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Effects of mitochondrial uncoupling on adipocyte intracellular Ca^{2+} and lipid metabolism

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Abstract

Previous data from this laboratory demonstrate that increased intracellular Ca^{2+} ($[Ca^{2+}]i$) coordinately regulates human and murine adipocyte lipid metabolism by stimulating lipogenesis and inhibiting lipolysis. However, recent data demonstrate metabolic uncoupling increases $[Ca^{2+}]i$ but inhibits lipogenesis by suppressing fatty acid synthase (FAS) activity. Accordingly, we have evaluated the interaction between mitochondrial uncoupling, adipocyte $[Ca^{2+}]i$, and adipocyte lipid metabolism. Pretreatment of 3T3-L1 cells with mitochondrial uncouplers (DNP or FCCP) amplified the $[Ca^{2+}]i$ response to depolarization with KCl by 2-4 fold ($p < 0.001$), while this increase was prevented by $[Ca^{2+}]i$ channel antagonism with lanthanum. Mitochondrial uncouplers caused rapid (within 4hr) dose-dependent inhibition of FAS activity ($p < 0.001$), while lanthanum caused a further additive inhibition. The suppression of FAS activity induced by uncoupling was reversed by addition of ATP. Mitochondrial uncouplers increased FAS expression significantly while $[Ca^{2+}]i$ antagonism with lanthanum decreased FAS expression ($P < 0.001$). In contrast, mitochondrial uncouplers independently inhibited basal and isoproterenol-stimulated lipolysis (20-40%, $p < 0.001$), while this inhibition was fully reversed by lanthanum. Thus, mitochondrial uncoupling exerted short-term regulatory effects on adipocyte $[Ca^{2+}]i$ and lipogenic and lipolytic systems, serving to suppress lipolysis via a Ca^{2+} -dependent mechanism and FAS activity via a Ca^{2+}-independent mechanism. © 2003 Elsevier Inc. All rights reserved.

1. Introduction

The discovery of novel homologues of the brown adipose tissue mitochondrial uncoupling protein (UCP), such as UCP2, has provoked intensive study of these mitochondrial proteins and the role that they may play in energy metabolism [1]. Since lipogenesis is dependent upon mitochondrial ATP production [2], mitochondrial uncoupling in adipocytes may contribute to control of lipid metabolism.

Previous data from our laboratory demonstrates that intracellular calcium ($[Ca^{2+}]i$) plays a key role in the regulation of adipocyte energy metabolism [3]. Increased $[Ca^{2+}]i$ stimulates the expression and activity of fatty acid synthase (FAS), a key enzyme in de novo lipogenesis, and inhibits basal and agonist-stimulated lipolysis in both human and murine adipocytes [4–5]. However, little is known of the mitochondrial regulation of $[Ca^{2+}]i$ and lipid metabolism in adipose tissue. Accordingly, in the present study, the role of mitochondrial uncoupling in regulating lipolysis and lipogenesis as well as intracellular calcium have been examined in differentiated 3T3 L1 adipocytes. We used two different mitochondrial uncouplers to induce uncoupling effect on adipocytes mitochondria [6–7]. We report that mitochondrial uncoupling increases $[Ca^{2+}]i$ and down-regulates lipolysis via a calcium-dependent mechanism. In contrast, the effect of mitochondrial uncoupling on fatty acid synthase is more complex, with up-regulation of FAS expression via a calcium-dependent mechanism, and suppression of FAS activity via a Ca^{2+}-independent mechanism.

2. Materials and methods

2.1. Culture and differentiation of 3T3 L1

3T3-L1 preadipocytes were incubated at a density of 8000 cells/cm^2 (10 cm [2] dish) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and antibiotics at 37°C in 5% CO_2 in air. Confluent preadipocytes were induced to differentiate with a standard differentiation medium consisting of DMEM-F10 (1:1, vol/vol) medium supplemented with 1% FBS, 1μM dexamethasone, IBMX (0.5 mM) and antibiotics (1% BSA). Preadipocytes were maintained in this differentiation medium for

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3 days and subsequently cultured in adipocyte medium. Cultures were refed every 2–3 days. Cell viability was measured via trypan blue exclusion.

