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In vivo effect of an antilipolytic drug (3,5′-dimethylpyrazole) on autophagic proteolysis and autophagy-related gene expression in rat liver

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Abstract

Autophagy is an intracellular pathway induced by starvation, inhibited by nutrients, that is responsible for degradation of long-lived proteins and altered cell organelles. This process is involved in cell maintenance could be induced by antilipolytic drugs and may have anti-aging effects [A. Donati, The involvement of macroautophagy in aging and anti-aging interventions, Mol. Aspects Med. 27 (2006) 455–470]. We analyzed the effect of an intraperitoneal injection of an antilipolytic agent (3,5′-dimethylpyrazole, DMP, 12 mg/kg b.w.), that mimics nutrient shortage on autophagy and expression of autophagic genes in the liver of male 3-month-old Sprague–Dawley albino rats. Autophagy was evaluated by observing electron micrographs of the liver autophagosomal compartment and by monitoring protein degradation assessed by the release of valine into the bloodstream. LC3 gene expression, whose product is one of the best known markers of autophagy, was also monitored. As expected, DMP decreased the plasma levels of free fatty acids, glucose, and insulin and increased autophagic vacuoles and proteolysis. DMP treatment caused an increase in the expression of the LC3 gene although this occurred later than the induction of authophagic proteolysis caused by DMP. Glucose treatment rescued the effects caused by DMP on glucose and insulin plasma levels and negatively affected the rate of autophagic proteolysis, but did not suppress the positive regulatory effect on LC3 mRNA levels. In conclusion, antilipolytic drugs may induce both autophagic proteolysis and higher expression of an autophagy-related gene and the effect on autophagy gene expression might not be secondary to the stimulation of autophagic proteolysis.

Keywords: 3,5′-Dimethylpyrazole; Antilipolytic drugs; LC3 gene expression; Autophagy; Autophagic proteolysis; Amino acid; Insulin; Glucose

Autophagy is the major catabolic pathway for long-lived proteins, cell organelles and is conserved from yeast to human [1]. Double membrane vesicles called autophagosomes enclose a portion of cytoplasm and deliver it to lysosomes for degradation and for eventual recycling [2]. Under stress stimuli, such as lack of nutrients or decreased lipolysis, autophagic proteolysis is triggered while nutrients and insulin were shown to act like negative regulators [3]. Autophagy has a key role in cell reshaping, maintenance, and repair in physiological and pathological conditions, including atrophy and hypertrophy [4], disease [5], and aging [6]. Antilipolytic drugs can induce autophagy [7], removal of altered mitochondria [8], and anti-aging effects [9].

In this study, we explored the effect of the induction of autophagy by the administration of 3,5'-dimethylpyrazole (DMP) and the suppression of DMP-induced effects by the injection of glucose on rat liver autophagic proteolysis and on LC3 expression. LC3 protein is one of the two ubiq-

Abbreviations: DMP, 3,5′-dimethylpyrazole; FFA, free fatty acids.

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uitin-like systems required for autophagosome formation and is currently the most commonly used maker to monitor autophagy [10,11].

Materials and methods

Animals. Groups of male Sprague–Dawley rats of 3 months of age were used. Animals were fed and watered ad libitum and raised on a 12 h/12 h photoperiod at 20–22 °C. By the age of 3 months, groups of five rats fed ad libitum, were fasted for 22 h and then injected with DMP (12 mg/kg p.c. in saline) intraperitoneally 2 h before sacrifice. The animals were sacrificed under nembutal anesthesia (50 mg/kg b.w., intraperitoneally) at 15, 30, 60, 90, and 120 min after the DMP injection in accordance with the italian law on the use of experimental rats, under veterinary control and approval by Local Bioethical Committee. Other groups were fasted for 22 h and injected with DMP, 2 h before sacrifice, and then given 2 g/kg b.w. glucose intraperitoneal, 42, 57, and 87 min after the DMP injection.

