Differential involvement of ERK in d-limonene induced activation of p70S6K and LC3 lipidation

C. Chiappini[1], L. Berliocchi[1], A. Adornetto[2], R. Russo[2], G. Bagetta[2], M.T. Corasaniti[1]

[1]Dept. of Health Sciences, "Magna Graecia" University of Catanzaro, Catanzaro, Italy
[2]Dept. of Pharmacy, Health and Nutritional Sciences, University of Calabria, Arcavacata di Rende (Cosenza), Italy

d-Limonene, a well-tolerated monoterpene mainly found in citrus peel, has been reported to inhibit carcinogenesis and promote tumour regression [1] and to exert anxiolytic-like activity [2], though the underlying mechanism remains controversial. Recently, we observed that d-limonene (125-750 μM) does not induce necrotic or apoptotic cell death [3] but it rapidly modulates autophagic markers in SH-SY5Y cells [4]. Autophagy is a tightly regulated intracellular degradative process ensuring adaptation to starvation and other stresses and involved in promoting removal of damaged organelles, misfolded and aggregate-prone proteins; importantly, its deregulation has been involved in a number of diseases including cancer and neurodegeneration [5]. The present study was undertaken for in depth investigating the cell signaling pathways involved in d-limonene actions. The experiments were performed in human SH-SY5Y cells exposed to d-limonene (125-750 μM) or vehicle (DMSO; 0.018-0.108%) to evaluate microtubule-associated protein light chain 3 (LC3) lipidation and the activation (phosphorylation) of Extracellular signal-Regulated Kinase 1/2 (ERK1/2) and p70 S6 kinase (p70S6K) by western blotting. d-Limonene (125-750 μM) caused a rapid, concentration- and time-dependent increase in the phosphorylated, activated form of ERK (p-ERK) which paralleled the increase in LC3-II levels (the lipidated form of LC3-I, that specifically associates with the membrane of expanding autophagosomes). By using the lysosomal inhibitor bafilomycin A1, autophagic flux assay indicated that enhancement of LC3-II levels by d-limonene is due to increased autophagosome formation rather than to a decreased autophagosomal turnover. To assess the possible involvement of ERK, we used the Mitogen-Activated Protein Kinase (MAPK) Kinase (MEK) inhibitor, PD98059 (20 μM). The latter inhibitor abrogated ERK phosphorylation but failed to affect increased LC3-II levels ruling out a role for ERK in the observed LC3 lipidation. To investigate whether d-limonene enhances LC3-II levels by repressessing mammalian target of rapamycin (mTOR), a negative regulator of autophagy, we examined the phosphorylation level of p70S6K (Thr389; p-p70S6K), a downstream target of mTOR kinase. d-Limonene rapidly and transiently increased the levels of p-p70S6K, a finding indicative of mTOR activation, and this effect was abolished by pharmacological inhibition of ERK. Our present results indicate that, at non cytotoxic concentrations, d-limonene induces ERK-mediated activation of p70S6K and ERK-independent increase of LC3 lipidation. These findings deserve further investigation to confirm the ability of d-limonene to stimulate autophagy despite mTOR pathway activation as it might be usefully exploited as a mechanism to obtain mTOR-mediated effects, such as increased synaptogenesis [6], devoid of the potential risk associated to inhibition of autophagy.

[1] Miller J.A. et al. (2011) Oncol Rev 5: 31–42.
[2] Lima N.G.P.B. et al. (2013) Pharmacol Biochem Behav 103: 450–454.
[3] Russo R. et al. (2013) Fitoterapia 89: 48-57.
[4] Russo R. et al. (2014) PLoS One 9(11): e113682.
[5] Levine B. Kroemer G. (2008) Cell 132: 27-42.
[6] Li N. et al. (2010) Science 329: 959–964.