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The Antidepressant Phenelzine Alters Differentiation of Cultured Human and Mouse Preadipocytes

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Running title: Antidepressant impact on adipogenesis in vitro

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ABBREVIATIONS:

ACC , acetyl-CoA carboxylase; aP2, adipocyte protein 2; C/EBP-α, CCAAT/enhancer binding protein-α; DOG, 1,2-deoxyglucose; ERK1/2, p42/p44 extracellular signal-regulated kinase; FAS, fatty acid synthase; G3PDH, glycerol-3-phosphate dehydrogenase; MAO, monoamine oxidase; MEF, mouse embryonic fibroblasts; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]; PPAR-γ, peroxisome proliferator-activated receptor-γ; SREBP-1c, sterol regulatory element-binding protein-1c; SSAO, semicarbazide-sensitive amine oxidase; UCP1, uncoupling protein-1.

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ABSTRACT

Change in body weight is a frequent side effect of antidepressants, and is considered to be mediated by central effects on food intake and energy expenditure. The antidepressant phenelzine (NARDIL®) potently inhibits both monoamine oxidase and semicarbazide-sensitive amine oxidase activities, two enzymes that are highly expressed in adipose tissue, raising the possibility that it could directly alter adipocyte biology. Treatment with this compound is rather associated with weight gain. The aim of this work was to examine the effects of phenelzine on differentiation and metabolism of cultured human and mouse preadipocytes, and to characterize the mechanisms involved in these effects. In all preadipocyte models, phenelzine induced a time- and dose-dependent reduction in differentiation and triglyceride accumulation. Modulation of lipolysis or glucose transport were not involved in phenelzine action. This effect was supported by the reduced expression in the key adipogenic transcription factors peroxisome proliferator-activated receptor-gamma (PPAR-gamma) and CCAAT/enhancer binding protein-alpha (C/EBP-alpha), which was observed only at the highest drug concentrations (30-100 µM). The PPAR-gamma agonists thiazolidinediones did not reverse phenelzine effects. By contrast both the reduction in cell triglycerides and in sterol regulatory element-binding protein-1c (SREBP-1c) was detectable at lower phenelzine concentrations (1-10 µM). Phenelzine effect on triglyceride content was prevented by providing free fatty acids to the cells, and was partially reversed by overexpression of a dominant positive form of SREBP-1c, showing the privileged targeting of the lipogenic pathway. When considered together, these findings demonstrate that an antidepressant directly and potently inhibits adipocyte lipid storage and differentiation, that could contribute to psychotropic drug side effects on energy homeostasis.

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INTRODUCTION

Weight change occurring during treatment of psychiatric disorders is a frequent side effect and may force patients to discontinue pharmacological treatment, thus putting them at the risk of relapse. Psychotropic drugs, including antidepressants, mood stabilizers, and antipsychotics, more often increase but sometimes reduce body weight. Considering their widespread use, concern has arisen regarding treatment-associated weight gain and obesity-associated morbidities, such as type 2 diabetes and cardiovascular diseases (Despres and Lemieux, 2006; Van Gaal et al., 2006).

However, the general mechanisms influencing body weight during treatment for mental disorders are still far to be elucidated, and likely include disease-related, improvement-related, and drug-related factors. Many psychiatric disorders are associated *per se* with weight changes, most often with weight loss. Thus, it was thought that drug-induced weight gain merely reflects the restoration of normal body weight, but this issue remains in debate (Zimmermann et al., 2003). Otherwise, it is well known that antidepressants can modulate weight by interfering with the function of specific central nervous system pathways involved in the regulation of appetite and food intake, but also through variations in resting energy expenditure (Zimmermann et al., 2003). Thus, alterations in weight equilibrium induced by the major classes of antidepressants such as tricyclic agents or selective serotonin reuptake inhibitors, but also by monoamine oxidase (MAO) inhibitors employed in a second line strategy for severe depression, appear mainly related to the regulation of food intake and/or energy expenditure at the central nervous system level.

However, to our knowledge, the possibility that antidepressants could directly modify the biology of peripheral tissues involved in body energy homeostasis has not been documented. In particular, given the high expression levels of MAO and semicarbazide-sensitive amine oxidase (SSAO) in fat cells, it is quite conceivable that the antidepressants of the MAO inhibitor family could exert direct and specific effects on adipose tissue development or metabolism. This is reinforced by the observation that these amine oxidases could regulate major physiological functions in adipocyte.

It is now well established that in different species, SSAO expression and activity are largely induced during the adipose conversion process (Fontana et al., 2001; Mercier et al., 2001). SSAO

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activation by exogenous substrates activates glucose transport (Enrique-Tarancon et al., 1998), adipocyte differentiation (Fontana et al., 2001), and inhibits lipolysis (Amant et al., 2005; Morin et al., 2001; Visentin et al., 2003). All these insulin-mimicking effects are related to SSAO-generated hydrogen peroxide production (Enrique-Tarancon et al., 2000). *In vivo*, SSAO substrates reduce hyperglycaemia and improve glucose tolerance in animal models of diabetes (Marti et al., 2001). Although less documented, MAO activity is also present in adipose tissue (Tong et al., 1979), and is predominantly expressed in human fat cells as MAO-A, with a minor amount of MAO-B (Pizzinat et al., 1999). Tyramine, a preferential substrate for MAO, partially reproduces insulin effects on Akt phosphorylation, glucose transport, and adipose conversion (Fontana et al., 2001). *In vivo* studies also support the view that amine oxidase activities could interact with the control of energy balance, and especially with lipid mobilization-deposition since mitigation of obesity has been observed in rodents treated with amine oxidase inhibitors (Carpene et al., 2008; Carpene et al., 2007; Prevot et al., 2007; Yu et al., 2004).

These observations raise the possibility that amine oxidase inhibitors directly alter adipose tissue development, and led us to examine the potential direct effects of several amine oxidase inhibitors on adipocyte differentiation or lipid accumulation in several preadipocyte culture models. We observed that in four independent models of adipocyte differentiation, the hydrazine derivative phenelzine (2-phenylethyhydrazine, NARDIL®), an antidepressant with a potent MAO and SSAO inhibitory activity, dramatically prevented cell triglyceride accumulation and adipose conversion, a phenomenon related to a reduced expression of several key adipogenic transcription factors, such as PPAR-γ, C/EBP-α, and SREBP-1c. These findings were in apparent discrepancy with body weight gain frequently observed during phenelezine treatment. Our findings demonstrate that in addition to its central effect on food intake and energy expenditure, the antidepressant phenelzine can directly act on a preadipocyte to limit its development.

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MATERIALS and METHODS

Subjects

Adipose tissue was obtained from mesenteric fat depots from men or women (16-77 years) undergoing abdominal surgery. Subjects were devoided of metabolic, endocrine, or cardiovascular diseases. The study was approved by local ethics committee, and an informed consent was given to the patients.

Cell culture

Murine 3T3-F442A and 3T3-L1 preadipocytes and the murine brown preadipocyte T37i cell line (Zennaro et al., 1998) were grown until subconfluence conditions as described in supplemental data published on the web site at http:// molpharm.aspetjournals.org.

Preadipocytes were obtained from human adipose tissue fragments as described (Dieudonne et al., 2004; Serazin-Leroy et al., 2000), then plated into cell culture dishes at a density of 2-4 x $10^4/cm^2$ in a DME/HamF12 medium with 10 % FCS and antibiotics. Following an overnight culture, cells were washed 4 times. After 48-72 h, adipocyte differentiation was induced in a chemically defined medium including DME/Ham F12, antibiotics, 80 nM insulin, 0.2 nM triodothyronine, 10 µg/ml transferrin, and for the first 2 days 2.2 µM troglitazone and 200 µM methyl-isobutylxanthine.

The mouse embryonic fibroblasts (MEF), that constitutively express peroxisome proliferator-activated receptor-γ (PPAR-γ) nuclear receptor (MEF-PPAR-γ) cells, were cultured as described (Davis et al., 2004). They were exposed or not to 10 µM pioglitazone from confluence (day 0) or day 4 post-confluence.

Unless otherwise specified, murine cells were exposed to phenelzine from confluence. Human preadipocytes were treated with the drug when shifted in the serum-free medium.

Morphological and biochemical determinations

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Morphological determination of cell lipid content, measurement of cell viability and proliferation, determination of triglyceride content and glycerol-3-phosphate dehydrogenase (G3PDH) activity in preadipose or adipose cells are described in supplemental data on line.

Western-blot analysis

For Western-blot analysis, cell extracts and protein electropheresis were performed as mentioned in appendix 1 and supplemental table 1 (published as supplemental data online).