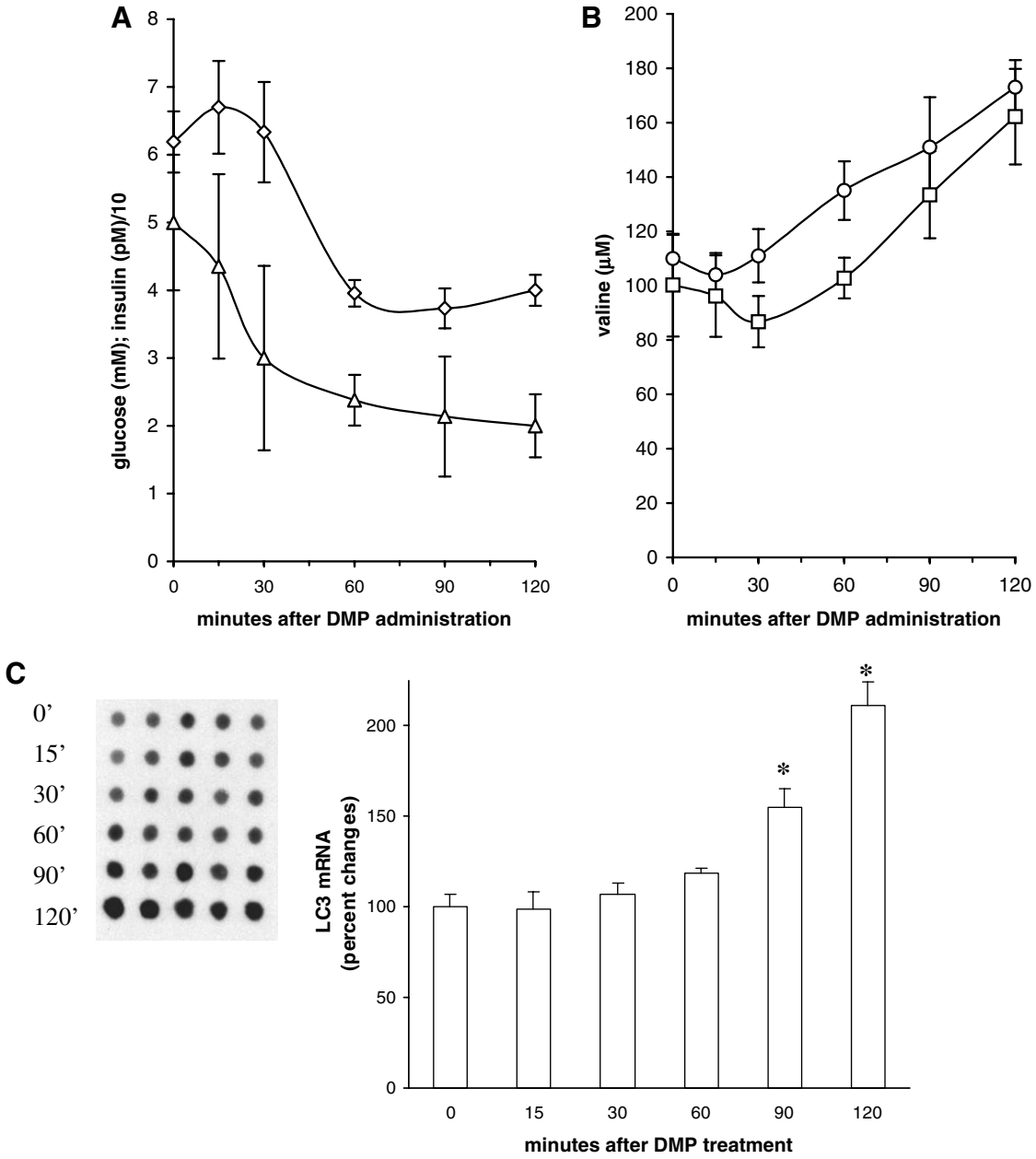

Fig. 1. Effects of DMP injection on plasma levels of glucose, insulin (A), valine (B), and on LC3 mRNA levels in the liver (C). (A) Effects of DMP on plasma levels of glucose (◇) and insulin (△). Statistical analysis (one-way ANOVA): The effect of DMP on plasma glucose was highly significant ($p < .0001$; Tukey test = 0, 15, 30 vs 60, 90, 120: p's $< .05$ all). The effect of DMP on plasma insulin was also highly significant ($p < .0002$; Tukey test = 0 vs 30, 60, 90, 120; 15 vs 90, 120: p's $< .05$ all). (B) Effects of DMP on the levels of valine in portal (○) and caval plasma (□). Statistical analysis (two-way ANOVA): the effect of DMP on both caval and portal plasma valine was highly significant ($p < .0001$; Tukey test = 0, 15, 30 vs 90, 120; 60 vs 120: p's $< .05$ all). The level of plasma valine was significantly higher in portal than in caval blood ($p < .01$). The interaction between the effect of DMP and the plasma source was not significant. (C) Effect of DMP on LC3 mRNA levels expressed as percent of the value at time 0. Dot-blot analysis (upper left) and relative expression levels (right) of the LC3 gene, in rats at time 0, 15, 30, 60, 90, and 120 min after DMP injection. Dots per time points are replicates of five distinct experiments. *Significant difference from gene expression at time 0 (Tukey test, $p < 0.05$).

