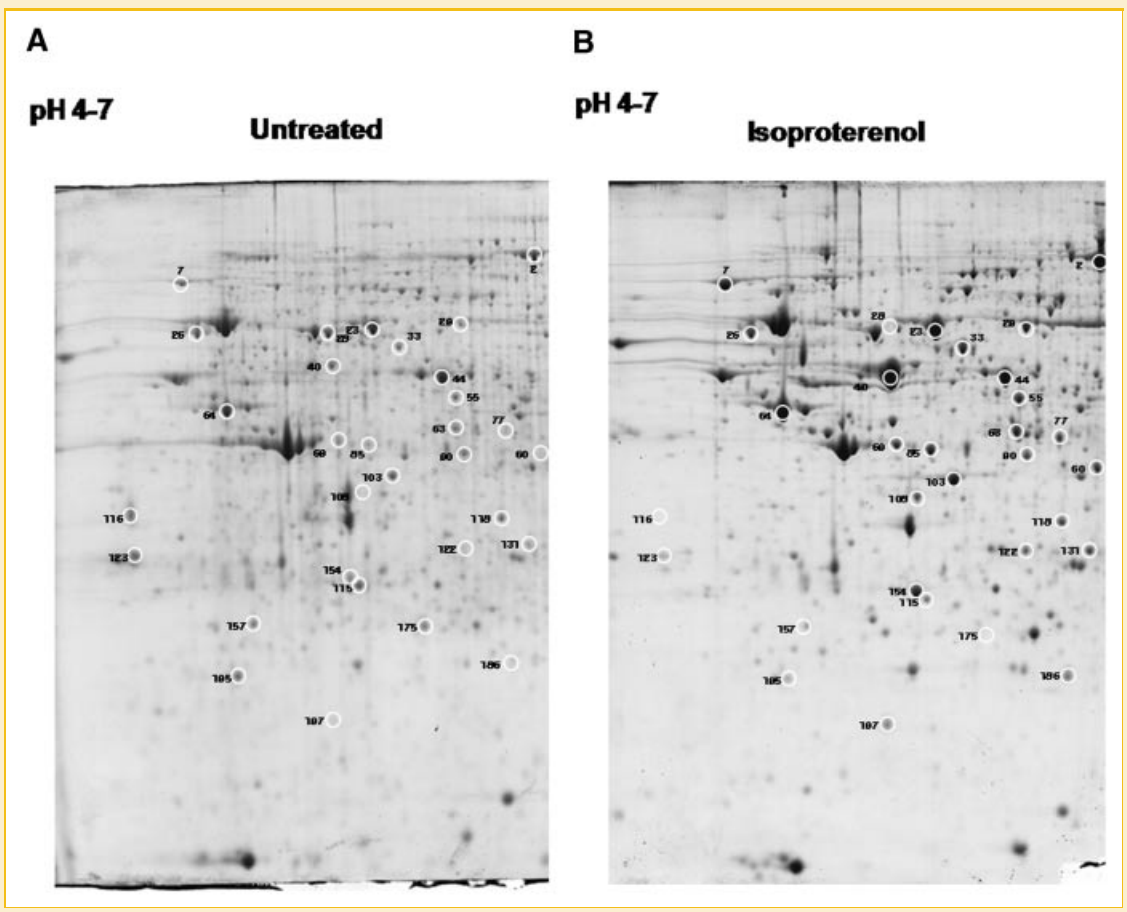

Fig. 2. 2-D map of mitochondrial protein of adipocytes. Mitochondrial protein (600 μg) from either isoproterenol- or TNF-α-treated or basal adipocytes were separated by 2-D-PAGE using pH 4–7 IPG strips for the first dimension and 12% SDS–PAGE for the second dimension. Proteins were detected by CBB 250. The numbered spots are those differentially expressed by lipolytic stimulation in adipocytes compared untreated cells. Duplicate gels were compared for each of three separate harvests (n = 6).