Lipolysis experiments

Lipolysis was assessed as glycerol release from adherent 3T3-F442A adipocytes. Adipocytes were cultured without insulin for 24 h, then shifted for 24 period in a serum-free medium including DME/HamF12, antibiotics, and 0.1 % fatty acid-free BSA. Thereafter, cells were cultured in the same medium in the absence or in the presence of 100 µM phenelzine.

Lipolysis experiments with or without 10 µM (-)-isoproterenol or 10 µM forskolin were performed as described in supplemental data.

Determination of 2-deoxyglucose uptake

Adipocytes were cultured and treated as for the lipolysis experiments. Uptake of glucose was determined using [1,2-^{3}H]-deoxyglucose ([^{3}H]DOG) (ICN Biochemicals, Orsay, France), a nonmetabolizable analog of glucose. Glucose uptake was described in supplemental data.

Quantitative real-time PCR.

Total RNA was extracted by the method of Cathala (Cathala et al., 1983), and quantitative real-time PCR was performed as described (Teixeira et al., 2006). Details on RNA analysis are given in appendix and supplemental table 2 (published as supplemental data online).

Adenofection experiments

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The adenovirus vector encoding for the dominant positive form of rat SREBP-1c, so called Ad. SREPB-1c DP, was generated as previously mentioned (Foretz et al., 1999). SREBP-1c gene expression was under control of a cytomegalovirus promoter, and a green fluorescent protein, co-expressed to monitor transfection efficiency. The adenovirus containing the major late promoter with no exogenous gene, called Ad.null, was used as a control. Adenoviral vectors were purified after propagation in the HEK293 cell line and stored at -80°C until use. At confluence, adenofection was performed under serum-free conditions for 90 min at a multiplicity of infection of 500 (500 plaque-forming units/cell) that is known to achieve an optimal infection efficiency in 3T3 adipocytes (Le Lay et al., 2002). Cells were then refed in a serum-containing differentiating medium in the absence or in the presence of 50 or 100 µM phenelzine. Similar efficiency in viral infection was assessed by green fluorescent protein expression. Microscopic analysis, cell triglyceride, G3PDH activity, FAS and adiponectin expression were analyzed at day 7 following confluence.

Statistical analysis

Results of quantitative analysis are presented as mean $\pm$ standard errors (S.E.), as specified in figure legends. In the dose-dependence and time course experiments (triglyceride and G3PDH assays), a separate one way analysis of variance (ANOVA) was employed with the selected concentrations or time as the independent variables and percentage between drug-exposed and control samples as the dependent variable. Comparison of mean values between groups was analyzed with post-hoc Tukey's HSD multiple comparison test. For lipolysis, glucose transport and RT-PCR experiments, the statistical significance of differences between means was analyzed with a two-sided Student's *t*-test. A p value < 0.05 was considered as the threshold of statistical significance.

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RESULTS

Phenelzine is a potent inhibitor of preadipocyte differentiation and triglyceride accumulation

Confluent 3T3-F442A cells were cultured for various periods of time in the absence or in the presence of the drug, within a concentration range compatible with that detectable in the plasma of treated patients (www.biopsychiatry.com/phenelzine/phenelzine-nardil). Following a 8-day treatment with various phenelzine concentrations, a slight decrease in lipid content was detectable from 1 µM of the compound, and was dramatic at 30 µM, reaching a maximal effect at 100 µM (Figure 1A). This was confirmed by microscopic analysis showing that a 8-day-exposure to 100 µM phenelzine dramatically decreased the number and size of fat vacuoles as compared to control cells (Figure 1B). Noteworthy, the reduction in lipid droplets occurs at concentrations where alterations in the spheric shape of adipocytes were not detectable. This observation suggested that at low concentrations, phenelzine preferentially altered the process of triglyceride accumulation, while a global reduction in the differentiation level occurred at higher concentrations. In agreement with this result, phenelzine induced a dose-dependent decrease in 3T3-F442A cell triglyceride content, that was significant from 1 µM of the drug, and then continued to markedly decline with increasing doses (Figure 1C), with a maximal 90 % reduction at 100 µM as compared with control cells, giving a half-maximal effect at 20 µM. Adiponectin and aP2 protein expressions were also examined by Western-blot analysis in 3T3-F442A cells treated with various phenelzine concentrations (Figure 1D). aP2 down-regulation was only observed at the highest 100 µM phenelzine concentration. Adiponectin expression levels were maintained until a concentration of 10 µM, and were decreased from 30 µM, with a maximal reduction at 100 µM. The dose-dependent phenelzine-induced decrease in G3PDH activity, that reflected the level of adipocyte differentiation had a pattern close to that of adiponectin (not shown).

Confluent 3T3-F442A cells were maintained from confluence in the absence or in the presence of 100 µM of the compound, and cell extracts were prepared at intervals. At day 6 following confluence, phenelzine provoked a marked 3-fold decrease in cell triglyceride content (Figure 1E). This effect was even more pronounced at day 8, with about a 6-fold reduction. A similar profile was observed for

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G3PDH activity, which was dramatically down-regulated by phenelzine in day 8 postconfluent 3T3-F442A cells (Figure 1F).

The reversibility of phenelzine effect was also tested in FT3-F442A cells (Figure 2). Chronic phenelzine exposure (100 µM) for 7 days from confluence markedly reduced cell triglyceride accumulation. When phenelzine treatment was pursued throughout the culture, cell triglyceride remained very low, and maximally represented less than 10 % of the control value at day 16 following confluence. On the contrary, phenelzine withdrawal from the culture medium at day 7 allowed 3T3-F442A cells to progressively recover a large part of their triglyceride content, though it remained significantly lower to that from control adipocytes.

In addition, we examined the impact on fully mature adipocytes (day 8 after confluence) of a 8-day treatment with various concentrations of the drug (1-100 µM). Under these culture conditions, phenelzine displayed no significant effect on cell triglyceride content (supplemental Table 3).

Similar effects of phenelzine were observed on three other models of adipogenesis, *i.e.* the murine 3T3-L1 white preadipose cell line, primary cultures of stromal vascular fraction derived from human subcutaneous abdominal adipose tissue, and the murine brown preadipose T37i cell line. For 3T3-L1 cells, triglyceride accumulation was markedly reduced by 100 µM phenelzine at day 6 following confluence, reaching a maximal 80 % reduction at day 9 (Figure 3A). For primary cultures of human preadipocytes cultures in a chemically defined medium, phenelzine induced a dose-dependent reduction in oil red O staining (Figure 3B), that was seen from 3 µM and was maximal between 10 and 30 µM. The effect of the drug on lipid accumulation was also illustrated by the dramatic decrease in the number and size and lipid droplets (Figure 3B). Finally, we also examined the influence of phenelzine during the course of differentiation of the brown preadipocyte T37i cell line (Zennaro et al., 1998). Phenelzine induced a dramatic and dose-dependent decrease in triglyceride storage of T37i cells (Figure 3C). In addition, the drug provoked a marked dose-dependent down-regulation of UCP1 (Figure 3C).

Taken together, the observations drawn from four distinct models of adipogenesis indicate that phenelzine strongly inhibits the adipocyte differentiation process, and has a preferential effect on cell triacylglycerol accumulation.

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Mechanisms involved in phenelzine effects on lipid accumulation and adipose conversion

We then attempted to elucidate the molecular mechanisms by which phenelzine altered adipose conversion and fat storage.

We first ensured that phenelzine did not exert a direct cytotoxic effect. Microscopic analysis did not reveal any detectable cell lysis, whatever the cultured cell model. Moreover, cell viability was assessed in 3T3-F442A cells by the MTT assay and indicated that cell exposure to 100 µM phenelzine for 8 days did not induce cytotoxicity (100 $\pm$ 2.9 % and 106.2 $\pm$ 2.8 % in control and phenelzine-treated cells, respectively; n=16). This was confirmed by Trypan blue exclusion test (not shown).

Phenelzine effects on adipocyte differentiation could implicate differences in cell proliferation rates. [^{3}H]-thymidine incorporation assays show that phenelzine exposure (100 µM) for 48 h during clonal expansion slightly decreased cell proliferation by 30 % (supplemental table 4). In addition, the same experiment performed in preconfluent cells did not reveal a significant phenelzine effect during the exponential growth phase.

The inhibitory effect of phenelzine on cell fat accumulation could be related to an activation of triacylglycerol hydrolysis, *i.e* lipolysis. Hence, mature 3T3-F442A adipocytes were treated for 48 h with 100 µM phenelzine, and lipolysis was tested under basal conditions or in response to an optimal concentration of the beta-adrenoceptor agonist (-)-isoproterenol (10 µM) or of the adenylyl cyclase effector forskolin (10 µM). As shown in Figure 4A, phenelzine did not modify basal lipolysis. However, it induced an almost 2-fold reduction in (-)-isoproterenol- or forskolin-stimulated glycerol production.