Detection of glucose, insulin, and valine plasma levels. At the given time, blood samples were taken and levels of free fatty acids (FFA) and glucose in plasma were assayed by the acyl CoA synthetase/oxidase and the glucose oxidase/peroxidase techniques, respectively, using commercially available kits (FFA: Free Fatty Acids Half Microtest, Boehringer–Manheim KK; glucose: Glucinet, Sclavo ISVT). Plasma insulin was measured by radioimmunoassay using rat insulin as a standard [13]. Induction of autophagy was assessed by the valine assay in peripheral and liver blood as previously reported [14].

RNA extraction. Total RNA was extracted from 10 mg of liver, using a commercial Kit (Nucleospin, Macherey–Negel) according to the manufacturer's protocol. The RNA concentration in each sample was quantified spectrophotometrically (A260) and only preparations exhibiting a ratio A260/A280 > 1.8 were used.

RT-PCR. Total LC3 cDNA was obtained by retrotranscription reaction; 5 ng of total RNA from rat liver was used as a template for reverse transcription (RT) reaction, using Oligo-dT and Superscript II RT (First Strand System for RT-PCR, Invitrogen). Total cDNA was then subjected to Polymerase Chain Reaction (PCR) with the following primers pair: forward 5′-catgccgtccgagaagacct-3′; reverse 5′-cagaggcagctctcactgag-3′. The reaction conditions were: 94 °C for 2 min, 30 cycles at the following temperatures: 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. Additional 5 min at 72 °C was set for the final extension. LC3 PCR product was further tested by digestion with the endonuclease Pst-1 (Promega) according to the manufacturer's instruction.

Radiolabeling. In order to probe total RNA samples for LC3 expression, LC3 PCR product was radiolabeled with α32dCTP (Perkin-Elmer) in