2.2. Fatty acid synthase (FAS) activity assay

Adipocytes were incubated for 4 h in the presence of 0-50 μM 2,4-dinitrophenol (DNP) or 0-5 μM carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) (Sigma, St Louis, MO) with or without insulin (30nM) and with or without lanthanum(1mM) (Sigma, St Louis, MO). Subsequently, the cells were returned to serum free medium (containing 1% BSA) for 48 hr. Fatty acid synthase activity was measured in cytosolic extracts by measuring theoxidation rate of NADPH, as described previously [8–10]. Adipocytes were then homogenized in 250 mmol/L sucrose solution containing 1 mmol/L ethylenediamine-tetraacticacid (EDTA), 1mmol/L dithiothreitol (DTT), and 100 μmol/L phenylmethylsulfonyl fluoride (PMSF) (pH 7.4). The homogenates were centrifuged at 18,500 X g for 1h and then incubated in phosphate buffer and acetyl-CoA, with or without ATP (10nM) (Sigma, St Louis, MO), for 15 min, and the infranatant was used for measuring oxidation of NADPH right after adding malonyl-CoA. The final FAS activity was normalized to nucleic acids, which was measured by CyQUANT cell proliferation assay kit (Parkard instrument Company, Inc., Downers Grove, IL) according to manufacturer's instruction.

2.3. Lipolysis assay

Adipocytes were incubated for 2 h in the presence of 0-50 μM DNP or 0-5 μM FCCP with or without insulin (30nM) and with or without lanthanum (1mM), and with or without isoproterenol (50nM). Glycerol released into the culture medium was determined as an indicator for lipolysis, using a one-step enzymatic fluorometric method [11]. Glycerol release data was normalized for cellular nucleic acids as described above.

2.4. $[Ca^{2+}]i$ measurement

$[Ca^{2+}]i$ in 3T3 L1 adipocytes was measured using a fura-2 dual-wavelength fluorescence imaging system. Preadipocytes were plated in 35-mm dishes (P35G-0-14-C, MatTek). Prior to $[Ca^{2+}]i$ measurement, cells were preincubated with or without different concentration 0-50 μM DNP or 0-5 μM FCCP with or with or without lanthanum(1mM) for one hour. Cells were then put in serum-free medium overnight and rinsed with HEPES balanced salt solution (HBSS) containing the following components (in mM): 138 NaCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 0.9 NaH_2PO_4, 4 $NaHCO_3$, 5 glucose, 6 glutamine, 20 HEPES, and 1% bovine serum albumin. Cells were loaded with fura-2 acetoxymethyl ester (fura-2 AM) (10 μM) in the same buffer for 2 h at 37°C in a dark incubator with 5% CO_2. To remove extracellular dye, cells were rinsed with HBSS three times and then postincubated at room temperature for an additional 1 h for complete hydrolysis of cytoplasmic fura-2 AM. The dishes with dye-loaded cells were mounted on the stage of Nikon TMS-F fluorescence inverted microscope with a Cohu model 4915 charge-coupled device (CCD) camera. Fluorescent images were captured alternatively at excitation wavelength of 340 and 380 nm with an emission wavelength of 520 nm. After establishment of a stable baseline, the response to 40mM KCl was determined. $[Ca^{2+}]i$ was calculated using a ratio equation as described previously [12]. Each analysis evaluated responses of 5 representative whole cells. Images were analyzed with InCyt Im2 version 4.62 imaging software (Intracellular Imaging, Cincinnati, OH). Images were calibrated using a fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR) to create a calibration curve in solution, and cellular calibration was accomplished using digitonin (25 μM) and pH 8.7 Tris-EGTA (100 mM) to measure maximal and minimal $[Ca^{2+}]i$ levels respectively.

2.5. Total RNA extraction

Total cellular RNA isolation kit (Ambion Inc., Austin, TX) was used to extract total RNA from 3T3 L1 cells according to manufacturer's instruction.

2.6. Quantitative real time PCR

3T3 L1 adipocyte FAS mRNA was quantitatively measured using a Smart Cycler Real Time PCR System (Cepheid, Sunnyvale, CA) with a TaqMan 1000 Core Reagent Kit (Applied Biosystems, Branchburg, NJ). The primers for mouse FAS are as follows. Forward primer: 5′-CCCAGAGGCTTGTGCTGACT-3′ reverse primer: 5′-CGAATGTGCTTGGCTTGGT-3′. Pooled 3T3 L1 adipocyte total RNA was serial-diluted in the range of 1.5625-25 ng and used to establish a FAS standard curve; total RNAs for unknown samples were diluted in this range. Reactions of quantitative RT-PCR for standards and unknown samples were performed according to the instructions of Smart Cycler System (Cepheid, Sunnyvale, CA) and TaqMan Real Time PCR Core Kit (Applied Biosystems, Branchburg, NJ). The FAS mRNA quantitation for each sample was normalized to 18s mRNA via real-time PCR using the following primers: forward, 5′-AGTCCCTGCCCTTTGTACACA-3′; reverse, 5′-GATCCGAGGGCCTCACTAAAC-3′.

2.7. Statistical analysis

All data are expressed as mean ± SE. Data were evaluated for statistical significance by one-way analysis of variance (ANOVA), and significantly different group means were then separated by the least significant difference test by using SPSS (SPSS Inc, Chicago, Ill.).