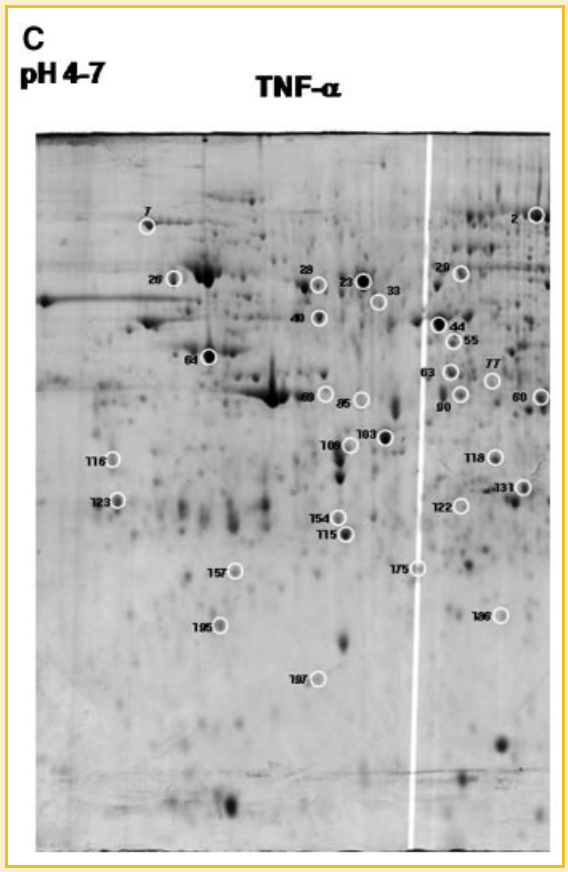

Fig. 2. (*Continued*).

digested with trypsin, and analyzed by LC-MS/MS. The expression data for the up- and down-regulated proteins are summarized in Table I. To confirm the expressional changes obtained by 2-D-PAGE and LC-MS/MS analysis, Western blot analysis was performed for several proteins (Fig. 3). The expression of aldehyde dehydrogenase 2, isocitrate dehydrogenase 3 (NAD+) alpha (IDH 3 alpha), and peroxiredoxin 4 had the similar patterns compared to 2-D map. In 2-D map, the expression level of 3-hydroxyl-CoA dehydrogenase in TNF-α-treated adipocytes was twofold higher than in isoproterenol-treated cells. The expression level of adenylate kinase 2 was up-regulated in only isoproterenol-treated cells. In Western blot analysis, the expression level of 3-hydroxyl-CoA dehydrogenase showed a similar increase between in isoproterenol-treated cells and in TNF-α-treated adipocytes. Adenylate kinase 2 was also up-regulated in TNF-α-treated adipocytes as although significantly less than in isoproterenol-treated cells.

PROTEINS ASSOCIATED WITH THE FATTY ACID OXIDATION

Fatty acids must be transported into mitochondria and activated to acyl-CoA form. The present study found an increase in the abundance of carnitin palmitoyltransferase 2 (CPTII, spot no. 4) in isoproterenol-treated adipocytes. CPT II mediates the transport of acyl-CoA across the inner mitochondrial membrane. Aldehyde dehydrogenase 3 (spot no. 60), acetyl-coenzyme A dehydrogenase, medium-chain (spot no. 78), 3-hydroxyacyl-CoA dehydrogenase (spot no. 148) and enoly coenzyme A hydratase, short chain 1 (spot no. 32) were up-regulated both in isoproterenol-treated and in TNF-α-treated adipocytes. In particular, fatty acid-coenzyme A ligase, long chain 2 (spot no. 3), glutaryl-coenzyme A dehydrogenase (spot no. 19), and acetyl-coenzyme A acetyltransferase (spot no. 112) were highly up-regulated in only isoproterenol-treated adipocytes. It was suggested that TNF-α and especially isoproterenol-induced lipolysis are accompanied by an increase in fatty acid oxidation to support ATP synthesis.