Another hypothesis was that phenelzine could alter glucose availability, leading to a decreased fatty acid and triglyceride synthesis. Thus we examined the influence of phenelzine on basal and insulin-stimulated glucose transport of 3T3-F442A adipocytes. Neither basal nor maximal insulin-activated [^{3}H]DOG transport were affected by a prior exposure to phenelzine (Figure 4B). In addition, the antidepressant did not modify the EC_{50} values of insulin for stimulating [^{3}H]DOG uptake.

It was questionable if phenelzine effect on cell triglyceride accumulation and adipocyte differentiation could be related to its inhibitory properties on amine oxidase activities. For this

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purpose, we compared the potencies of various amine oxidase inhibitors to modulate cell triglyceride content. While pargyline and semicarbazide are MAO- and SSAO-selective inhibitors, respectively, iproniazide, hydrazine, and phenelzine inhibit both MAO and SSAO activities. Drugs were used at a concentration of 100 µM, since it has been shown that it maximally inhibits the related amines oxidase activity(-ies) (Mercier et al., 2003; Moldes et al., 1999). Iproniazide did not display a significant effect on cell lipid accumulation (Figure 5). Pargyline, semicarbazide and hydrazine only provoked a weak but significant decrease in lipid fat stores. Thus, there was no clear relationship between the pharmacological properties of a given compound on specific amine oxidase activities, and its capacity to modulate adipocyte lipid storage.

We also investigated the influence of phenelzine on the intimate control of the adipocyte differentiation program. We tested the variations of the key adipogenic transcription factors PPAR-γ, C/EBP-α and SREBP-1c following a 8-day exposure from confluence to various drug concentrations (1-100 µM) (Figure 6A). PPAR-γ expression was affected only at the two highest phenelzine concentrations (30 and 100 µM), with a maximal 50 % decrease. Likewise, C/EBP-α was down-regulated at 30 and 100 µM. Of interest, the decrease in the immature large form of SREBP-1c was observed from 10 µM phenelzine. The reduction in the transcriptionnally active mature form of SREBP-1c was even detectable from 3 µM, with virtually no expression from 30 µM. As expected, the down-regulation in SREBP-1c expression induced a clear reduction of the lipogenic enzymes FAS and ACC, whose genes represent direct targets for the SREBP-1c transcription factors. As an internal control, no significant change in the p42/p44 ERK1/2 was detected in the presence of phenelzine. Moreover, phenelzine caused a clear dose-dependent decrease in SREBP-1c mRNA steady state levels, with a profile close to that of protein expression (Figure 6B). However, there was only about a maximal 3-fold reduction in SREBP-1c mRNA abundance, thus raising the possibility of translational or post-translational regulations of the transcription factor by the compound. As expected, the drug induced a reduction in FAS expression, whose gene represents a typical target gene of SREBP-1c (Figure 6 A and B).

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We also tested if the inhibitory effect of phenelzine on cell triglyceride accumulation could be circumvented by directly providing exogenous fatty acids to the cells. Confluent 3T3-F442A cells were cultured with or without phenelzine, and in the absence or in the presence of Intralipid (100 mg/L) as an exogenous source of fatty acids (Figure 7). As expected, Intralipid alone provoked a moderate increase in cell triglyceride content. Overall, the phenelzine-induced decrease in cell triglycerides was prevented in the presence of Intralipid. Interestingly, Intralipid could not restore the inhibitory effect of the drug on G3PDH activity (Figure 7B). These results suggest that while the phenelzine-induced blockade of the lipogenic pathway has likely a key role in the decrease in fat strores, other drug-mediated events also contribute to the alterations in the adipose conversion process.

We then examined if the phenelzine-induced down-regulation in SREBP-1c expression was responsible, at least in part, for the reduction in cell triglyceride content. The ability of an adenovirus-driven expression of SREBP-1c to prevent the effects of phenelzine on cell lipid storage was investigated in 3T3-F442A cells. As observed previously in non infected cells, phenelzine caused in 3T3-F442A cells infected with the Ad-null vector a dramatic and dose-dependent decrease in triglyceride accumulation (Figure 8 A and B), G3PDH activity (Figure 8C), and adiponectin and FAS expression (Figure 8D). In cells infected with Ad-SREBP-1c DP cultured without phenelzine, and in agreement with previous works (Le Lay et al., 2002), there was a slight but not significant increase in lipid vacuoles and triglyceride accumulation (Figure 8 A and B), that could be related to the induction of the lipogenic pathway reflected by the induction of FAS expression (Figure 8D, lane 2 compared to lane 1). When cells were exposed to the maximal dose of 100 µM phenelzine, infection with Ad.SREBP-1c only weakly reversed the drug effects on the morphological and biochemical markers of adipocyte differentiation. By contrast, when cells were treated with the intermediate concentration of 50 µM, adenofection with Ad.SREBP-1c DP prevented the main part of the inhitory effect of phenelzine on the adipose conversion process.

We also examined the possibility that the down-regulation of the “master” adipogenic transcription factor PPAR-γ could exert a key role in mediating phenelzine effects. In a first set of experiments, we attempted to reverse phenelzine-induced repression of several adipocyte differentiation markers by a parallel supplementation from confluence with the PPAR-γ agonist

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troglitazone. As shown in Figure 9A, concomitant exposure of 3T3-F442A differentiating cells to phenelzine and troglitazone did not restore the expression of ACC, C/EBP-α, and adiponectin proteins. This was associated with a similar pattern of morphological and biochemical parameters (not shown). However, it was conceivable that the absence of troglitazone efficiency to reverse phenelzine action was due to the decreased expression levels of PPAR-γ in the presence of the drug. We thus investigated in a second set of experiments the influence of a chronic phenelzine exposure on MEF-PPAR-γ stably expressing the PPAR-γ nuclear receptor. In these cells, adipose conversion could be amplified by thiazolidinedione exposure, as assessed by oil red O staining and the induction FAS, ACC, C/EBP-α, and adiponectin expression in response to pioglitazone (Figure 9B). However, even in MEF-PPAR-γ cells, phenelzine still dramatically down-regulated adipose conversion, a phenomenon that could not be reversed by pioglitazone.

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DISCUSSION

MAO inhibitors represent important pharmacological tools to treat severe depressions. However, these compounds are also known to exert significant side effects on weight gain and feeding behaviour. Though it is generally considered that these metabolic complications are essentially related to the central effects of MAO inhibitors, it must be emphasized that these antidepressants can also inhibit MAO activity in peripheral tissues, including key players of energy balance such as liver, skeletal muscle, and adipose tissue. Moreover, several MAO inhibitors can also inhibit other amine oxidases. Although use of phenelzine is limited to severe and resistant depression, this compound is a prototypical drug, since it potently inhibits both MAO and SSAO activities (Lizcano et al., 1996). It is thus conceivable that the metabolic side effects of several MAO inhibitors reflect both their central effects on food intake or energy expenditure, and a direct targeting of peripheral tissues such as the main tank of energy stores, white adipose tissue. In our work, we document for the first time that an antidepressant of the MAO-inhibitor family, phenelzine, potently alters adipose tissue development in several distinct cellular models of adipocyte differentiation. Slight differences between these cellular models exist in the potency of phenelzine to repress triacylglycerol accumulation during adipose conversion. While T37i seem more resistant to phenelzine effect, low concentrations of the drug (1-10 µM) already reduce cell triglyceride content in 3T3-F442A cells and primary cultures of human preadipocytes. These concentrations are compatible with therapeutic plasma phenelzine concentrations (Lichtenwalner et al., 1988; Lichtenwalner et al., 1995; Waring and Wallace, 2007). Nevertheless, in order to obtain a clear cut effect, studies of the mechanisms that underlie phenelzine effects during adipogenesis were performed at high drug concentrations (30-100 µM).

Surprisingly, while phenelzine does not influence basal lipolysis, the antidepressant inhibited rather than stimulated isoproterenol- and forskolin-stimulated lipolysis. Thus, an increased lipolytic activity was not involved in phenelzine-induced down-regulation in cell triglyceride content. Though elucidation of the mechanisms responsible for this modulation of effector-stimulated lipolysis is beyond the scope of our study, it will be of interest to determine which step(s) of the catecholamine-sensitive pathway is targeted by the drug, including hormone sensistive lipase and adipocyte

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triglyceride lipase (Langin, 2006). Whatever the mechanism involved in the modulation of lipolysis by phenelzine, it cannot account for the limitation of triacylglycerol accumulation in differentiating preadipocytes. Finally, we also excluded the possibility that phenelzine could reduce basal and insulin-stimulated glucose transport, resulting in decreased substrate availability for *de novo* fatty acid synthesis. This finding is in agreement with previous data showing that basal and insulin-stimulated glucose transport is not altered in adipocytes obese Zucker rats receiving phenelzine or other amine oxydase inhibitors (Carpene et al., 2008; Carpene et al., 2007; Prevot et al., 2007).