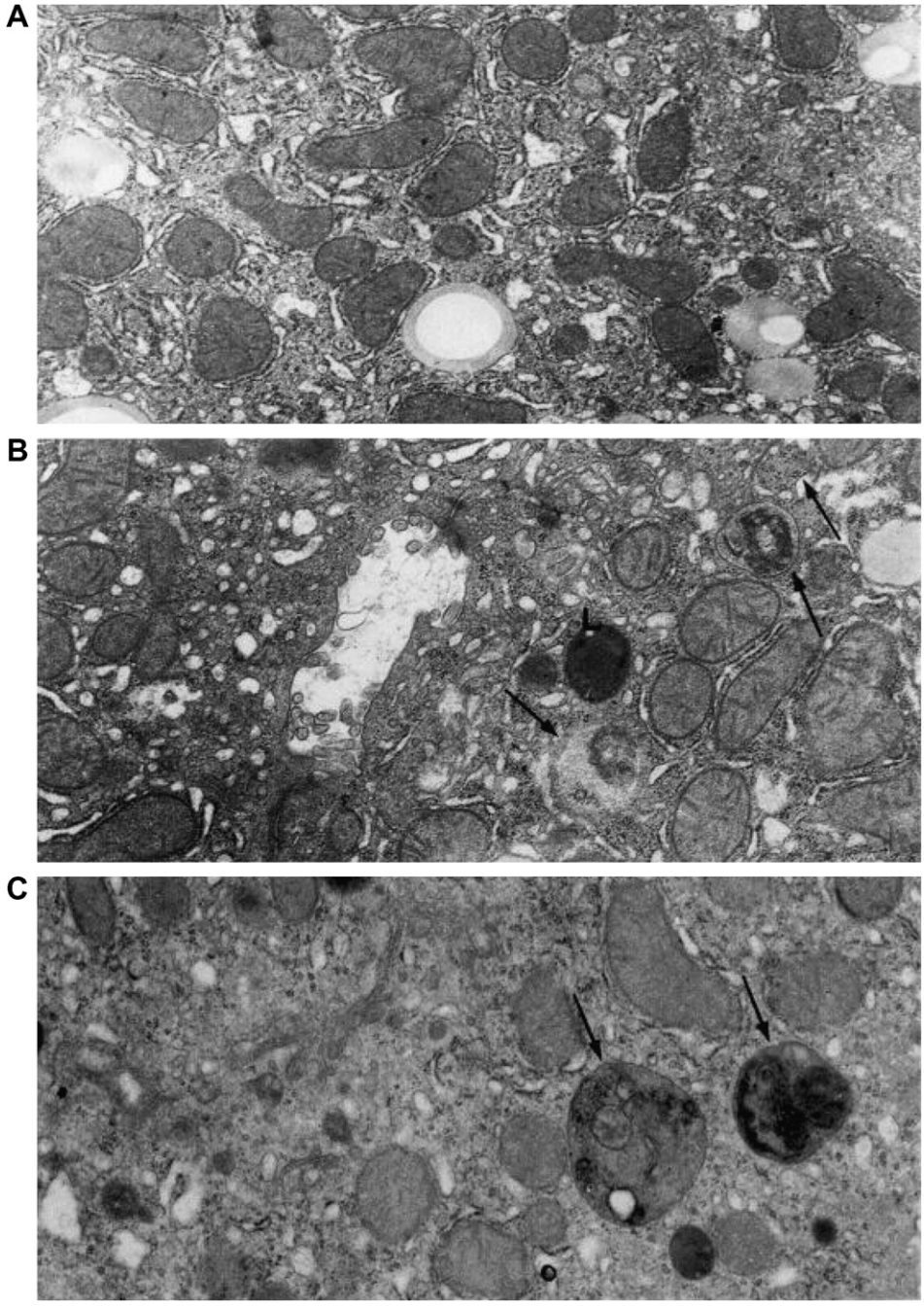

Fig. 2. Representative electron micrographs (18400×) of rat liver cells treated with DMP. Samples were taken at 0 (A), 60 (B), and 120 (C) minutes after DMP treatment. Black arrows show autophagosomal structures induced by DMP treatment at 60 and 120 min. Compare (A) to (B) and (C). L, lysosomes.

a transcription reaction using Megaprime DNA Labelling System kit (Amersham-Biosciences).