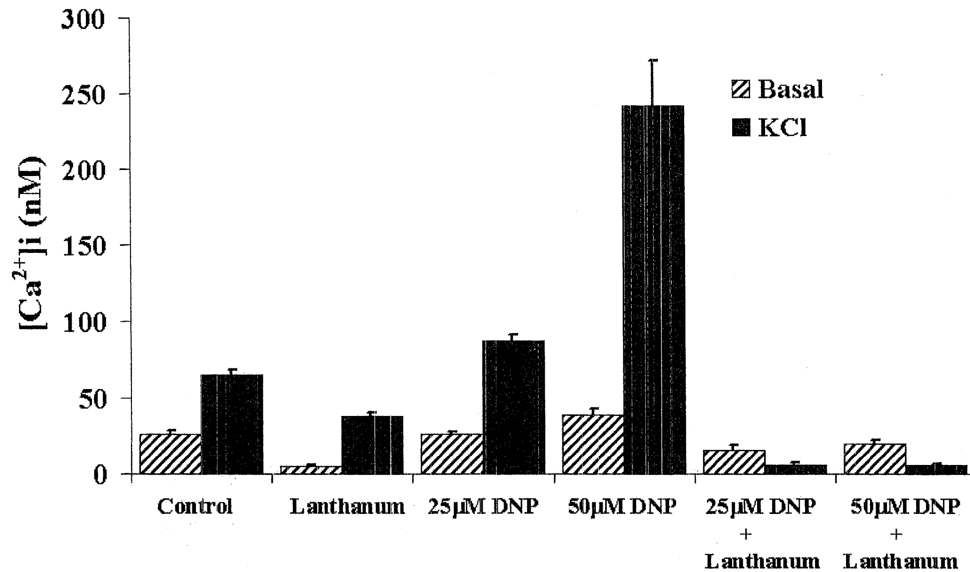

Fig. 1. The effect of DNP on intracellular calcium in 3T3 L1 adipocytes. Differentiated 3T3 L1 adipocytes were treated with or without DNP(25μM, 50μM), DNP plus KCl (40mM) or DNP plus lanthanum(1mM). The intracellular calcium levels were measured by Fura-2 dual wavelength fluorescence imaging system, which was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n = 12). Difference letters above the bars indicate a significant difference at level of $p < 0.05$)

3. Results

Both mitochondrial uncouplers increased KCl-stimulated $[Ca^{2+}]i$ ($p < 0.001$). Antagonism of calcium channels with the non-specific inhibitor lanthanum decreased both basal and stimulated $[Ca^{2+}]i$ significantly ($p < 0.001$) and reversed the stimulation of $[Ca^{2+}]i$ induced by mitochondrial uncouplers (Fig. 1 and Fig. 2).

Mitochondrial uncoupling with DNP or FCCP significantly inhibited lipolysis (Figs. 3 and 4). This effect was attenuated by insulin while lanthanum completely reversed the suppression by mitochondrial uncouplers or/and insulin (Figs. 3 and 4). These results indicate that metabolic uncouplers suppress lipolysis via a calcium dependent mechanism.

In short-term (4 hr) treatment with DNP, FAS activity was decreased significantly by mitochondrial uncoupling ($p < 0.001$) (Fig. 5). However, lanthanum was unable to reverse this short-term suppression, indicating that this short-term suppression of FAS activity by DNP was calcium-

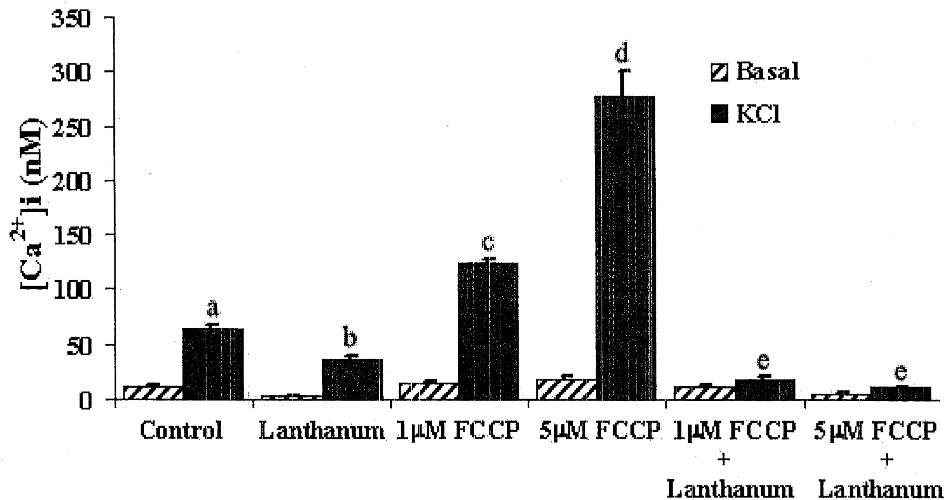

Fig. 2. The effect of FCCP on intracellular calcium in 3T3 L1 adipocytes. Differentiated 3T3 L1 adipocytes were treated with or without FCCP (1μM, 5μM), FCCP plus KCl (40mM) or FCCP plus lanthanum(1mM). The intracellular calcium levels were measured by fura-2 dual wavelength fluorescence imaging system, which was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n = 12). Difference letters above the bars indicate a significant difference at level of $p < 0.05$).