Many of our experimental data converge to suggest that phenelzine markedly alters the adipocyte differentiation program, and has a preferential inhibitory effect on the lipogenic pathway. Interestingly, phenelzine effects on cell triacylglycerol accumulation are detectable at lower concentrations than those required to down-regulate adipocyte differentiation, as assessed by well-characterized markers of adipose conversion, aP2 or adiponectin. The concentration gap between these two biological effects, together with the exclusion of the mechanisms mentioned above, suggest that at low or intermediate phenelzine concentrations, phenelzine preferentially acts through its anti-lipogenic properties. In line with this, phenelzine effects on cell triglyceride stores can be completely prevented when the lipogenic pathway is by-passed by directly providing fatty acids to the cells. The potent phenelzine-induced down-regulation of SREBP-1c, the key transcription factors that drives the emergence of the lipogenic pathway, is also in agreement with a privileged alteration in *de novo* fatty acid synthesis from glucose. In the basal state, SREBP-1c is associated with endoplasmic reticulum. When activated, it processes to the Golgi apparatus where it undergoes a proteolytic cleavage that allows to translocate to the nucleus to modulate the transcription rate of its target genes. Noteworthy, we observed that the phenelzine-induced decrease in the nuclear mature 68 kDa SREBP-1c protein was detectable at lower concentrations that those decreasing the immature high molecular weight form. Though phenelzine-induced SREBP-1c down-regulation could be the consequence of the decrease of the related mRNA levels, we cannot exclude the possibility that the antidepressant also modulates the proteolytic maturation and/or nuclear translocation of this transcription factor. Elucidation of the transcriptional or post-transcriptional mechanisms that underlie phenelzine-induced SREBP-1c decrease represents another issue. Finally, the involvement of SREBP-1c down-regulation

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by phenelzine in the prevention of cell triacylglycerol accumulation is also largely supported by the observation that at a submaximal drug concentration, adenovirus-driven expression of SREBP-1c restores triglyceride stores and expression of adipose conversion biochemical and molecular markers.

At the highest concentrations (30-100 μM), phenelzine also alters the magnitude of the adipocyte differentiation process, as reflected by the decrease in the levels of the two major adipogenic transcription factors, PPAR-γ and C/EBP-α. It was thus conceivable that PPAR-γ, generally considered as the master effector of adipogenesis (Lehrke and Lazar, 2005), could play a pivotal role in the anti-adipogenic effects of phenelzine. However, two complementary approaches do not support this view. First we show that troglitazone, a PPAR-γ selective agonist, does not prevent phenelzine effects on adipocyte differentiation. Though the significant residual PPAR-γ expression following phenelzine exposure suggests that thiazolidinediones can remain efficient, troglitazone was unable to antagonize the anti-adipogenic properties of the antidepressant. However, one could raise the proviso that the absence of thiazolidinedione efficiency to counteract phenelzine effects in 3T3-F442A adipocytes could be related to the lower PPAR-γ expression levels induced by the antidepressant. Nevertheless, thiazolidinediones were also unable to block the anti-adipogenic effects of the drug in MEF that constitutively express PPAR-γ. Thus, it seems unlikely that alterations in PPAR-γ expression and/or function could represent a key mechanism that mediates phenelzine action.

Adipogenesis is associated with the induction of numerous secreted factors, named adipokines, some of which influence energy balance and insulin sensitivity. Adiponectin is thus a major adipokine with insulin-sensitizing and anti-inflammatory properties. Experimental and clinical investigations converge to demonstrate that obesity-associated adiponectin deficiency could be involved in metabolic and cardiovascular complications (Kadowaki and Yamauchi, 2005). Of interest, phenelzine strongly down-regulates adiponectin expression. It it thus tempting to speculate that modulation of adiponectin could represent an important mechanism for long term side effects of antidepressants.

Interestingly, and in contrast to preadipocytes, mature adipocytes are unsensitive to the lipid-depleting effects of phenelzine. A putative explanation for the differential efficiency of phenelzine between preadipocyte and adipocyte could also reflect differentiation-dependent variations in drug metabolism. Phenelzine is extensively metabolized primarily by oxidation *via* MAO. Since expression

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and activity of MAO is induced during adipogenesis (Bour et al., 2007), one could suggest that the degree of fat cell differentiation may influence the magnitude of cell response to phenelzine. Alternatively, one can point out that in adipocytes, phenelzine has apparent opposite effects on key mechanisms that govern homeostasis of triacylglycerol storage. On one hand, phenelzine inhibits adipocyte differentiation and the lipogenic pathway, thus preventing cell triglyceride accumulation. On the other hand, the antidepressant reduced catecholamine- or forskolin-stimulated lipolysis, a phenomenon that promotes cell lipid engorgment. In addition, through UCP1 down-regulation, phenelzine could promote an anti-thermogenic effect. These findings illustrate that the drug effects on adipocytes are complex and non univocal. Thus the net effect of phenelzine on adipocyte triglyceride accumulation likely involves a subtle balance between the anti-adipogenic, anti-lipogenic, and anti-lipolytic effects of phenelzine at various stages of adipose conversion. It will be a major challenge to investigate in vivo the exact impact of phenelzine on adipose tissue development and metabolism.

Since it is generally considered that the differentiation and proliferation processes are inversely correlated, some phenelzine effects could involve an increased proliferation rate of preadipocytes. However, phenelzine did not induce cell proliferation. By extension, the development of fat mass generally observed in patients under phenelzine treatment is unlikely related to a direct effect of the drug to expand the population of adipocyte precursors.

Body weight gain is generally considered to be more frequent in patients treated with tricyclic antidepressants than with selective serotonin reuptake inhibitors (Vieweg et al., 2008; Zimmermann et al., 2003). MAO inhibitors, including phenelzine, are also rather associated with weight gain to an extent comparable to tricyclic antidepressants even if the contribution of increased adiposity remains unknown (Balon et al., 1993; Remick et al., 1989). This apparent discrepancy between clinical observation and our experimental data must be analyzed cautiously. As mentioned above, phenelzine effects on adipocyte metabolism are not univocal: while this compound largely prevents lipid accumulation during the adipocyte differentiation process, it displays anti-lipolytic properties in mature adipocytes. In addition, as regards to the wide tissue distribution of amine oxidases, phenelzine could also impact the function of other organs or tissues that have pivotal roles in the regulation of energy homeostasis. Since amines exert an essential role in the regulation of food intake (McIntyre et

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al., 2001), some phenelzine effects on energy homeostasis are likely mediated by targeting the central nervous system. In a more general manner, the net effect of a psychotrop on body weight and adiposity probably reflects the balance between central and peripheral effects, and involves phenomenons that prevent or promote energy storage. Identification of the direct peripheral effects of these compounds on liver, skeletal muscle, pancreas and adipose tissue certainly represents a major challenge to reduce the metabolic side effects of these largely prescribed drugs.

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FIGURE LEGENDS

Figure 1: Dose-dependent and time-dependent inhibitory effect of phenelzine on 3T3-F442A differentiation. Confluent 3T3-F442A cells (day 0) were cultured for 8 days in the absence or in the presence of various concentrations of phenelzine. At day 8, cells were stained with oil Red O and photographed macroscopically (**A**) and under microscopy (**B**). **C,** at day 8 cell triglyceride content was tested. Results represent the mean ± S.E. of 8 separate determinations and are expressed as the percentage of the control value (mean 0.34 g/L). **D,** at day 8 cell extracts were prepared and aP2 and adiponectin protein expression was tested by Western blot analysis. **E,** 3T3-F442A cells were cultured from confluence (day 0) in the absence or in the presence of phenelzine (100 µM). At day 2, 4, 6 and 8 cell triglyceride content was tested. **F,** Cytosolic extracts were prepared in parallel, and G3PDH activity was measured. Results represent the mean ± S.E. of 4 separate determinations, and are expressed as the percentage of the control value at day 8 (0.52 g/L and 566 nmol/min/mg for triglyceride content and G3PDH activity, respectively).

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, phenelzine-treated *versus* control.

Figure 2: Spontaneous reversibility of phenelzine effect on 3T3-F442A adipocyte differentiation. Confluent 3T3-F442A cells (day 0) were cultured in the absence or in the presence of phenelzine (100 µM). At day 7, in cells previously exposed to phenelzine, the compound was either omitted (PZ-) or maintained (PZ+). At day 7, 9, 13 and 16, cell triglyceride content was assayed. Results represent the mean ± S.E. of 5-6 separate determinations, and are expressed as the percentage of the control value at day 16 (mean 2.18 g/L). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, phenelzine-treated *versus* control cells, whatever the sequence to drug exposure; °°°, $p < 0.001$, (PZ-) cells as compared to (PZ+) cells.