Dot-blot analysis. Total RNAs from each rat liver were spotted onto a positively charged nylon membrane in a Dot-blot apparatus (Bio-Rad). Five micrograms of RNA was dissolved in 20 μl of a mixture containing 50% formamide, 7% formaldehyde, and 1× SSC buffer [15] and denatured during 15 min at 68 °C. Samples were loaded onto the membrane and a gentle suction was applied through the manifold. The RNA was linked to the membrane by baking at 80 °C for 30 min and by UV light for 2 min. Membranes containing RNA were incubated in a prehybridization solution containing 50% unionized formamide, 5× SSC, 1× PE buffer [15], 5× Denharts, and 75 μg/ml Salmon Sperm DNA, for 24 h at 42 °C. Hybridization was performed at 42 °C for 24 h in a solution (50% unionized formamide, 5× SSC, 1× PE, 5× Denharts, and 75 μg/ml Salmon Sperm DNA) containing the LC3 radiolabeled probe (α32dCTP-LC3). To avoid background noise and get more stringent conditions, the membrane was rinsed at 68 °C in a low-saline solution (2× SSC + 0.1% SDS) for 20 min and exposed on X-Omat KodaK films for 3 days at −80 °C.

The images were captured, digitized and the intensity of the spots, resulting from the annealing of radiolabeled LC3 probe with total RNAs, was quantified using the software Scion Image.

Statistical analysis. ANOVA test was used for evaluation of the different multiple conditions (One- or two-way analysis of variance). If it was positive, Tukey–Kramer test was performed to determine statistic significance. $p < 0.05$ values were considered to be significant.

Table 1
Effect of glucose load (2 g/kg b.w.) on glucose and insulin plasma levels in control and DMP-treated rats

Time after DMP injection	Glucose (mM)				Insulin (pM)			
	CTRL		*DMP*		*CTRL*		*DMP*	
	–	*+GLU*	–	*+GLU*	–	*+GLU*	–	*+GLU*
0′	6.2 ± 0.36	6.0 ± 0.64	6.4 ± 0.41	5.9 ± 0.80	45 ± 8.5	52 ± 7.4	54 ± 11.2	49 ± 9.0
5′	5.9 ± 0.45	29.7 ± 0.82	6.1 ± 0.51	25.1 ± 0.70	54 ± 4.9	337 ± 14.0	29 ± 4.3	127 ± 17.2
10′	5.4 ± 0.40	27.8 ± 0.96	6.2 ± 0.66	25.3 ± 1.56	51 ± 8.6	366 ± 36.6	28 ± 8.9	156 ± 30.2
20′	5.8 ± 0.72	19.5 ± 3.65	5.8 ± 0.72	18.5 ± 1.80	67 ± 3.5	321 ± 47.4	28 ± 4.3	239 ± 19.4
60′	5.6 ± 0.28	9.7 ± 0.60	3.8 ± 0.32	8.3 ± 0.92	52 ± 8.6	104 ± 38.8	29 ± 8.6	86 ± 11.6

Decrease in plasma levels of insulin and glucose caused by DMP administration is prevented by a glucose load.

