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Title: Activation of pannexin-1 mediates triglyceride-induced macrophage cell death

Author's name: Byung Chul Jung[1,2], Sung Hoon Kim[2,3], Jaewon Lim[2,4,*], and Yoon Suk Kim[2,*]

Affiliation: [1] Department of Nutritional Sciences and Toxicology, University of California, Berkeley, State of California 94720, United States
[2] Department of Biomedical Laboratory Science, College of Health Sciences, Yonsei University, Wonju, Gangwon-do 26493, Republic of Korea
[3] Department of Biomedical Laboratory Science, Korea Nazarene University, Cheonan, Chungcheongnam-do 31172, Republic of Korea
[4] Department of Biomedical Laboratory Science, College of Medical Sciences, Daegu Haany University, Gyeongsan, Gyeongsangbuk-do 38610, Republic of Korea

Running Title: Pannexin-1 in TG-induced macrophage cell death

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Corresponding Author's Information: *To whom correspondence should be addressed:
Yoon Suk Kim, Department of Biomedical Laboratory Science, College of Health Science, Yonsei University, Wonju, Gangwon-do, 26493, Republic of Korea
Tel : +82-33-760-2860, Fax: +82-33-760-2195, E-mail : yoonsukkim@yonsei.ac.kr
Jaewon Lim, Department of Biomedical Laboratory Science, College of Medical Sciences, Daegu Haany University, Gyeongsan, Gyeongsangbuk-do 38610, Republic of Korea
Tel : +82-53-819-1352, Fax: +82-53-819-1353, E-mail : jaewon330@dhu.ac.kr

MATERIALS AND METHODS

Materials

Lipofundin® MCT/LCT 20% (B. Braun Melsungen AG; Melsungen, Germany) was used to deliver TG into cells, as previously described (1). Hereafter, Lipofundin® MCT/LCT 20% will be referred to as TG for convenience. The caspase-1 substrate, Ac-YVAD-pNA was purchased from Biomal (Plymouth Meeting, PA, USA). The caspase-2 substrate, Ac-VAVAD-pNA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Glyburide, apyrase, and probenecid were purchased from Sigma-Aldrich. The caspase-1 specific inhibitor, z-YVAD-FMK, and caspase-2 specific inhibitor, z-VDVAD-FMK, were purchased from BioVision (Mountain View, CA, USA). ATP was purchased from Sigma-Aldrich. Antibodies specific for caspase-1, -3, -7, -8, and -9, as well as PARP, were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies specific for pannexin-1 were purchased from R&D systems (Minneapolis, MN, USA).

Cell culture

The THP-1 human acute monocytic leukemia cell line (ATCC, Manassas, VA, USA) was grown in RPMI 1640 media supplemented with 10% fetal bovine serum and penicillin-streptomycin and maintained at 37°C in a humidified atmosphere with 5% CO_2. To induce differentiation of THP-1 cells into macrophages, cells were seeded in 6-well plates at a density of 1×10^6 cells/well and treated with 200 nM phorbol 12-myristate 13-acetate (PMA) for 48 h.

Atomic absorption spectroscopy for measurement of K^+ concentration

To assess the concentration of K^+ efflux, THP-1 macrophages (1×10^6 cells per well) were incubated with or without TG for 24 h. Cells were washed with PBS and cultured in serum-free medium another 6 h at 37°C. The concentration of K^+ in each sample was quantified using an atomic absorption spectrometer (AA7000F, Shimadzu, Tokyo, Japan).

Trypan blue dye exclusion assay

To enumerate viable cells, cells were trypsinized and 10 μL of 0.4% trypan blue

stain solution was mixed with 10 μL of the trypsinized cell suspension. Non-stained cells in the resulting mixture were counted using a hemocytometer (Marienfeld, Lauda-Königshofen, Germany).

Measurement of caspase activity

The activities of caspase-1 and caspase-2 were determined as previously described (2). Briefly, cells were lysed with PBS buffer containing 1% Triton X-100 and centrifuged at 19,000 × *g* for 10 min at 4°C. The supernatant was collected and the total protein concentration was quantified. To detect caspase-1 activity, 90 μg of the protein sample was combined with 200 μM of Ac-YVAD-pNA in 150 μL PBS. To detect caspase-2 activity, 90 μg of the protein was combined with 200 μM of Ac-VAVAD-pNA in 150 μL PBS. Reactions were incubated for 3 h at 37°C, and the activity was determined by measuring the absorbance at 405 nm.

Western blot analysis

Cells were washed with PBS and lysed at 4°C with a lysis buffer containing 1% Triton X-100, protease inhibitor cocktail (Sigma-Aldrich), phosphatase inhibitor cocktail (Roche, Mannheim, Germany), and PBS. Lysates were clarified and the supernatants were subjected to western blotting as described previously (3).

Ethidium bromide (Et-Br) uptake assay and measurement

Dye uptake experiments were performed as described previously (4). Briefly, THP-1 cells were seeded on glass coverslips and treated with 200 nM PMA for differentiation into macrophages. Then, the cells were incubated with or without 1 mg/mL of TG for 24 h. Differentiated THP-1 cells were carefully washed three times with Dulbecco's phosphate buffered saline (DPBS) devoid of Ca^{2+} and loaded with pre-warmed DPBS containing Et-Br (final concentration 25 μg/mL) for 5 min at 37°C. The supernatant was discarded and cells washed twice with pre-warmed DPBS and fixed in 2% paraformaldehyde for 5 min. After fixation, cells were washed twice with DPBS and incubated with a DAPI stain solution (Vector Lab, Burlingame, CA, USA) in the dark for 10 min. Intracellular Et-Br fluorescence in cell monolayers was observed using a laser confocal scanning microscope (LSM 710,

Zeiss, Heidenheim, Germany). Et-Br fluorescence intensity was measured using Image J (National Institute of Health, Bethesda, MD, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). The *p*-values were calculated using Student's *t*-test. Data are shown as the mean and standard error of the mean (SEM). Each experiment was conducted three times and the data were pooled for analysis. Differences were considered to be statistically significant at $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$.

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