Supplemental Digital Content 1: Supplemental Results

Autophagy-related protein 5 (atg5) knockdown aggravated procaine myotoxicity

C2c12 cells were treated with procaine at different concentrations (1.75~7.0 mM) for 24 h. The results of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay demonstrated that the cell viability was decreased dose-dependently by 18.9, 42.7, 66.8 and 72.0% in the cells treated with 1.75, 3.5, 5.25 and 7 mM of procaine, respectively, compared with untreated controls (0 mM) (fig. 1). Therefore, a concentration of 3.75 mM of procaine was selected in the following experiments.

Next, C2c12 cells were treated with 3.5 mM procaine for 6 h. Cells were collected for immunoblotting analysis for microtubule-associated protein light chain 3 (LC3) conversion and p62 protein levels. As shown in figure 2, procaine administration increased both LC3-II/LC3-I ratio by 120.2% and p62 protein levels by 28.7%, respectively, compared with the untreated controls ($P < 0.01$). The data suggest that procaine at a myotoxic dosage impaired autophagic flux.

Finally, C2c12 cells were transfected with atg5 small interfering RNA for 48 h and followed by challenging with procaine (3.5 mM) for 24 h. The results of MTT assay demonstrated that the procaine-induced decrease in cell viability was further decreased by 36.5% by atg5 knockdown ($P < 0.0001$) (fig. 3).

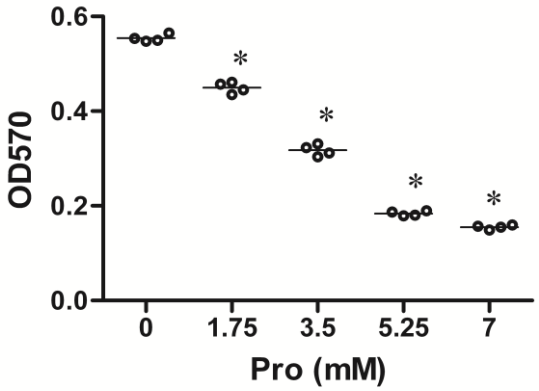

Fig. 1. Dose-effects of procaine on myotoxicity of C2c12 cells.

C2c12 cells were treated with procaine (pro) at the indicated concentrations for 24 h. MTT assay was performed. * $P < 0.01$ *vs.* untreated controls (0 mM). n = 4 per group.

MTT = 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide.

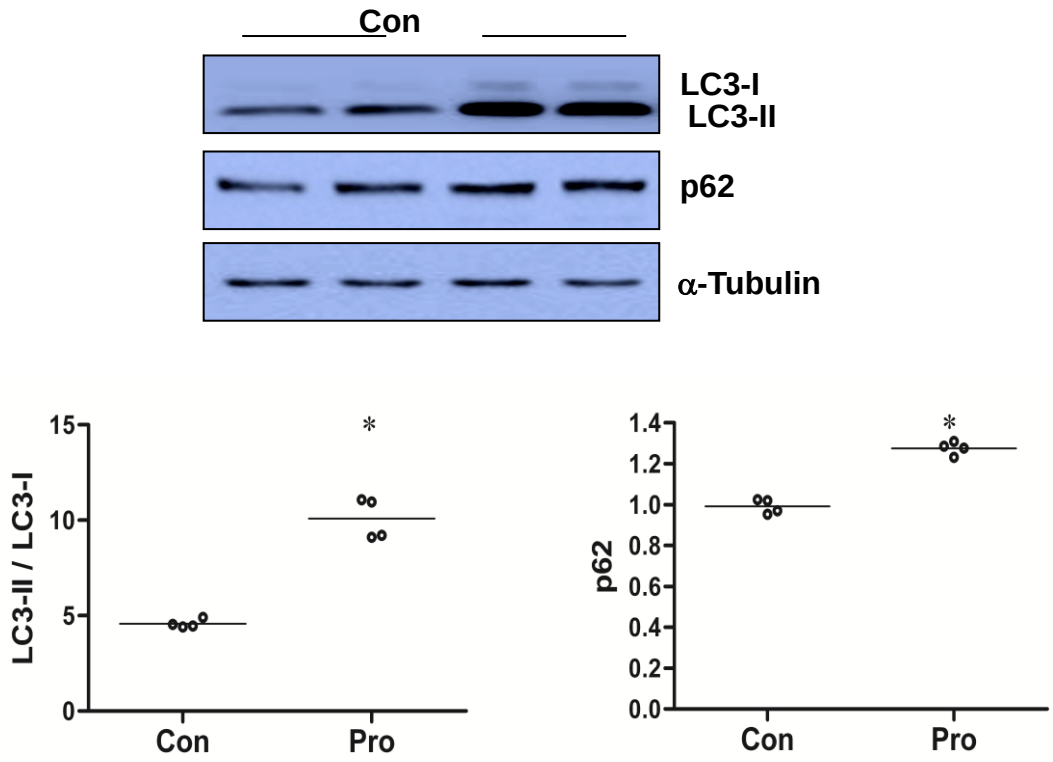

Fig. 2. Procaine increased LC3-II/LC3-I ratio and p62 protein levels in C2c12 cells.

C2c12 cells were treated with procaine (3.5 mM) for 6 h. Cells were collected and cellular extracts were prepared for western blot for LC3 and p62. The same membrane was blotted with an α-tubulin antibody as a loading control.

* *P* < 0.01 *vs.* Con group. n = 4 per group.

Con = untreated control; LC3 = microtubule-associated protein light chain 3; Pro = procaine.

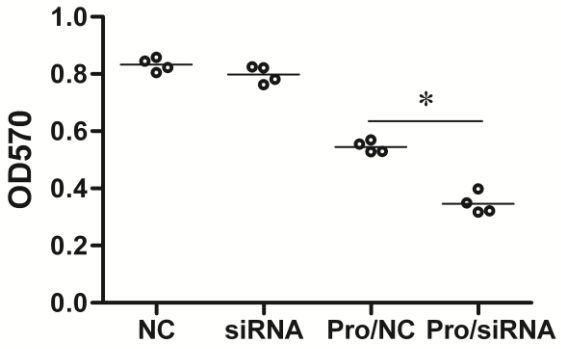

Fig. 3. Inhibition of autophagy with atg5 knockdown aggravated the myotoxicity of procaine in C2c12 cells.

C2c12 cells were exposed to procaine (3.5 mM) at 48 h after atg5 small RNA interfering. MTT assay was performed 24 h after procaine exposure. * $P < 0.01$. n = 4 per group.

atg 5 = autophagy-related protein 5; Con = untreated control; MTT = 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NC = negative controls (cells transfected with scrambled RNA); Pro = procaine; siRNA = small interfering RNA.