Aspirin induces apoptosis through mitochondrial cytochrome *c* release

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Abstract Aspirin and other non-steroidal anti-inflammatory drugs induce apoptosis in many cell types. Although the involvement of caspases has been demonstrated, the mechanism leading to caspase activation remains unknown. We have studied the role of the mitochondrial pathway in aspirin-induced apoptosis. The apoptotic effect of aspirin was analyzed in different cell lines (Jurkat, MOLT-4, Raji and HL-60) showing induction of mitochondrial cytochrome *c* release and caspases 9, 3 and 8 processing. Furthermore, early aspirin-induced cytochrome *c* release was not affected by the caspase inhibitor Z-VAD·fmk and preceded loss of mitochondrial membrane potential. Therefore, aspirin-induced apoptosis involves caspase activation through cytochrome *c* release. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Aspirin; Non-steroidal anti-inflammatory drug; Apoptosis; Cytochrome *c*; Mitochondrion; Caspase

1. Introduction

Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) induce apoptosis in several cell types, including human colorectal tumor cell lines [1–4], fibroblasts [5,6], B-cell chronic lymphocytic leukemia cells [7] and myeloid leukemia cell lines [8]. Furthermore, administration of NSAIDs induces apoptosis of colon cancer cells in vivo [9,10].

The apoptotic action of NSAIDs was inhibited by the caspase inhibitor Z-VAD·fmk, demonstrating the involvement of caspases [4,7,8]. However, the pathway leading to caspase activation remains unknown. Caspases play a central role in the execution of programmed cell death [11]. The two most well-studied pathways of caspase activation include the surface death receptor pathway and the mitochondrion-initiated pathway [12]. In the mitochondrial pathway, cytochrome *c* and other apoptogenic proteins (e.g. apoptosis-inducing factor [13]) are released from the intermembrane space to the cytosol [14,15]. Once released, cytochrome *c* binds to Apaf-1 and induces activation of caspase 9 [16].

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Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; $\Delta\Psi_m$, mitochondrial membrane potential; PT, permeability transition; PI, propidium iodide; ECL, enhanced chemiluminescence

In the present study, we examined the role of the mitochondrial pathway in aspirin-induced apoptosis. In particular, the relationship between cytochrome *c* release, caspase activity and loss of mitochondrial membrane potential ($\Delta\Psi_m$) was analyzed. Our results indicate that aspirin triggers cytochrome *c* release as an early event preceding caspase activation and loss of $\Delta\Psi_m$.

2. Materials and methods

2.1. Cell culture

The human cell lines Jurkat and MOLT-4 (acute T leukemia), Raji (B-cell Burkitt lymphoma) and HL60 (promyelocytic leukemia) were from the European Collection of Cell Cultures. Cells were grown in RPMI 1640 medium (Biological Industries, Beit Haemek, Israel) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL, Paisley, UK), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere at 5% carbon dioxide.

2.2. Reagents

Aspirin (acetylsalicylic acid) and propidium iodide (PI) were from Sigma Chemicals Co. (St Louis, MO, USA). Annexin V-FITC was from Bender MedSystems (Vienna, Austria). Z-VAD·fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) was from Bachem AG (Bubendorf, Switzerland). JC-1 was from Molecular Probes Europe BV (Leiden, The Netherlands).

2.3. Analysis of viability by phosphatidylserine exposure and PI uptake

Cell viability was determined by analyzing phosphatidylserine exposure and membrane integrity by double staining with annexin V-FITC and PI, prior to flow cytometric analysis (FACSCalibur, Becton Dickinson, Mountain View, CA, USA), as previously described [4]. Data analysis was performed with CellQuest software (Becton Dickinson). Cell viability was measured as the percentage of annexin V- and PI-negative cell population.

2.4. Western blot analysis of caspases

Whole cell protein extracts were obtained by lysing cells with Laemmli sample buffer [17]. Protein concentration was measured with the Micro BCA Protein Assay Reagent kit (Pierce, Rockford, IL, USA). For Western blot, protein extracts (50 μg) were subjected to reduction conditions before being subjected to electrophoresis on a 12% polyacrylamide gel and then transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Membranes were incubated with the polyclonal antibody against cleaved caspase 9 (New England Biolabs) or monoclonal antibodies against caspase 3 (Transduction Laboratories, Lexington, KY, USA) or caspase 8 (Cell Diagnostica, Münster, Germany). Antibody binding was detected with a horseradish peroxidase-coupled anti-mouse or anti-rabbit secondary antibody (Amersham, Bucks, UK) and the enhanced chemiluminescence (ECL) detection kit (Amersham).