PROTEINS ASSOCIATED WITH THE TCA CYCLE

TCA cycle is a series of chemical reactions of central importance in all living cells that use oxygen as part of cellular respiration. Acetyl-CoA, the end product of each round of fatty acid oxidation, enters the TCA cycle where it is further oxidized to CO_2 and H_2O with the concomitant generation of three moles of NADH, one mole of $FADH_2$ and one mole of ATP. The expression of pyruvate carboxylase (spot no. 2), aconitase 2 (spot no. 1), dihydrolipoamide dehydrogenase (spot no. 15), citrate synthase (spot no. 20), dihydrolipoamide *S*-succinyltransferase (e2 component of 2-oxo-glutarate complex, spot no. 55), acetyl-coenzyme A dehydrogenase medium chain/P-5 pending protein (spot no. 90), isocitrate dehydrogenase 3 (NAD+) alpha (spot no. 103), malate dehydrogenase (spot no. 118) and citrate lyase beta (spot no. 131) were increased both in isoproterenol-treated and in TNF-α-treated adipocytes as compared with untreated cells. Succinate-coenzyme A ligase, GDP-forming beta subunit (spot no. 85), succinate dehydrogenase Fp subunit (spot no. 29), dihydrolipoamide s-acetyltransferase (E2 component of pyruvate dehydrogenase complex, spot no. 33), phosphoenolypyruvate carboxykinase 2 (spot no. 14), and aldehyde dehydrogenase 2 (spot no. 13) levels were higher in isoproterenol-treated adipocytes than in controls but not in TNF-α-treated adipocytes.

PROTEINS ASSOCIATED WITH THE ATP AND OXIDATIVE PHOSPHORYLATION

Mitochondria are responsible for meeting 80–90% of cellular energy needs through the process of the oxidative phosphorylation of ADP. The expression of adenlyate kinase 2 (spot no. 126), which catalyzes the phosphorylation of AMP to ADP and thereby helps to regulate the cellular pools of ADP and ATP, was up-regulated in isoproterenol-stimulated adipocytes but not in TNF-α-treated adipocytes. Electron transferring flavoprotein alpha, beta, (spot nos. 121 and 125) and NADH dehydrogenase, ubiquinone Fe-S protein 8 (spot no. 186) were also found to be up-regulated only by isoproterenol-stimulation in adipocytes. ATP synthase H+ transporting mitochondrial F1 complex, alpha (spot no. 9) expression was increased both in isoproterenol-treated and in TNF-α-treated adipocytes.

PROTEINS ASSOCIATED WITH THE BRANCHED CHAIN AMINO ACID DEGRADATION

The branched-chain amino acids (BCAAs) consist of L-leucine, L-isoleucine, and L-valine. They are considered essential as they cannot be synthesized de novo and must be obtained. The catabolism of L-leucine, L-isoleucine, and L-valine initially involves

TABLE I. List of Up- or Down-Regulated Proteins in Either Isoproterenol- or TNF-α-Treated Adipocytes After 2-D Followed by LC MS/MS Analysis