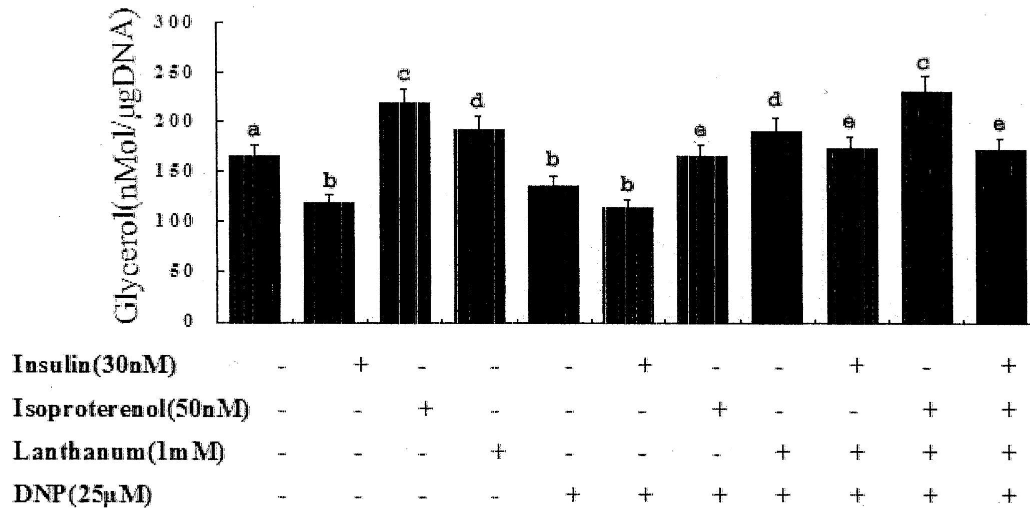

Fig. 3. The effect of DNP on lipolysis in 3T3 L1 adipocytes. Differentiated 3T3 L1 adipocytes were treated with or without DNP (25μM), DNP plus insulin (30nM), DNP plus isoproterenol (50nM) or DNP plus lanthanum(1mM). Lipolysis was determined as glycerol release by enzymatic fluorescence method, which was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n = 12). Difference letters above the bars indicate a significant difference at level of $p < 0.05$).

independent. Similar results were observed with a long-term (48 hr) treatment with both mitochondrial uncouplers, with lanthanum causing a further suppression (Figs. 5 and 6). However, both mitochondrial uncouplers significantly increased FAS expression and this effect was reversed by lanthanum (Fig. 7). Thus, metabolic uncoupling has a dual effect on FAS: to decrease activity via a Ca^{2+}-independent mechanism and to increase FAS expression via a Ca^{2+}-dependent mechanism. The addition of ATP reversed the DNP-induced suppression of FAS activity, indicating that this effect is secondary to ATP depletion (Fig. 8).

4. Discussion

Several studies have reported that mitochondrial uncoupling regulates lipid metabolism both in vivo and in vitro [13–15]. However, the mechanism mediating this effect remains unclear. Our previous data suggested intracellular calcium is an important regulator of both lipogenesis and lipolysis. Accordingly, We investigated whether there is an effect of mitochondrial uncoupling on intracellular calcium level and whether $[Ca^{2+}]i$ is the mediator for the regulation of lipid metabolism by mitochondrial uncoupling.

