鎘誘導粒線體 DNA 缺損細胞經粒線體-鈣離子-calpain 路徑進行 caspase 非依存性細胞凋亡

Cadmium Toxicity toward Caspase-Independent Apoptosis through Mitochondria-Calcium-Calpain Pathway in mtDNA-Depleted Cells

中文摘要

粒線體 (mitochondria)除了負責細胞內能量生成外，也已被證實是調控細胞凋亡的主要胞器。本論文利用去除粒線體去氧核核糖核酸(mitochondrial DNA, mtDNA)的人類骨癌細胞株 (osteosarcoma cell line)，以下稱 Rho zero cell (0 cell)，以重金屬鎘 (cadmium, Cd)，作爲細胞凋亡 (apoptosis)誘導物質，探討在粒線體喪失正常生理功能下，鎘毒性及細胞凋亡機轉。以流式細胞技術，首先進行 PI (propidine iodine)單染色法及 annexinV/PI 雙染色法，分別進行 25 M Cd 之從時(time course)實驗及作用 24 小時之劑量相關(dose-dependent)實驗。結果顯示在鎘對 0 cell 之細胞毒性具有從時及從量(time and dose-dependent)的關係，25 μM 鎘作用 24 小時約有 50%的細胞凋亡比例。使用廣泛性 caspase 抑制劑 (Z-VAD.fmk)無法防止鎘所造成的細胞凋亡，續以 caspase 3 之螢光受質 (DEVD-AFC)測定 caspase 3 活性，亦顯示 caspase 3 未被活化，由此判定鎘誘導 0 cell 進行 caspase 非依存性 (caspase-independent)細胞凋亡。另外使用粒線體膜電位專一性染劑 JC-1 偵測粒線體膜電位 (mitochondrial membrane potential, m)，結果指出粒線體膜電位隨時間增加有去極化 (depolarization)現象，由時間先後順序顯示粒線體傷害發生於細胞凋亡之前。但使用共軛焦螢光顯微鏡技術(confocol microscopy)並未發現凋亡因子(pro-apoptotic factors)由粒線體釋放，包括: AIF (apoptosis inducing factor)、Endo G (endonuclease G)、Cyt c (cytochrome c)。同時使用不同的活性氧分子專一性染劑，均無法測得活性氧分子 (reactive oxygen species, ROS)有濃度增加的現象， 顯示 0 cell 與一般細胞凋亡過程不同。爲進一步瞭解鈣離子是否參與調控 Cd 所造成之細胞凋亡。以 Fluo-3 AM 染劑觀察細胞內鈣離子 (Ca2+ i)的變化，發現相較於控制組，鎘處理 30 分鐘後鈣離子有 9 倍左右的上升，隨即降低至 4 倍，但作用後 2 小時反而有 11 倍的上升，顯示鎘可以導致胞內鈣離子有劇烈波動現象。接著，使用細胞內鈣離子螯合劑(BAPTA-AM)預處理細胞，結果發現可以完全抑制鎘誘導的細胞凋亡，證實鈣離子確實參與細胞凋亡。續以粒線體鈣離子通道抑制劑 (mitochondrial calcium uniporter inhibitor) RR (ruthenium red)處理細胞，實驗結果顯示 RR 可明顯降低鎘引起的細胞內鈣離子波動，且可以抑制鎘所誘導之細胞凋亡，推測(1) RR 阻斷鈣離子進入粒線體，(2)鎘進入粒

線體的通道受阻而減低了鎘導致的粒線體傷害，此外，也證明了細胞內鈣離子恆定與細胞凋亡的密切關係。 Ca2+ i 提昇可能會活化其下游與 apoptosis 相關的蛋白水解酶 calpain，以 calpain substrate (suc-LLVY-AMC)，利用螢光測定儀偵測不同鎘作用時間下的 calpain 活性，結果發現在鎘作用後 2 小時後 calpain 的比活性 (specific activity, RFU/mg/sec)會由控制組的 88.05 增加至 168.05 (p=0.043)，但在加入 RR 作用後 calpain 比活性則降低至 34.10 (p=0.023)，進一步以 50 μM calpain inhibitor I (ALLN)和 50 μM calpain inhibiotor II (ALLM)前處理細胞，發現 calpain inhibitor II (ALLM)可以完全阻斷鎘誘導的細胞凋亡，此實驗證明鎘是經由影響細胞內鈣離子而活化 calpain 進而引發細胞凋亡。綜合上述實驗結果，本論文說明了即使在粒線體功能缺損下，細胞仍可以進行凋亡，另外也說明了鎘是透過粒線體-鈣-calpain 路徑誘導細胞進行 caspase-independent apoptosis。

英文摘要

Mitochondria were believed to be integrators and coordinators of programmed cell death next to their respiratory function. Using mitochondrial DNA (mtDNA)-depleted osteosacoma cell (0 cell) as a cell model, we investigated the apoptogenic signaling pathway of cadmium (Cd) under a condition of mitochondrial dysfunction. The apoptotic percentage was around a platue of 50.0% after 24-h exposure of 25 μM Cd using flow cytometry co-staining with Annexin V and propidium iodine (PI). Pretreatment of Z-Val-Ala-DL-Asp(OMe)—fluoro -methylketone (Z-VAD.fmk), a broad spectrum of caspase inhibitor, was found fail to prevent apoptosis from suffering of Cd toxicity. Moreover, Cd was unable to activate caspase 3 using a fluromicrotiter plate reader with benzyloxy-carbonyl-Asp-Glu-Val-Asp-7-amino-4- trifluoromethyl -coumarin (DEVD-AFC) as a substrate, indicating that Cd induced caspase-independent apoptosis in mtDNA-depleted cells. JC-1 staining demonstrated that mitochondrial membrane depolarization was a prelude of apoptosis. It was noted, however, that we could not detect the release of cytochome c (cyt c); apoptosis-inducing factor (AIF), and endonuclease G (Endo G) from mitochondria by using confocol microscopy. Additionally, Cd treated were unable to induced intracellular oxidative stress using ROS-specific fluorescence dyes as indicators. The intracellular calcium concentration was oscillated to a 11-fold elevation after 2-h exposure of Cd. More importantly, the apoptogenic activity of Cd was almost abolished by BAPTA-AM, an intracellular calcium chelator. This implied that calcium might play a crucial role in cadmium-induced apoptosis. In addition, mitochondrial calcium uniporter blocker, ruthenium red (RR), could prevent both intracellular calcium oscillation and cell death from cadmium toxicity. This observation suggested that RR could block the

calcium influx into mitochondria and/or prevent Cd toxicity toward mitochondria, resulting in escaping of apoptosis from Cd treatment. Moreover, using suc-LLVY-AMC as substrate to detect calpain activity by fluromicrotiter plate reader, we found the calpain specific activity (RFU/mg/sec) was provoked from 88.01 to 168.01 ($p=0.043$) after 2-h exposure of Cd. Interestingly, RR could reduce the calpain specific activity from 168.01 to 34.10 ($p=0.023$). Pretreatment of calpain inhibitor I and II (ALLN and ALLM) for 1 h reduced the apoptotic percentage (from 54.8 to 32.6 and from 54.8 to 10.0, respectively). This observation leads us to conclude that, the mtDNA-depleted mitochondria provide an alternative athway for Cd to conducting a caspase-independent apoptosis through mitochondria -calcium-calpain mechanism.