Spot ID	PI	MW (kDa)	Description	ACC. No.	Matched pep. seq.	Sequence coverage (%)	Regulation Iso/TNF-α*
Proteins involved in fatty acid oxidation							
3	7.25	77.9	Fatty acid coenzyme A ligase, long chain 2	31560705	26	50.79	Up 161%/−**
4	8.49	72.7	Carnitin palmitoyltransferase 2	6753514	14	34.19	Up 60.2%/−
112	8.37	45.9	Acetyl coenzyme A acetyltransferase	21450129	17	64.15	Up 120%/−
19	8.1	44.5	Glutaryl-coenzyme A dehydrogenase	6679959	8	36.14	Up 90%/−
78	7.77	41.6	Acetyl coenzyme A dehydrogenase, medium chain	28488948	6	13.54	Up 79%/32.1%
60	6.59	39.0	Aldehyde dehydrogenase 3	110825702	24	55.11	Up 220%/200%
32	8.76	32.0	Enoyl coenzyme A hydratase, short chain 1	20846515	19	73.45	Up 160%/112%
148	7.3	30.0	Hadhsc protein 3-hydroxyacyl-coA dehydrogenase	20379935	18	82.7	Up 116%/312%
Proteins involved in TAC cycles							
2	6.44	108.3	Pyruvate carboxylase	6679237	10	21.68	Up 270%/125%
1	7.84	85.6	Aconitase 2	18079339	22	41.54	Up 185%/161%
29	6.41	78.1	Succinate dehydrogenase Fp subunit	54607098	29	62.95	Up 139.2%/−
33	5.84	73.9	Dihydrolipoamide s-acetyltransferase (E2 component of pyruvate dehydrogenase complex)	21594641	5	14.49	Up 131.1%/−
14	6.87	64.0	Phosphoenolypyruvate carboxykinase 2	28077029	17	37.5	Up 97.9%/−
55	6.09	59.9	Dihydrolipoamide *S*-succinyltransferase (e2 component of 2-oxo-glutarate complex)	21313536	19	47.92	Up 132.4%/21%
15	7099	54.0	Dihydrolipoamide dehydrogenase	31982856	23	62.48	Up 37%/177%
13	6.71	51.6	Aldehyde dehydrogenase 2	6753036	26	61.31	Up 61.3%/−
85	5.71	48.7	Succinate-coenzyme A ligase, GDP-forming beta	28175163	21	71.83	Up 187.7%/−
90	6.52	47.1	Acetyl-coenzyme A dehydrogenase medium chain/P-5 pending protein	6680618	10	36.34	Up 81.1%/12%
20	8.26	45.8	Citrate synthase	13385942	18	42.89	Up 110%/111%
118	6.29	35	Malate dehydrogenase	31982186	5	52.36	Up 20.6%/20.7%
103	5.82	42.6	Isocitrate dehydrogenase 3 (NAD+) alpha	18250284	20	48.18	Up 142.5%/143%
131	6.69	30.3	Citrate lyase beta	12844088	4	22.84	Up 97.9%/95%
Proteins involved in oxidative phosphorylation							
9	8.19	60.0	ATP synthase H+ transporting mitochondrial F1 complex, alpha subunit, isoform 1	6680748	20	52.08	Up 167.7%/165%
121	7.5	31.7	Electron transferring flavoprotein, alpha polypeptide	31981826	18	66.97	Up 92.5%/−
125	8.44	23.4	Electron transferring flavoprotein, beta-subunit	29351581	22	84.93	Up 37.9%/−
126	7.8	22.6	Adenylate kinase 2	8392883	8	43.53	Up 107.2%/−
186	5.28	20.1	NADH dehydrogenase, ubiquinone Fe-S protein 8	27661165	15	83.42	Up 38.3%/−
Proteins involved in branched chain amino acid degradation							