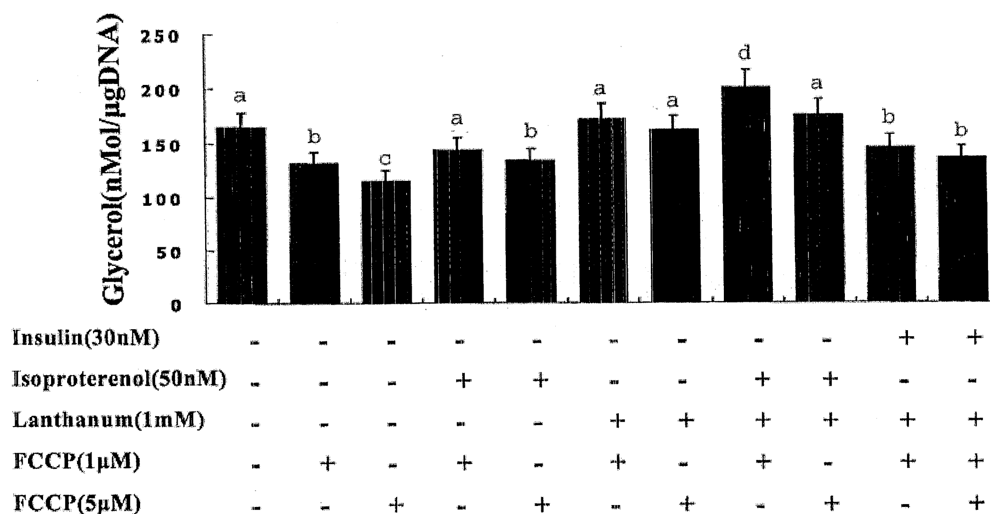

Fig. 4. The effect of FCCP on lipolysis in 3T3 L1 adipocytes. Differentiated 3T3 L1 adipocytes were treated with or without FCCP (1μM, 5μM), FCCP plus insulin (30nM), FCCP plus isoproterenol (50nM) or FCCP plus lanthanum(1mM). Lipolysis was determined as glycerol release by enzymatic fluorescence method, which was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n = 12). Difference letters above the bars indicate a significant difference at level of $p < 0.05$).

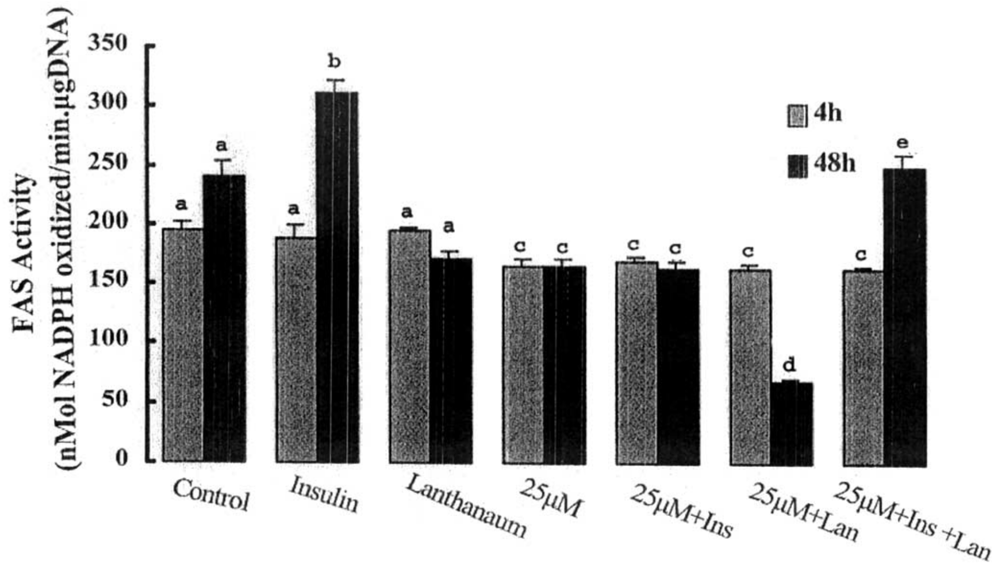

Fig. 5. The effect of DNP on fatty acid synthase (FAS) activity in 3T3 L1 adipocytes. Differentiated 3T3 L1 adipocytes were treated with or without DNP (25μM), Insulin (30nM), Lanthanum (1mM), DNP plus insulin or DNP plus lanthanum. FAS activity was measured as the oxidative rate of NADPH, which was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n = 12). Difference letters above the bars indicate a significant difference at level of p <0.05.

The role of the mitochondria in regulation of $[Ca^{2+}]$i homeostasis has been widely investigated. Several observations suggest that $[Ca^{2+}]$i is regulated by mitochondria in an energy-linked process [16–18]. In addition, experimental evidence indicates a positive association between mitochondrial calcium and mitochondrial ATP production [19–22]. Our results suggest that mitochndrial uncoupling contributes to the control of membrane calcium channel activity in differentiated 3T3 L1 adipocytes. We report that mitochondrial uncouplers dose-responsively increase $[Ca^{2+}]$i in adipocytes. This regulation of $[Ca^{2+}]$i level by mitochondrial uncouplers in adipocytes appears to involve membrane calcium channels, as this effect can be completely blocked by non-specific calcium channel antagonist (lanthanum). Although Makowska et al. previously reported that mitochondrial uncoupler depressed the rate of calcium influx in vitro

