Core G subcellular fractionation of RAW 264.7 cells

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Version 01

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Tissue culture

RAW264.7 mouse macrophage-derived cells (ATCC, cat # TIB-71) were maintained between passages 4 and 24 at 37ºC and 10% CO_2. The medium was composed of high glucose- and L-glutamine-containing DMEM (Cellgro, cat.#10-013) supplemented with 10% heat inactivated fetal calf serum with low endotoxin content (Hyclone SH30071.03 ANG19242), 100 units/ml penicillin, and 100 ug/ml streptomycin. For an experiment, 5 T-150 flasks of cells were plated at the density of 36 x 10_6 cells per flask in 24 ml of the same medium. At 24 hrs after plating they were treated (or left untreated) with 100 ng/ml KDO_2-Lipid A for another 24 hrs followed by subcellular fractionation.

Subcellular fractionation

Cultured medium was removed and used for eicosanoid analysis to confirm activated state of the cells. In these experiments, eicosanoid profiles and levels are consistent with the results of whole cell experiments [ref].

The cells were harvested by scraping in PBS (total of 35 ml), pelleted at 200 g, 7 min, resuspended in 35 ml of isolation medium (250 mM sucrose, 10 mM HEPES-KOH, pH 7.4, 1 mM EGTA) and pelleted again to remove salts. For effective homogenization, the cells were subjected to mild osmotic shock by resuspending in 35 ml of hypotonic medium (same as the isolation medium above but with only 100 mM sucrose) and pelleted. Supernatant was set aside; cell pellet was carefully transferred into 7 ml glass Dounce homogenizer, homogenized in 10 ml of supernatant by 40 strokes of tight fitting pestle and recombined with the supernatant. Osmotic shock and details of homogenization are essential for effective cell lysis, organelle separation and the final yield.

Homogenate was brought to isotonicity by addition of 3.2 ml of hypertonic medium (same as the isolation medium above but with 1.78 M sucrose) and supplemented with 2 mM $MgCl_2$, essential for preservation fo nuclei throughout the preparation. Differential centrifugation parameters were as follows: 200 g for 10 min to pellet nuclei/unbroken cells („nuclear“ pellet), 5,000 g for 10 min to pellet mitochondria and 100,000 g for 1 hr to pellet microsomes. Postnuclear and postmitochondrial supernatants were additionally spun at 300 g and 5,000 g for 10 min, respectively, to additionally purify from residual nuclei and mitochondria. First nuclear and mitochondrial pellets were additionally washed by resuspension/pelleting in Mg^{2+}-containing and Mg^{2+}-free media, respectively. Supernatant from 100,000 g spin was retained as cytosolic fraction.

Nuclear, mitochondrial, and microsomal pellets were additionally separated in stepwise gradients of iodixanol (OptiPrep™, Axis-Shield; available through Sigma) in SW-41 bucket rotor. All gradient media were prepared according to manufacturer's instructions based on isolation medium above; media for nuclear preparation were supplemented with 5 mM $MgCl_2$.

Nuclei were purified according to manufacturer-suggested protocol; briefly, nuclear pellet was brought to 25% iodixanol (12 ml), iodixanol gradient was build from bottom up in three 12 ml tubes (4 ml 10%, 4 ml nuclei in 25%, 2.5 ml 30%, 1.5 ml 35%) and spun at 10,000 g for 20 min. Nuclei band at 30/35% interface.

Mitochondrial and microsomal pellets were brought to 35% iodixanol (6 ml) and fractionated by flotation for 2 hours at 50,000 g in three 12 ml tubes each. The following iodixanol gradient was used: 2ml 10%, 4 ml 17.5%, 4 ml 25%, and 2 ml of corresponding pellet in 35% iodixanol. Mitochondria band at 17.5/25% interface; plasma membrane and ER band at 10/17.5% and 17.5/25% interfaces, respectively.
All samples were frozen and stored at -80ºC.