63	6.34	56.9	Dihydrolipoamide branched chain transacylase E2	6753610	7	21.7	Up 129.5%/100%
17	7.12	53.8	Leucine aminopeptidase 3	31981147	24	59.73	Up 52%/50%
77	6	51.6	2-Oxoisovalerate dehydrogenase alpha	1709439	17	59.73	Up 117.3%/12%
73	8.12	36.8	Branched chain aminotransferase 2 protein	17389266	12	42.55	Up 91%/−
84	8.39	32.5	Methylenetetrahydrofolate dehydrogenase	6678952	7	41.64	Up 69.2%/−
Proteins involved in oxidative stress dissipation							
11	7.48	44.7	NADP-dependent isocitrate dehydrogenase	27370516	13	49.43	Down 71%/69%
166	8.25	23.6	Peroxiredoxin 4	7948999	15	65.69	Down 82%/80%
176	8.95	22	Peroxiredoxin 3, anti-oxidant protein 1	6680690	5	29.22	Down 34.1%/25%
140	7.27	17.0	Alkyl hydroperoxide reductase, thiol specific antioxidant enzyme	12846314	21	43.82	Down 17.3%/15%
Proteins involved in folding							
7	4.9	96.6	Tumor rejection antigen 96	6755863	20	48.45	Up 289.9%/200%
28	5.54	78.4	dnaK-type chaperone hsc70	476850	21	46.69	Down 108%/81%
23	5.91	73.5	Heat shock protein 74	6754256	24	60.21	Up 112%/33%
40	5.55	68.4	Gro EL precursor	72957	4	37.74	Up 512%/100%
141	7.41	12.5	Peptidylprolyl isomerase C	6679441	3	72.20%	Down 10.2%/−
50	6.32	62	Chaperonin subunit 2	6671700	7	32.10%	Up −/61.2%
Others							
26	4.96	78.9	FK506 binding protein 9 precursor	23396603	25	41.85	Up 94%/72%
69	5.56	54.4	Capping protein (actin filament) muscle Z-line	6671672	10	32.08	Up 152.7%/−
18	8.22	53.9	Serine hydrooxymethyl transferase 2	21312298	17	43.45	Up 57.1%/50%
108	5.78	39.2	Dimethylarginine dimethylaminohyrolase 1	20878745	12	52.23	Up 114%/51%
115	5.62	37.0	GTP binding protein subunit alpha1	120975	8	39.71	Down 12.2%/up 150%
116	4.9	35.9	Annexin A5	13277612	10	46.67	Down 62.2%/−
123	4.68	32.5	Tropomyosin 1 alpha	31560030	21	62.72	Down 39.1%/−
154	5.67	26.3	Prohibitin	6679299	4	20.96	Up 151.8%/−
157	4.8	25.6	Tyrosine 3-monooxygenase	6756041	6	26.02	Down 29.1%/−
175	5.21	22.1	Rho GDP dissociation inhibitor (GDI) alpha	31982030	4	41	Down 13.5%/−
135	8.58	19.1	RNA, member RAS oncogene homolog	27692688	6	19.34	Up 69.3%/35%
197	5.56	14.2	Ribosomal protein L12	27690912	8	34.42	Up 65.3%/down 40%
195	5.14	17	Phosphatidylethanolamine-binding protein	29840839	4	32.50%	Down 11.5%/−
127	7.4	23	Phosphoglycerate mutase homolog	20823772	4	40%	−/Down 62%
64	5.1	57	ATP synthase H+ transporting	7949003	15	74.30%	Up 50%/32%
122	6.05	32	Carbonic anhydrase VB	30581072	6	65.20%	Down 43.2%/42%
44	6.03	64	Glucose regulated protein 58 kDa, phospholipase C, alpha	6679687	11	79.40%	Up 26.4%/−