Figure 3: Phenelzine inhibits differentiation of other preadipocyte models. A, confluent 3T3-L1 cells were cultured from (day 0) in the absence or in the presence of phenelzine (100 µM). Cell triglyceride content was tested at day 3, 6 and 9. Results represent the mean ± S.E. of 4-5 separate

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determinations, and are expressed as the percentage of the control value at day 9 (mean 0.37 g/L). **B,** human preadipocytes were cultured in the absence or in the presence of various concentrations of phenelzine. At day 8 in a chemically defined medium, cells were photographed macroscopically after staining with oil Red O (B-1) and under microscopy without any coloration (B-2). **C,** the murine brown preadipose T37i line was cultured in the absence or in the presence of phenelzine (100 µM). At day 7 cells were stained with Oil Red O and photographed macroscopically, tested for triglyceride content (mean control value 0.81 g/L), and UCP1 expression. **, $p < 0.01$; ***, $p < 0.001$, phenelzine-exposed *versus* control cells.

Figure 4: Effect of phenelzine on lipolysis and glucose uptake in mature 3T3-F442A cells. A, day 8 post-confluent 3T3-F442A cells were cultured in the absence or in the presence of phenelzine (100 µM) for 48 h. Lipolysis was measured by determination of glycerol release in the culture medium under basal conditions or in response to an optimal concentration of (-)-isoproterenol (10 µM) or forskolin (10 µM). Results represent the mean $\pm$ S.E. of 6 separate determinations, are expressed as the percentage of the basal control value (mean 65.2 µM). **B,** day 8 post-confluent 3T3-F442A cells were cultured in the absence or in the presence of phenelzine (100 µM) for 48 h. [^{3}H]DOG uptake was measured under basal conditions or in response to various concentrations of insulin. Results represent the mean $\pm$ S.E. of 4 separate determinations, and are expressed as the percentage of the control value (mean 35 pmol/min/well). §, p=0.052; *, $p < 0.05$, phenelzine-exposed *versus* control cells.

Figure 5: Effect of chronic exposure various amine oxidase inhibitors on triglyceride accumulation of differentiating 3T3-F442A adipocytes. Confluent 3T3-F442A cells (day 0) were cultured for 8 days in the absence or in the presence of the indicated amine oxidase inhibitor (100 µM). Cell extracts were prepared and assayed for triglyceride content. Results represent the mean $\pm$ S.E. of 10 separate determinations, and are expressed as the percentage of the control value (mean 0.72 g/L). *, $p < 0.05$; ***, $p < 0.001$, drug-exposed *versus* control cells.

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Figure 6: Dose-dependent inhibitory effect of phenelzine on protein expression and mRNA levels of adipogenesis markers in 3T3-F442A cells. Confluent 3T3-F442A cells (day 0) were cultured for 8 days in the absence or in the presence of various concentrations of phenelzine. **A,** at day 8 cells extracts were prepared and PPARγ, C/EBPα, SREBP-1c (mature and immature forms), FAS, ACC, and ERK1/2 protein expression was tested by Western blot analysis, using specific antibodies. **B,** at day 8 total RNA was prepared, and SREBP-1c and FAS mRNA levels were determined by real time RT-qPCR analysis and are normalized to the 18S RNA levels. Results represent the mean ± S.E. of 4-6 separate determinations, and are expressed as the percentage of the control value.*, $p < 0.05$; ***, $p < 0.001$, phenelzine-exposed *versus* control cells.

Figure 7: Fatty acids prevent the inhibitory effect of phenelzine on triglyceride accumulation, but not on G3PDH activity. Confluent 3T3-F442A cells (day 0) were cultured for 8 days in the absence or in the presence of phenelzine (30 μM), and in the absence or in the presence of Intralipid (100 mg/L). At day 8 following confluence cell extracts were prepared and tested for triglyceride content (**A**) and G3PDH activity (**B**). Results represent the mean ± S.E. of 5-8 separate determinations, and are expressed as the percentage of the control value (mean 0.79 g/L and 2026 nmol/min/mg for triglyceride content and G3PDH activity, respectively). *, $p < 0.05$; **, $p < 0.01$, phenelzine-exposed *versus* control cells.

Figure 8: Adenoviral expression of a constitutively active SREBP-1c isoform reverses phenelzine effects on the phenotype of 3T3-F442A differentiating cells. At confluence, 3T3-F442A cells were infected either with Ad.null or with Ad.SREBP-1c DP, then immediately refed in a fresh differentiation medium in the absence or in the presence of 50 or 100 μM phenelzine. Similar efficiency in viral infection was assessed by green fluorescent protein expression. Microscopic analysis (**A**), cell triglyceride content (**B**), G3PDH activity (**C**), and adiponectin and FAS expression (**D**) were analyzed at day 7 following confluence. Results represent the mean ± S.E. of 4 separate determinations, and are expressed as the percentage of the control value (0.23 ± 0.02 g/L and 2212 ± 282 nmol/min/mg protein for triglyceride content and G3PDH activity, respectively). *, $p < 0.05$; **, p

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< 0.01: ***, $p < 0.005$, *versus* control value (Ad.null without phenelzine) ; #, $p < 0.05$; ##, $p < 0.005$, phenelzine-exposed and Ad. SREBP1c DP *versus* phenelzine-exposed and Ad. Null.

Figure 9: PPAR-γ is not a primary target of phenelzine-induced inhibition of adipose conversion

(**A**) Confluent 3T3-F442A cells were cultured in the absence or the presence of phenelzine (30 or 100 µM), and in the absence or the presence of 10 µM troglitazone. At day 8 following confluence, cells extracts were prepared and tested in Western blot analysis for ACC, C/EBP-α, and adiponectin expression. (**B**) Confluent MEF-PPAR-γ cells were cultured in the absence or the presence of 100 µM phenelzine, while pioglitazone was added either from confluence (day 0), or from day 4 post-confluence. At day 8 following confluence, cells were stained with oil red O, and protein extracts were prepared and tested in Western blot analysis for FAS, ACC, C/EBP-α, and adiponectin expression.