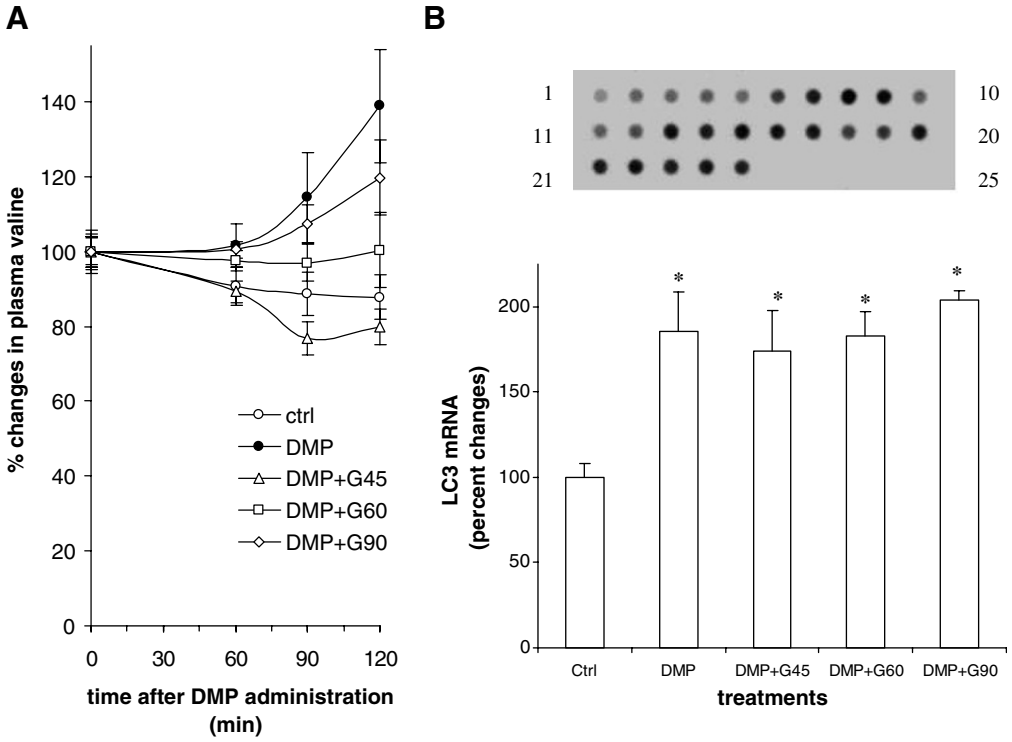

Fig. 3. Changes in valine plasma levels (A) and in LC3 gene expression (B) after the stimulation of autophagic proteolysis by the injection of DMP and the inhibition of stimulation by the injection of glucose. Glucose was administered at different time points: 42 (DMP + G45), 57 (DMP + G60), and 87 (DMP + G90) minutes after DMP administration. (A) Percent changes in caval valine plasma levels after the administration of DMP and glucose. Valine plasma levels (μM) at time 0 are: control 104 ± 4.8, DMP 114 ± 6.5, DMP + G45 105 ± 4.2, DMP + G60 105 ± 3.7, and DMP + G90 99 ± 4.0. Statistical analysis (two-way ANOVA): effect of treatments: $p < .0001$ (Tukey test = control vs DMP, DMP + G90; DMP vs DMP + G45; DMP + G60; DMP + G45 vs DMP + G60, DMP + G + 0: $p < .05$ all); effect of time after the DMP injection: not significant. Interaction between treatments and time: $p < .04$. (B) Effect of DMP and glucose on liver LC3 gene expression expressed as percent of the control. Dot-blot analysis (upper left) and relative expression levels (right) of LC3 gene. Total RNA is from animals treated with glucose at different time after DMP injection. Dots per treatment point are replicates of five distinct experiments. Spots 1–5: controls injected with physiologic solution, spots 6–10: injected only with DMP, spots 11–15: glucose at 42 min, spots 16–20: glucose at 57 min, spots 20–25: glucose at 87 min. *Significant difference from gene expression of control (Tukey test, $p < 0.05$).

Results

The administration of antilipolytic drugs to 3-month-old overnight fasted rats induced significant changes in plasma FFA (levels fell from 0.45 to 0.10 mM in less than 15 min, not shown), glucose (from 6.7 to 3.9 mM in 60 min, Fig. 1A), and insulin (from 50 down to 25 pM by the same time); valine levels in liver blood increased significantly after 60 min (from 100 to 170 μM; Fig. 1B) earlier than in caval blood. The concentration of regulatory amino acids [2,15] in plasma exhibited unbalanced changes: glutamine showed an early, significant decrease from 604 ± 39.2 μM (by 0 min) down to 516 ± 46.7 (by 15 min); 534 ± 33.9 (by 30 min); 504 ± 35.7 (by 60 min); 493 ± 31.5 (by 120 min); all other regulatory amino acids did not show any early decrease but increased significantly after 60 min, together with most plasma amino acids. Electron micrographs indicate changes in the lysosomal compartment (but no formation of autophagosomes) were observed by 30 min and autophagic vacuoles were seen in