Regulation values are mean (n = 6) lipolytic-stimulated values as a percentage of untreated values.
P-values of less than 0.05 were considered significant.
*Iso/TNF-α means the percent induction or inhibition by isoproterenol and TNF-α respectively.
**'−' Indicates that the difference in the protein expression levels between treated and untreated cells is not statistically significant. Statistical analysis using the Student's *t*-test function was performed.

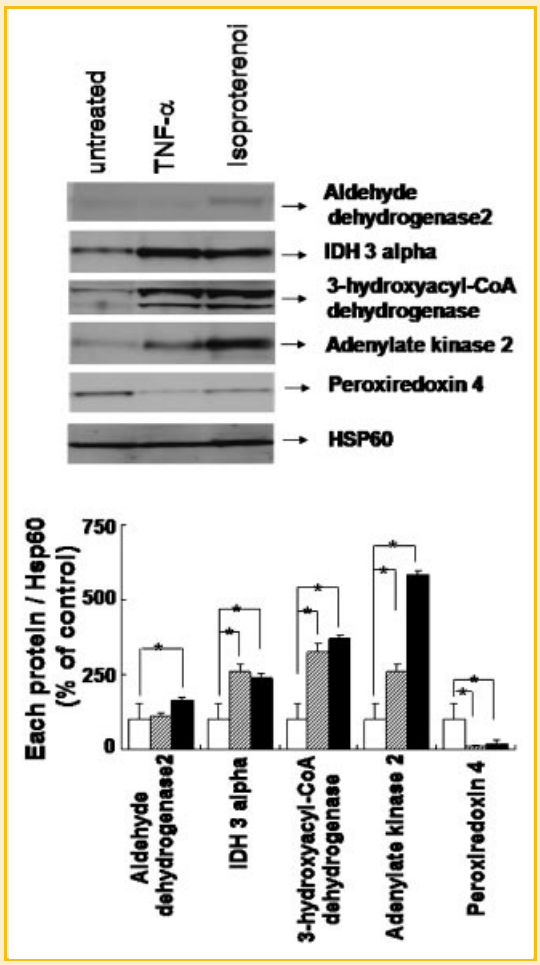

Fig. 3. Effect of either isoproterenol or TNF-α on levels of mitochondrial proteins. Fully differentiated adipocytes were treated without or with either 10 μM isoproterenol for 3 h or 10 ng/ml TNF-α for 24 h, and mitochondria were isolated as described in Materials and Methods Section. Equal amounts of mitochondrial proteins were analyzed by immunoblotting with antibodies to 3-hydroxyacyl-CoA dehydrogenase, aldehyde dehydrogenase 2, isocitrate dehydrogenase 3 (NAD+) alpha, adenylate kinase 2, and peroxiredoxin 4. Mitochondrial Hsp60 levels are shown as loading controls. Data were normalized to the protein expression levels of Hsp60. Data shown are means ± SD and were obtained from two independent experiments. $^{*}P < 0.05$ compared with untreated cells.

the same three reactions: the conversion of the amino acids to their corresponding alpha-keto acids; the conversion of the alpha-keto acids to their corresponding acyl-CoA thioesters and carbon dioxide; and the conversion of the acyl-CoA thioesters to their corresponding alpha, beta-unsaturated acyl-CoA thioesters. The BCAAs appear to be preferentially taken up by skeletal muscle, where they undergo similar catabolic reactions to those described above. Skeletal muscle appears to be the major site of both BCAA transamination and oxidation in humans. The expression of dihydrolipoamide branched chain transacylase E2 (spot no. 63) and leucine aminopeptidase 3 (spot no. 17) were up-regulated both in isoproterenol-treated and in TNF-α-treated adipocytes.

Isoproterenol increased the expression of 2-oxoisovalerate dehydrogenase alpha (spot no. 77), and branched-chain amino-transferase (spot no. 73), whereas TNF-α did not.

PROTEINS INVOLVED IN OXIDATIVE STRESS DISSIPATION

Mitochondria are the major organelles that produce ROS and the main target of ROS-induced damage. Production of NADPH is required for the regeneration of glutathione, which scavenges mitochondrial ROS. Mitochondrial NADP (+)-dependent isocitrate dehydrogenase is a major NADPH producer in mitochondria and thus plays a key role in the cellular defense against oxidative stress-induced damage [Jo et al., 2001]. In lipolytic stimulated adipocytes, NADP (+)-dependent isocitrate dehydrogenase (spot no. 11) was found to be down-regulated. In addition, alkyl hydroperoxide reductase (thiol specific antioxidant enzyme, spot no. 140), and peroxiredoxin 3, 4 (spot nos. 166 and 176) were down-regulated by lipolitic stimulation in adipocytes. Alkyl hydroperoxide reductase is the primary scavenger of endogenous H_2O_2 [Seaver and Imlay, 2001]. Peroxiredoxin-3 and 4 are the mitochondrial member of the antioxidant family of thioredoxin peroxidases that uses mitochondrial thioredoxin-2 (Trx2) as a source of reducing equivalents to scavenge hydrogen peroxide (H_2O_2) [Watabe et al., 1997; Rhee et al., 2005].

PROTEINS INVOLVED IN FOLDING

Protein folding is the physical process by which a polypeptide folds into its characteristic three-dimensional structure. Specialized proteins called chaperones or heat shock proteins assist in the folding of other proteins. To combat protein-related homeostatic disruption, such as oxidative stress, cells respond by synthesizing a family of highly conserved proteins termed heat shock proteins [Bruce et al., 2003].

The expression of tumor rejection antigen 96 (spot no. 7), heat shock protein 74 (spot no. 23), and Gro EL precursor (spot no. 40) were up-regulated both in isoproterenol-treated and in TNF-α-treated adipocytes. However, the expression of dnaK-type chaperone hsc70 (spot no. 28) was down-regulated by lipolitic stimulation in adipocytes.

OTHERS

Prohibitin is a highly conserved protein involved in mitochondrial biogenesis and function [Merkwirth and Langer, 2008]. It serves as a chaperone protein to stabilize mitochondrial respiratory enzymes [Nijtmans et al., 2002]. Also, it protects against oxidative stress in intestinal epithelial cells [Theiss et al., 2007; Schleicher et al., 2008]. Prohibitin was significantly up-regulated in isoproterenol-treated adipocytes but not in TNF-α-treated adipocytes. GTP binding protein subunit alpha1 was down-regulated in isoproterenol-treated adipocytes but up-regulated in TNF-α-treated adipocytes. Ribosomal protein L12 was up-regulated in isoproterenol-treated adipocytes but down-regulated in TNF-α-treated adipocytes.