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FIGURE 1

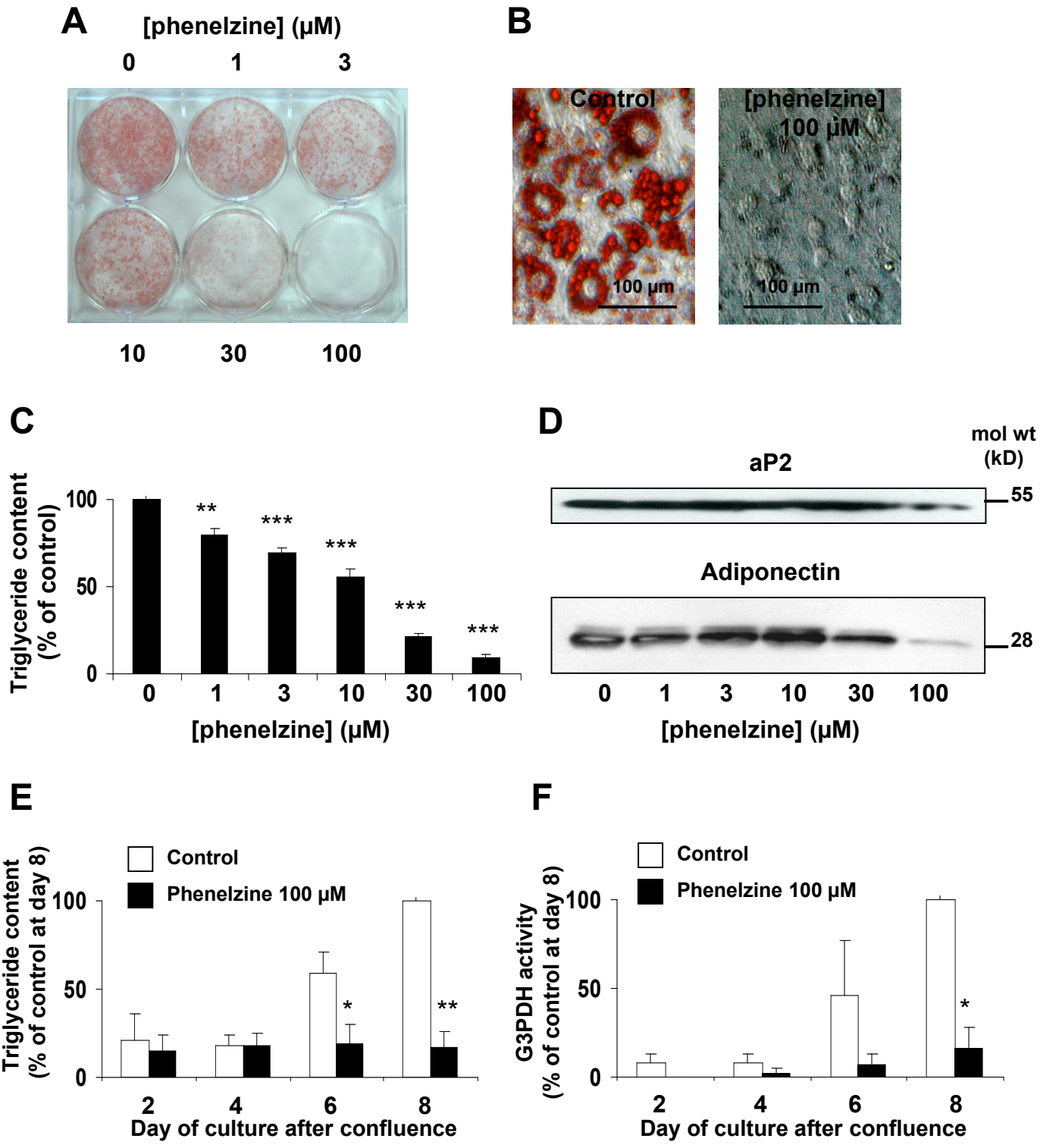

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FIGURE 2

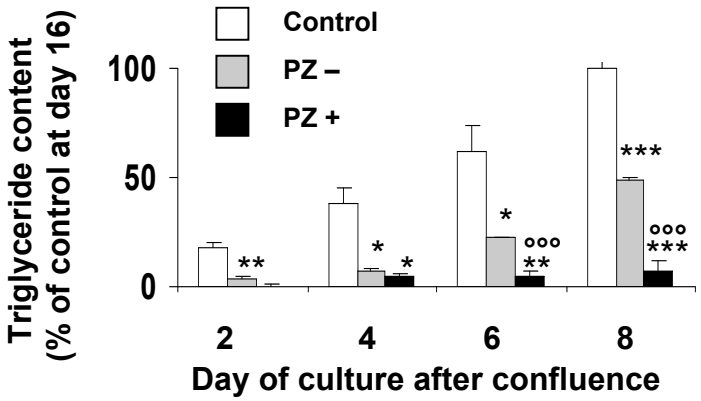

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FIGURE 3

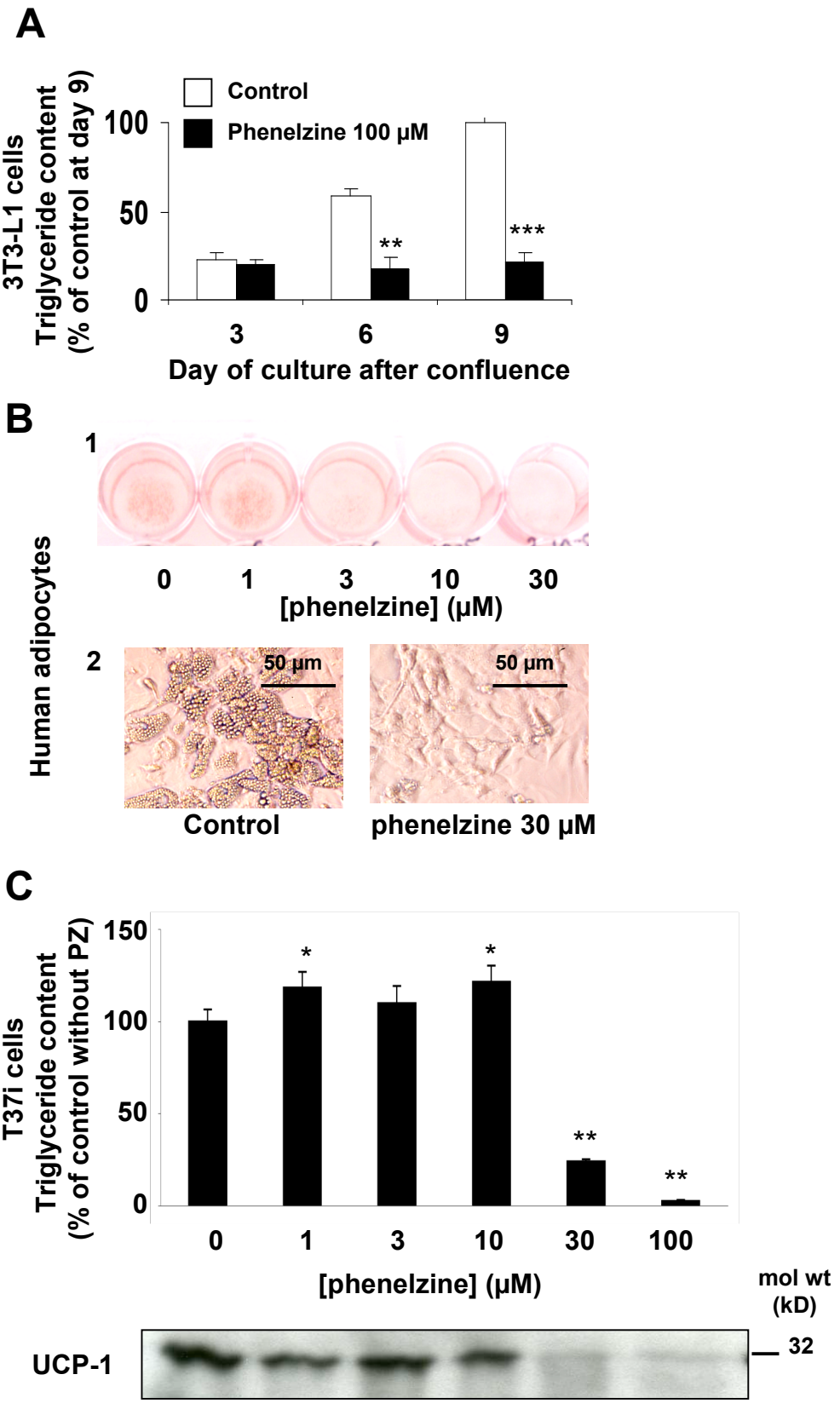