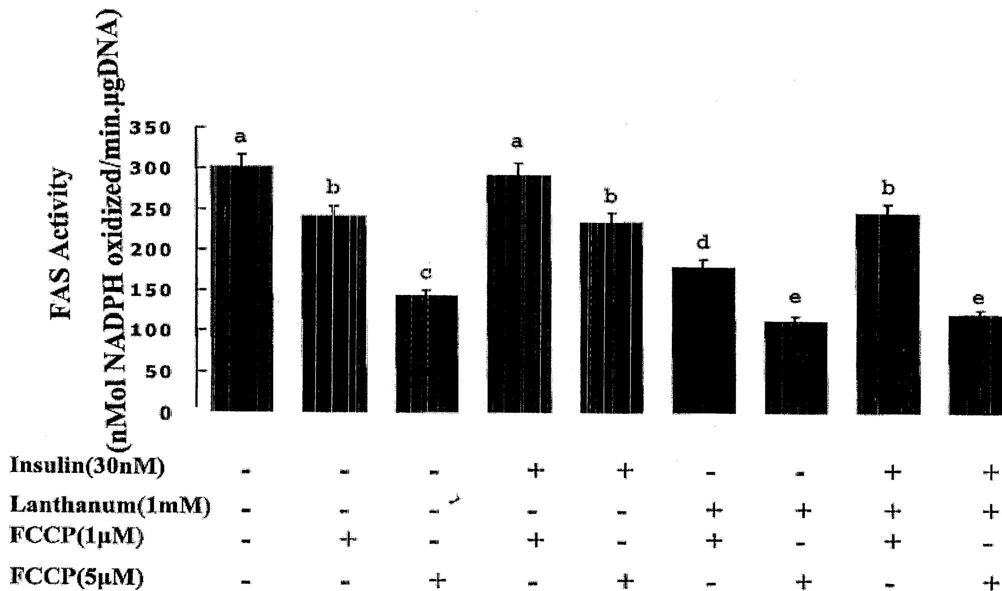

Fig. 6. The effect of FCCP on fatty acid synthase(FAS) activity in 3T3 L1 adipocytes. Differentiated 3T3 L1 adipocytes were treated with or without FCCP (1μM, 5μM), Insulin (30nM), Lanthanum (1mM), FCCP plus insulin or FCCP plus lanthanum. FAS activity was measured as the oxidative rate of NADPH, which was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n = 12). Difference letters above the bars indicate a significant difference at level of p <0.05.

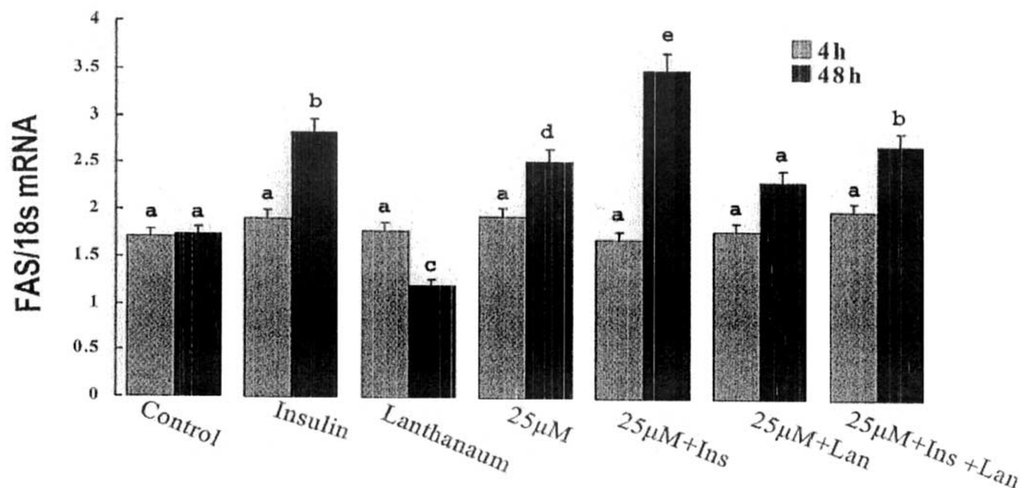

Fig. 7. The effect of DNP on fatty acid synthase (FAS) expression in 3T3 L1 adipocytes. Differentiated 3T3 L1 adipocytes were treated with or without DNP (25μM), Insulin (30nM), Lanthanum (1mM), DNP plus insulin or DNP plus lanthanum. FAS mRNA was measured by real time PCR, which was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n = 6). Difference letters above the bars indicate a significant difference at level of $p < 0.05$.

[23], this inhibitory effect of uncoupler on intracellular calcium was observed on store-operated calcium channel rather than voltage-gated calcium channel (VGCC). However, an important relevant role of VGCCs is to serve as highly regulated mechanisms to deliver calcium ions into specific intracellular locales for a variety of calcium-dependent processes [24], Thus, the observation from present work demonstrated that mitochondrial uncoupler regulates VGCC by increasing the calcium influx. Additionally, Lynch et al have shown that calcium was released from mitochondrial as a result of DNP dissipation of the H^+ gradient [25]. Consequently, we reasoned that intracellular calcium may be a trigger in the effect of mitochondrial uncoupling on lipid metabolism.