DISCUSSION

Mitochondria produce most of the energy required by cells. Thus, mitochondrial dysfunction may be a candidate that can explain changes in energy expenditure, body composition, and energy intake.

Lipolytic stimulation of adipocytes would increase circulating free fatty acid and thus lead to increased fatty acid oxidation. TNF-α and isoproterenol both increase adipocyte lipolysis by activating cAMP-dependent protein kinase A through elevation of intracellular cAMP content. Isoproterenol also up-regulates TNF-α expression in differentiated 3T3-L1 adipocytes [Fu et al., 2007]. Therefore, we may expect similar lipolysis and fatty acid oxidation profiles from TNF-α and isoproterenol treated cells. However, as shown in Figure 1, TNF-α-stimulated lipolysis was accompanied by a decrease in fatty acid oxidation, whereas isoproterenol-induced lipolysis was accompanied by an increase in fatty acid oxidation in differentiated 3T3-L1 adipocytes. This observation suggests that there would be significant differences in the effect of isoproterenol on energy production from that of TNF-α. TNF-α increases intracellular cAMP content by suppression of cAMP-specific phosphodiesterase through activation of mitogen-activated protein kinase kinase (MEK) and extracellular signal-related kinase (ERK), whereas isoproterenol increases intracellular cAMP content by activating adenylyl cyclases through β-adrenoreceptor. TNF-α is a well-known proinflammatory cytokine and is known to increase oxidative stress and to induce damages in mitochondrial functions and consequently to inhibit energy production, whereas isoproterenol is known to exert beneficial effects by suppressing lipopolysaccharide-induced production of TNF-α and inflammatory cytokines although it also increases phorbol myristyl acetated-induced production of TNF-α and inflammatory cytokines [Schulze-Osthoff et al., 1992; Szabo et al., 1997; Wlodek and Gonzales, 2003; Szelenyi et al., 2006]. However, little is known about the cellular mechanisms and alteration of the mitochondrial protein underlying this different lipolytic process induced by either isoproterenol or TNF-α. In this work, a portion of the mitochondrial proteome is characterized from cultured 3T3-L1 adipocytes incubated under both basal conditions that foster triacylglycerol storage and stimulated conditions when lipolysis is activated and fatty acids are released from the cells. The expression of several mitochondrial proteins that are involved in energy production, including fatty acid oxidation, TCA cycle, oxidative phosphorylation and the expression of prohibitin which is involved in mitochondrial structural and functional integrity [Nijtmans et al., 2002; Theiss et al., 2007; Schleicher et al., 2008] were remarkably increased in adipocytes following an isoproterenol treatment. However, in TNF-α-treated adipocytes, the expression of prohibitin was not altered compared with untreated cells and the expression of enzymes for fatty acid oxidation, TCA cycle and oxidative phosphorylation were either increased to a much lesser extent or not altered compared with untreated cells. In contrast, the expressions of four proteins involved in oxidative stress dissipation were decreased both in isoproterenol- and in TNF-α-treated adipocytes. These results suggest that both isoproterenol and TNF-α-treated mitochondria experience similar type of oxidative damage from lipolytic stimulation but that isoproterenol treated mitochondria may overcome this damage through the high expression of prohibitin and the proteins associated with energy