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FIGURE 4

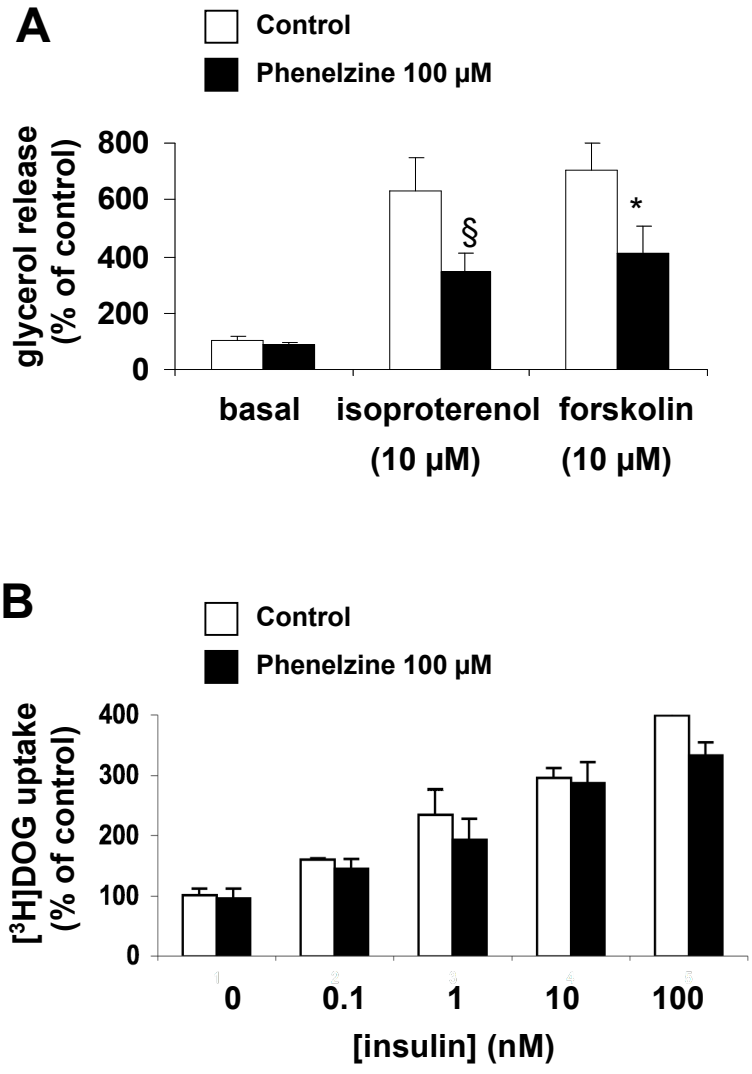

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FIGURE 5

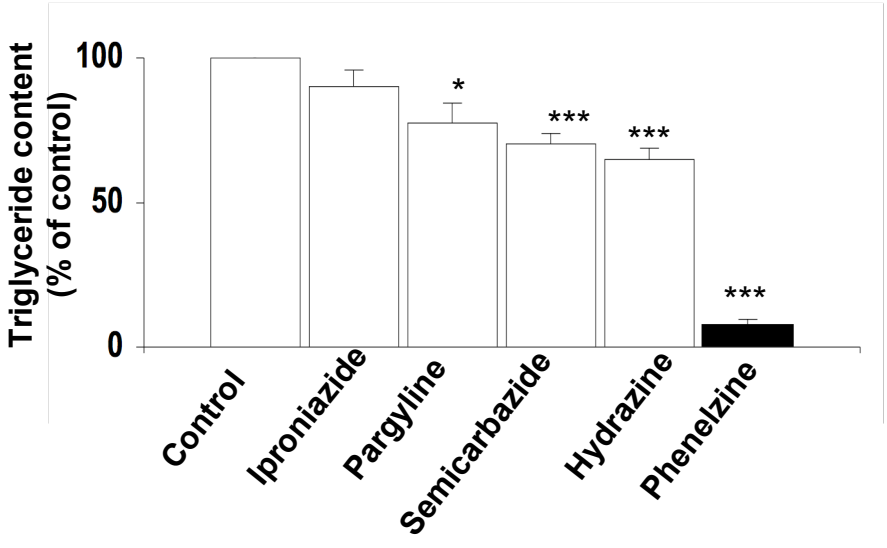

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FIGURE 6

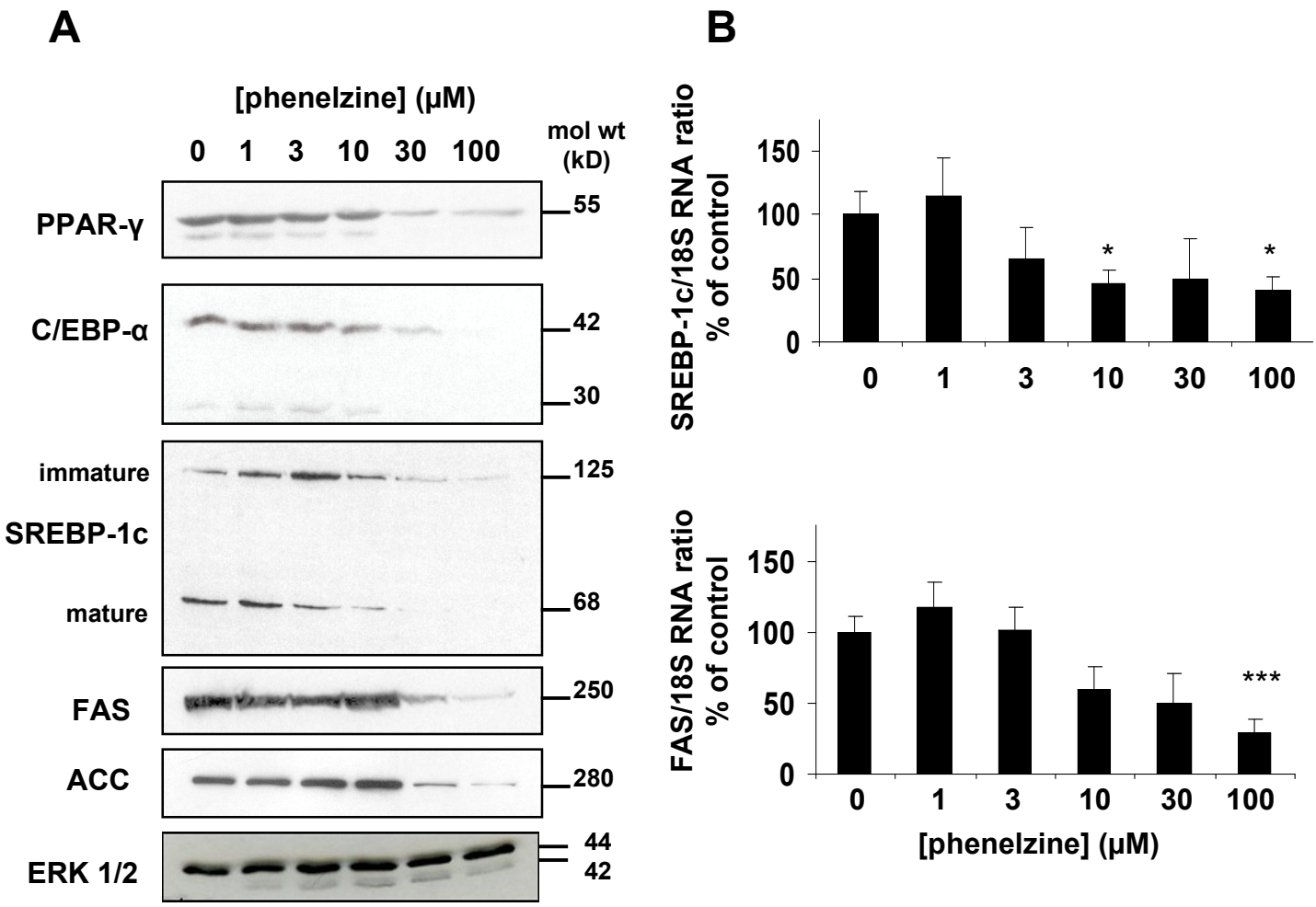

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FIGURE 7