Previous studies have demonstrated that lipolysis depends on the energy status of adipocytes [26–27]. A decrease in intracellular ATP elicited in white adipocytes*in vitro* by inhibitors of the mitochondrial respiratory chain

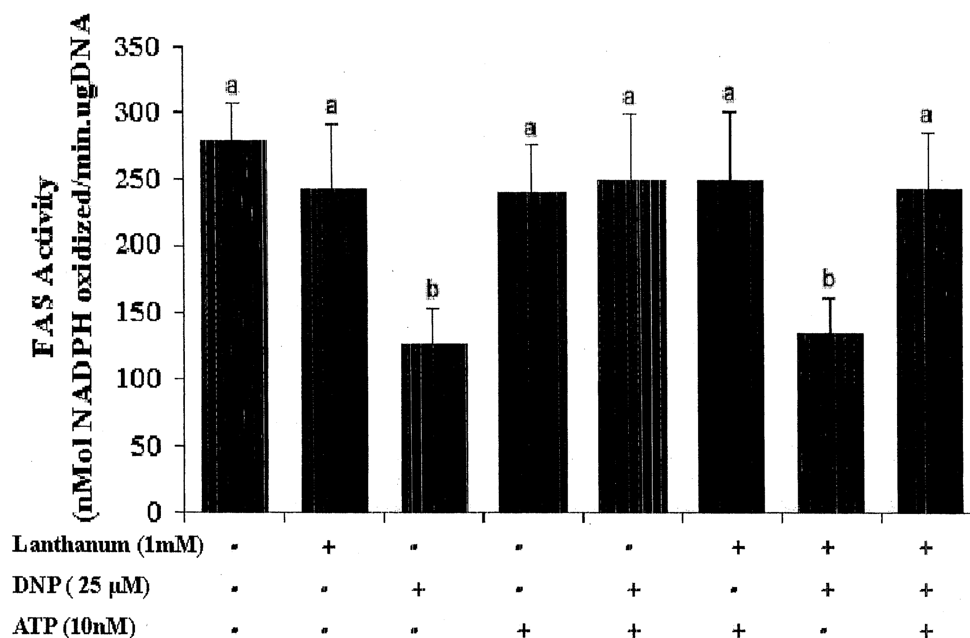

Fig. 8. The effect of DNP on fatty acid synthase (FAS) activity in 3T3 L1 adipocytes. Differentiated 3T3 L1 adipocytes were treated with or without DNP (25μM), ATP (10nM), Lanthanum (1mM), DNP plus ATP, DNP plus lanthanum or Lanthanum plus ATP. FAS activity was measured as the oxidative rate of NADPH, which was conducted as described in Materials and Methods. Data are expressed as mean ± SE (n = 6). Difference letters above the bars indicate a significant difference at level of $p < 0.05$.

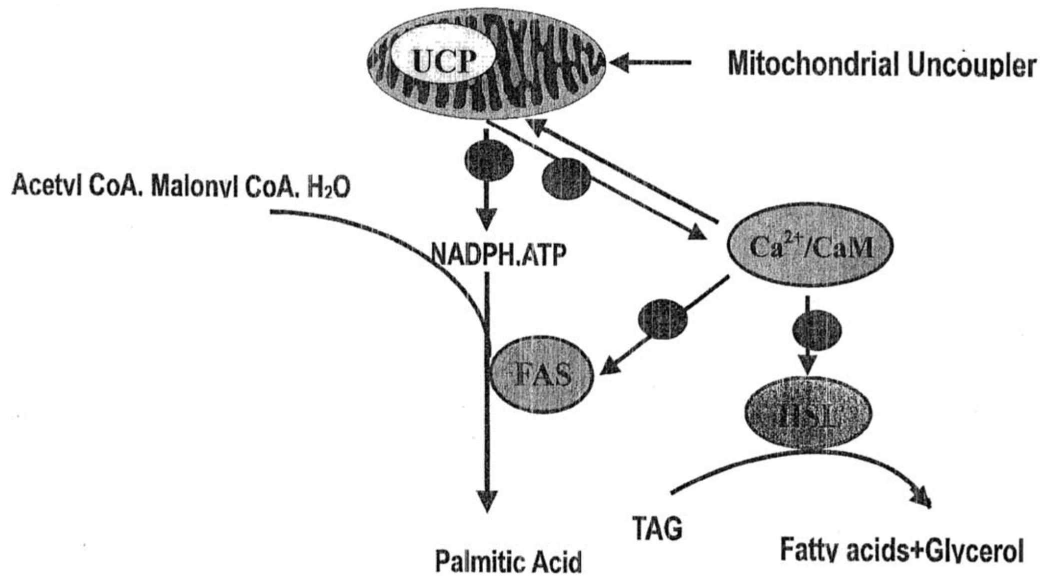

Fig. 9. A proposed scheme for the integration of the regulation of lipid metabolism by mitochondrial uncoupling. Mitochondrial uncoupling increases $[Ca^{2+}]$i by activating membrane calcium channels and thereby inhibits lipolysis via a Ca^{2+}-dependent pathway. Mitochondrial uncoupling has dual effects on lipogenesis: it inhibits FAS activity via a Ca^{2+}-independent mechanism and stimulates of FAS expression via a Ca^{2+}-dependent metabolism.