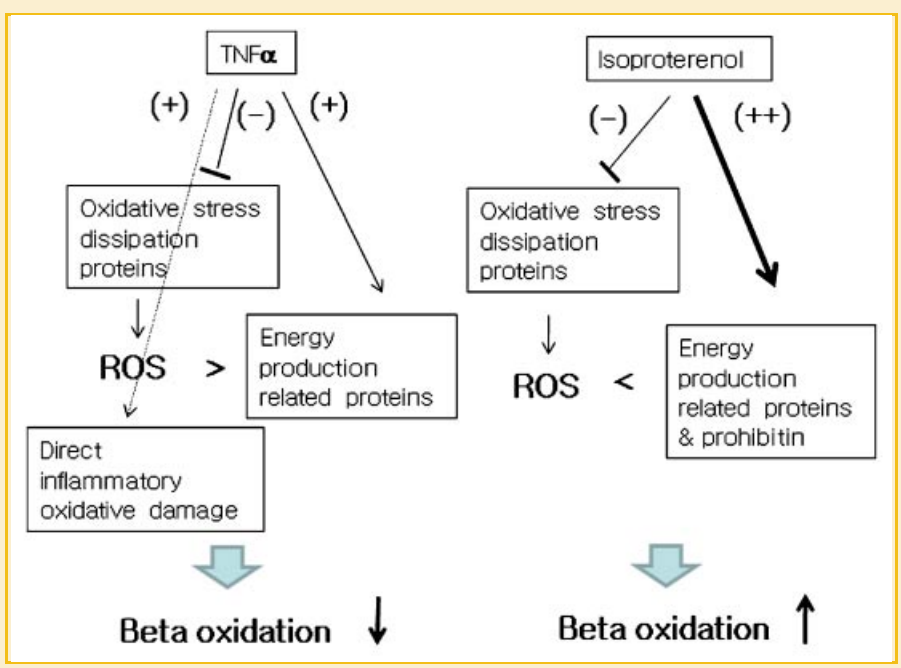

Fig. 4. Schematic representation of the effect of either isoproterenol or TNF-α on mitochondrial function. Isoproterenol- and TNF-α-treated mitochondria experience similar type of oxidative damage from lipolytic stimulation but that isoproterenol treated mitochondria may overcome this damage through the high expression of prohibitin and the proteins associated with energy production. (−), inhibition; (+), weak activation; (++), strong activation.

production. However, TNF-α-treated mitochondria may not be able to overcome this damage due to an insufficient increase in the expression of prohibitin and the proteins associated with energy production. In addition, previous reports have also shown that proinflammatory cytokines, such as TNF-α and interleukin 1, inhibit mitochondrial aconitase activity and disturb TCA cycle, resulting in decreased ATP production in the cells and these decreased energy levels cause and sustain obesity [Tatsumi et al., 2000; Wlodek and Gonzales, 2003]. Therefore, as shown in Figure 4, both isoproterenol and TNF-α decreased the expressions of proteins involved in oxidative stress dissipation, and consequently would increased ROS production. The insufficient effect of TNF-α upon increases of mitochondrial functional and structural integrity-related prohibitin expression and energy production-related mitochondrial protein expression, compared with isoproterenol treated cells, to overcome oxidative damage induced by lipolytic stimulation as well as the well-known direct inflammatory oxidative damage of TNF-α, might be associated with a decrease in fatty acid oxidation. In our experiment, the structural integrity of isoproterenol and TNF-α-treated mitochondria was not examined but an indication of functional integrity of mitochondria, the fatty acid oxidation was examined. It is also still uncertain whether the overexpression of prohibitin could restore, in part, the mitochondrial functional integrity in TNF-α-treated cells. Further well designed studies are needed to investigate the exact molecular mechanism underlying the expression of prohibitin and the difference of isoproterenol or TNF-α-mediated fatty acid oxidation.

In summary, the mitochondrial proteomes of untreated adipocytes were compared with those of lipolytically stimulated with either isoproterenol or TNF-α-treated cells using 2-D-PAGE-based proteomic tools. Differential expression analysis showed significant changes in 70 proteins. These include the proteins associated with energy production, including fatty acid oxidation, TCA cycle, and oxidative phosphorylation. Isoproterenol markedly enhanced the expression of prohibitin and proteins associated with energy production, whereas TNF-α increased the expression of only some of those proteins expression and to a much lesser extent. Proteins involved in oxidative stress dissipation were down-regulated both in isoproterenol and TNF-α-treated adipocytes. These differentially expressed proteins are expected to provide valuable information toward an understating of the pathophysiologic mechanism of mitochondrial dysfunction and insulin resistance associated with intracellular ROS production in TNF-α- or isoproterenol-treated adipocytes.

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