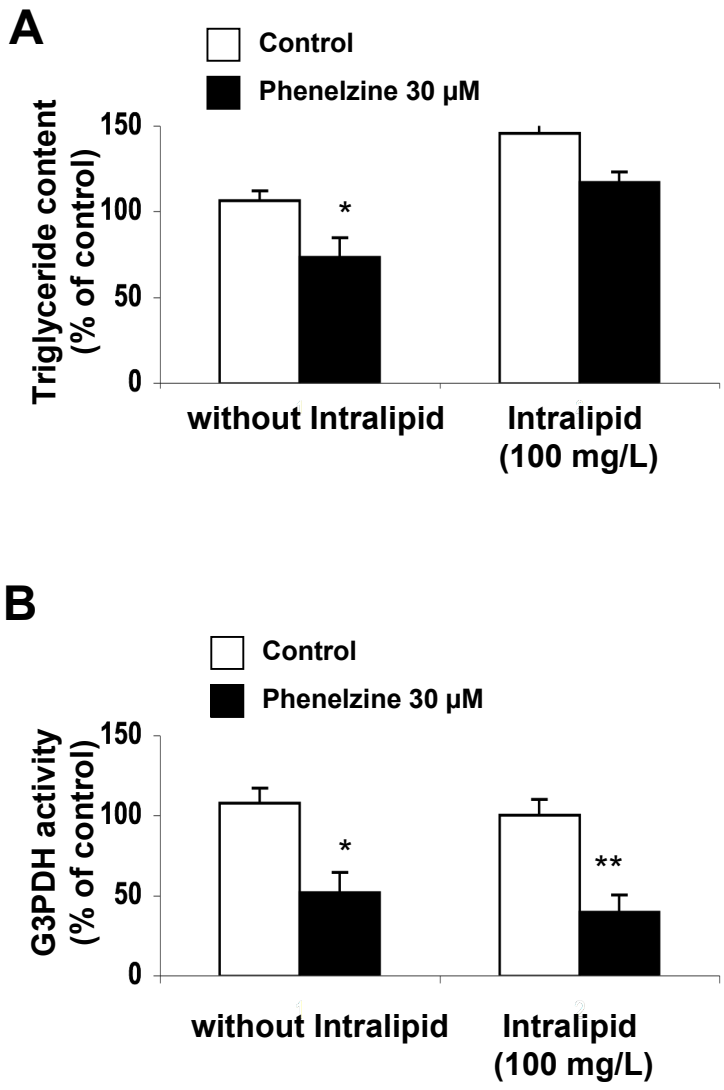
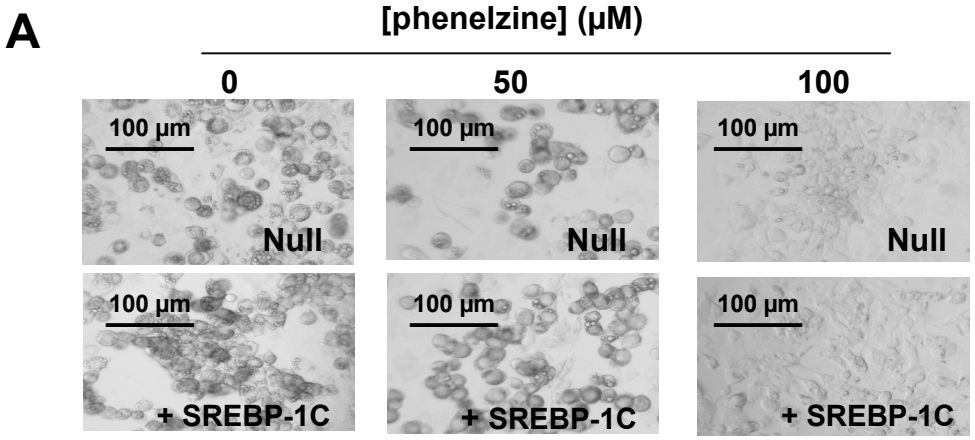
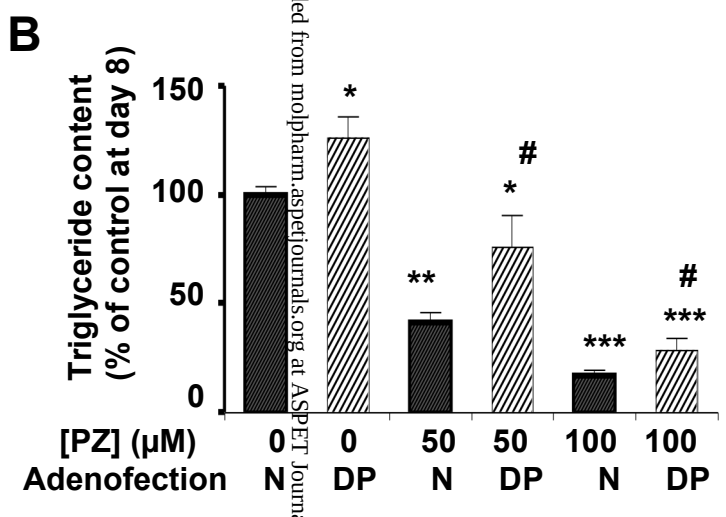
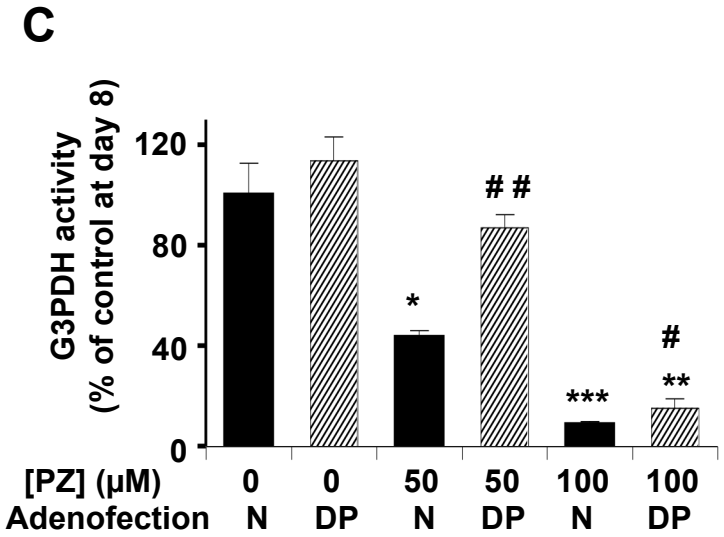
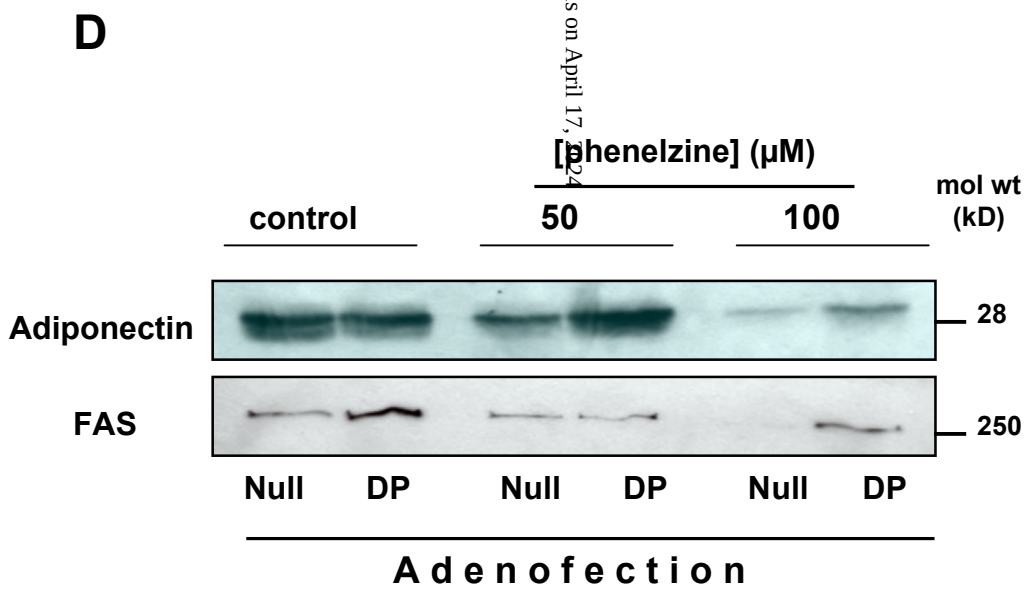


FIGURE 8









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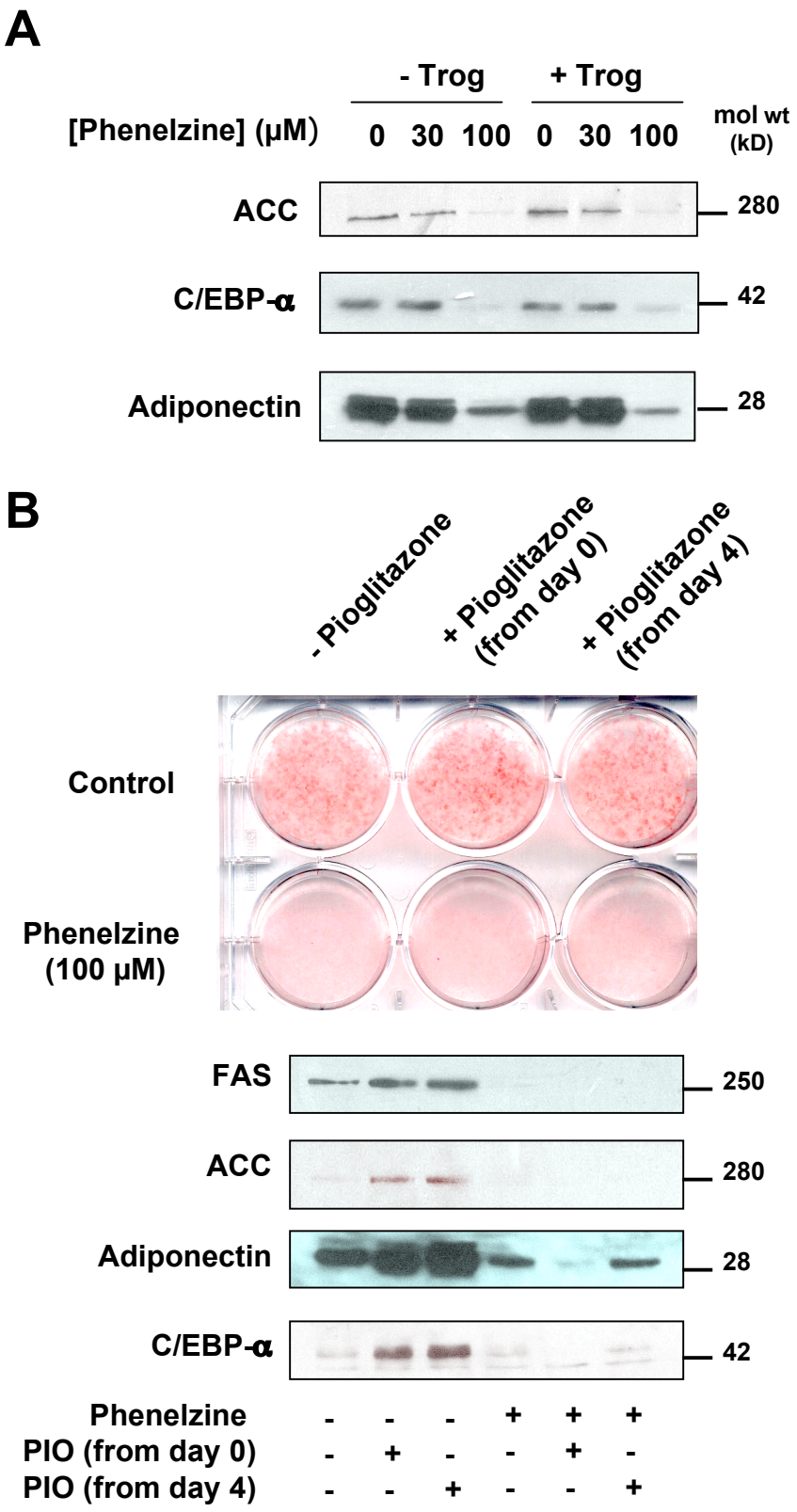
Figure 9