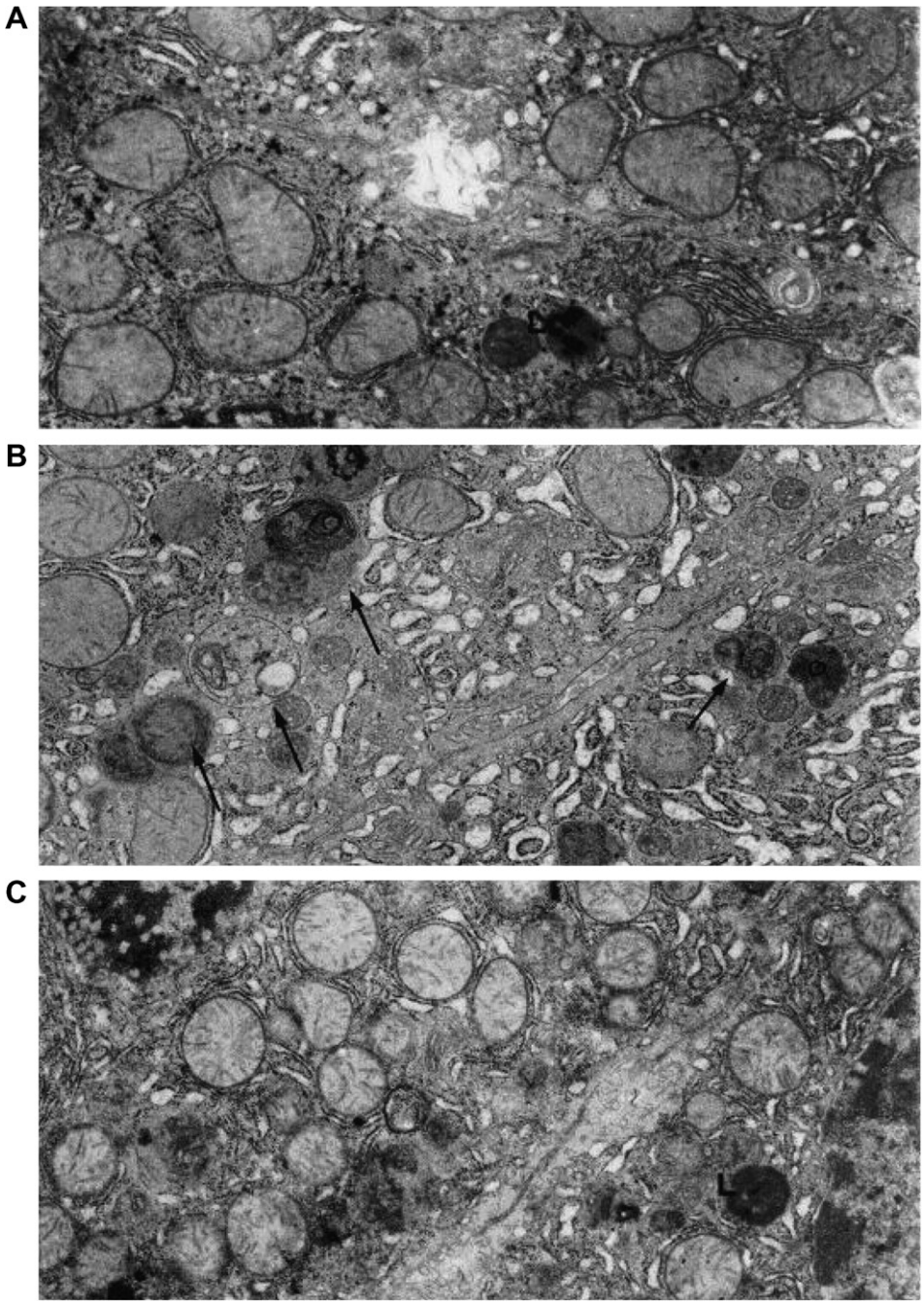

Fig. 4. Representative electron micrographs (16000×) of rat liver cells treated with DMP in the presence or absence of glucose. (A) Control, (B) treated with DMP for 120 min, (C) treated with DMP plus glucose. Several autophagic vacuoles (arrows) are visible in the peribiliar area of DMP-treated rats but not of rats given DMP plus glucose. L, lysosomes.