reversed the stimulation of lipolysisby catecholamines [28]. More recently, ATP was shown to be required for translocationof HSL from cytoplasm to the surface of lipid storage dropletsand for phosphorylation of HSL and other proteins that are involvedin lipolysis in adipocytes [29]. Incubation of isolated adipocytes with lipolytic hormones resulted in up to 50% decrease of their intracellular ATP level and in inhibition of lipolysis itself [29]. In addition, The intracellular ATP is also essential for insulin signaling [30–31], which is altered at the level of the interaction of insulin with its receptor [32]. In present study, we also showed that mitochondrial uncoupling inhibits lipolysis and this effect is via a calcium dependent mechanism. Previous data from our laboratory demonstrate that increasing $[Ca^{2+}]$i inhibits adipocyte lipolysis. This effect is primarily mediated by activation of adipocyte phosphodiesterase 3B and a reduction of cAMP levels, leading to a decrease in hormone sensitive lipase phosphorylation and, consequently, an inhibition of lipolysis [32]. In the present study, increased $[Ca^{2+}]$i and decreased lipolysis were observed concomitantly with two individual mitochondrial uncouplers, while inhibition of Ca^{2+} influx prevented the suppression of lipolysis. Therefore, $[Ca^{2+}]$i appears to mediate the effect of mitochondrial uncouplers on lipolysis. In addition, the mitochondrial uncoupling results in reduced adipocytes lipolysis in response to isoproterenal or insulin, suggesting a reduction in adipocytes cAMP levels.

We also found that mitochondrial uncoupling has dual effects on a key lipogenic system, fatty acid synthase. The suppression of FAS activity induced by short-term treatment with mitochondrial uncouplers indicates a non-genomic effect of mitochondrial uncoupling on FAS. This effect is a calcium-independent since lanthanum is unable to reverse the suppression. The stimulation of FAS expression was induced by long-term treatment with mitochondrial uncouplers and this stimulation of FAS expression can be fully reversed by lanthanum, indicating that a genomic effect of mitochondrial uncoupling on FAS expression is $[Ca^{2+}]$i-dependent, consistent with our previous reports of $[Ca^{2+}]$i regulation of FAS expression [8–9]. To our surprise, FAS activity was suppressed by long-term treatment with mitochondrial uncouplers and addition of lanthanum induced a further suppression on FAS activity, which suggests that there is both a calcium dependent and a non-genomic, Ca^{2+}-independent effect similar to that observed in the short-term treatment. This non-genomic effect of suppression on FAS activity by mitochondrial uncoupling may result from the shortage of the substrates (e.g. NADPH and ATP) secondary to reduced mitochondrial energy with mitochondrial uncouplers [33]. For each molecule ofacetate incorporated into long-chain FA, two molecules of NADPHare necessary in the reaction catalyzed by FA synthase. Moreover, mitochondrial production of ATP is required for synthesis of oxalacetate by carboxylation of pyruvate and subsequent synthesis of citrate in mitochondria and for FA synthesis and esterification in cytoplasm [34–35]. The ATP/ADP ratio has been demonstrated to affect pyruvate carboxylase activity directly [35–36]. Although the *in* vitro assay system used in this study provides acetyl-CoA, malonyl -CoA and NADPH, it does not provide exogenous ATP. Therefore, reduced availability of intramitochondrial ATP and the a reduced ATP: ADP ratio secondary to uncoupling may be responsible for the observed reduction in activity [37–38]. To further test this hypothesis, we found that addition of ATP reversed the uncoupler-induced suppression on FAS activity, indicating that inhibition of FA syn-

thesis by uncouplers probably resulted from limited availabilityof intramitochondrial ATP.

In conclusion, mitochondrial uncoupling participates in the regulation in lipid metabolism and $[Ca^{2+}]i$. Fig. 9 depicts a proposed scheme for the integration of this regulation, as follows. Mitochondrial uncoupling increases $[Ca^{2+}]i$ by activating membrane calcium channels and thereby inhibits lipolysis via a Ca^{2+}-dependent pathway. Mitochondrial uncoupling has dual effects on lipogenesis: it inhibits FAS activity via a Ca^{2+}-independent mechanism and stimulates of FAS expression via a Ca^{2+}-dependent metabolism (Fig. 9).

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