the peribiliar area of liver cells by 60 min after DMP injection (Fig. 2). To investigate whether DMP-dependent stimulation of autophagy could affect the expression of autophagy-related genes, mRNA levels of LC3 were monitored (Fig. 1C). The administration of DMP increased LC3 mRNA levels by 90 min and a twofold increase was seen 120 min after the injection.

The effects of DMP on blood glucose and insulin levels can be counteracted by the administration of glucose (2 g/kg b.w., Table 1). Glucose administration can inhibit DMP-dependent stimulation of autophagic proteolysis detected as a decrease in the release of the amino acid valine in the blood (Fig. 3A). In order to see how inhibition of autophagy affects LC3 gene expression, glucose (2 g/kg b.w.) was administered 42, 57, and 87 min after DMP injection (the timepoints for glucose administration were selected according to the time-course of the metabolic and endocrine events after the injection of DMP and according to the results obtained from electron micrographs after DMP treatment e.g. by 45 min the induction of autophagy is at its earliest steps; by 60 min, the process is fully active; by 90 min, activation of autophagy is maximal and LC3 gene expression is enhanced). Fig. 3A showed that the DMP-induced increase in blood valine levels was reduced by early glucose injection; and was inhibited only in part if glucose was injected after full activation of autophagy. Electron micrographs reveal no signs of activation of autophagy by DMP if glucose was injected close to the time of (42 min) DMP injection, and increasing numbers of autophagic vacuoles and residual bodies were seen in the liver cells of the DMP-treated rats if glucose was injected at a later time (Fig. 4). Surprisingly, Fig. 3B indicates that glucose did not counteract the DMP-induced increase of LC3 mRNA levels suggesting that expression of autophagic genes can be separated from the activation of autophagic proteolysis.

Discussion

In this report, we show that the antilipolytic agent 3,5′-dimethylpyrazole (DMP) can increase both autophagic proteolysis and autophagic gene expression in mammalian liver cells *in vivo*. Increased liver protein degradation may be secondary to lack of glucose and lower insulin and higher glucagon levels in DMP-treated rats [6]. Electron microscopy revealed a dramatic increase in autophagic vacuoles in the peribiliar area of liver cells in less than 1 h after DMP treatment. The expression level of LC3 autophagy-related gene was enhanced after DMP treatment with a longer latency than autophagic proteolysis. LC3 is considered the most reliable biochemical marker of autophagy, and it is localized onto autophagosomes double membrane during membrane elongation, completion, and fusion with lysosomes [16] and represents an ubiquitin-like system thought to act in protein recruiting for autophagosome vesicle formation. Previous reports in yeast showed that the induction of autophagy may take place in the absence of protein synthesis, but formed autophagic vacuoles are smaller [17].

Increased LC3 mRNA levels after the induction of autophagy might help to compensate for LC3 consumption during this process. A similar increase was seen with Beclin-1 and AMPK-alpha (unpublished data), two other genes involved in autophagy [18–21].

In mammals, levels of amino acids were shown to be the primary regulators of autophagy. The stimulatory effect of antilipolytic drugs on autophagic proteolysis *in vivo* may be secondary to concerted changes in insulinemia and glucagonemia [22]. Inhibition of the DMP-induced stimulation of autophagy by glucose injection may be in line with this hypothesis. To our knowledge, the effects of antilipolytic drugs on the regulation of autophagy gene expression have not been studied to date. Our results demonstrate that antilipolytic drugs can enhance both autophagic proteolysis and the expression of the autophagy-related gene LC3 with a different latency, and that stimulation of autophagy gene expression might not to be secondary to stimulation of autophagic proteolysis since these processes are distinct and can be separated temporally.

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