2.5. Cytochrome c release measurements

Release of cytochrome *c* from mitochondria to cytosol was measured by Western blot as previously described [18] with some modifications. Cells (5×10^6) were harvested, washed once with ice-cold

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phosphate-buffered saline and gently lysed for 30 s in 80 μl ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 0.05% digitonin, 25 mM Tris, pH 6.8, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at $12\,000 \times g$ at 4°C for 3 min to obtain the supernatants (cytosolic extracts free of mitochondria) and the pellets (fraction that contains mitochondria). Supernatants (50 μg) and pellets (40 μg) were electrophoresed on a 15% polyacrylamide gel and then analyzed by Western blot using anti-cytochrome *c* antibody (7H8.2C12, from Pharmingen, San Diego, CA, USA) and cytochrome oxidase subunit II antibody (Molecular Probes) and ECL, as described above.

2.6. Analysis of changes in $\Delta\Psi_m$

Changes in $\Delta\Psi_m$ were determined by staining the cells with JC-1 (Molecular Probes) prior to flow cytometry analysis (FACSCalibur, Becton Dickinson), as previously described [19]. Data analysis was performed with CellQuest software (Becton Dickinson) by measuring both the green (530 ± 15 nm, FL-1) and red (585 ± 21 nm, FL-2) JC-1 fluorescence. The loss in $\Delta\Psi_m$ is seen as a shift to lower JC-1 red fluorescence accompanied by an increase in JC-1 green fluorescence. At least 10 000 events were collected per sample.

3. Results

3.1. Aspirin induces mitochondrial cytochrome c release and caspase 9, 3 and 8 processing

In order to study the mechanism of aspirin-induced apoptosis we first tested its effect on different cell lines. Jurkat, MOLT-4, Raji and HL-60 cells were incubated with 10 mM aspirin for 12 h and cell viability was quantified as the percentage of annexin V- and PI-negative cells. As shown in Fig. 1A, aspirin induced about 50–60% decrease in cell viability in all cell lines tested. Furthermore, an increase in the percentage of cells with low DNA content was also detected (data not shown). To analyze the involvement of cytochrome *c* release in aspirin-induced apoptosis, cytosolic and mitochondrial fractions were obtained and analyzed for the presence of cytochrome *c* by Western blot. As shown in Fig. 1B, aspirin induced the appearance of cytochrome *c* in the cytosolic fractions of Jurkat and Raji treated cells and its disappearance from the mitochondrial fraction of the four treated cell lines, indicating that aspirin induced mitochondrial cytochrome *c* release. As a control for mitochondrial protein loading, cytochrome oxidase subunit II was also analyzed in the mitochondrial fraction by Western blot.

Cytosolic cytochrome *c* binds to Apaf-1 and induces activation of caspase 9 [16]. To demonstrate that this pathway was activated in aspirin-induced apoptosis, caspase 9 was analyzed by Western blot in whole cell extracts. Aspirin induced processing of caspase 9 in the four cell lines, as shown by the appearance of caspase 9 intermediate cleavage product (37 kDa) and the disappearance of the precursor form. Caspases 3 and 8 have been described to be activated downstream of caspase 9 [20]. Aspirin induced processing of caspases 3 and 8, as shown by the appearance of the caspase 8 intermediate cleavage product (43/41 kDa) and the disappearance of the precursor forms of caspases 3 and 8 in the four cell lines (Fig. 1C).

3.2. Aspirin-induced cytochrome c release precedes caspase activation and loss of $\Delta\Psi_m$

Cytochrome *c* release can be dependent on or independent of caspase activity [12]. Thus, we analyzed the time kinetics of cytochrome *c* release and its dependence on caspase activity in Jurkat cells. Cells were treated with 10 mM aspirin in the presence or absence of the caspase inhibitor Z-VAD·fmk (100 μM) for the indicated times and cytosolic extracts and whole cell extracts were obtained for cytochrome *c* release and caspase processing analysis, respectively. As shown in Fig. 2A, aspirin induced cytochrome *c* release in a time-dependent manner. Cytosolic cytochrome *c* levels were slightly increased as early as 3 h and markedly increased at 4 h. Aspirin-induced cytochrome *c* release was not modified by the presence of Z-VAD·fmk until 6 h. At longer times, Z-VAD·fmk partially inhibited the accumulation of cytochrome *c* in the cytosol. These results demonstrate that aspirin induces cytochrome *c* release in two stages, corresponding to an early cytochrome *c* release independent of caspase activity and a late cytochrome *c* release dependent on caspase activity. As shown in Fig. 2A, aspirin induced processing of caspase 9 at 3 h, simultaneous to cytochrome *c* release. Cell viability decreased markedly at 6 and 12 h. The presence of Z-VAD·fmk inhibited both the

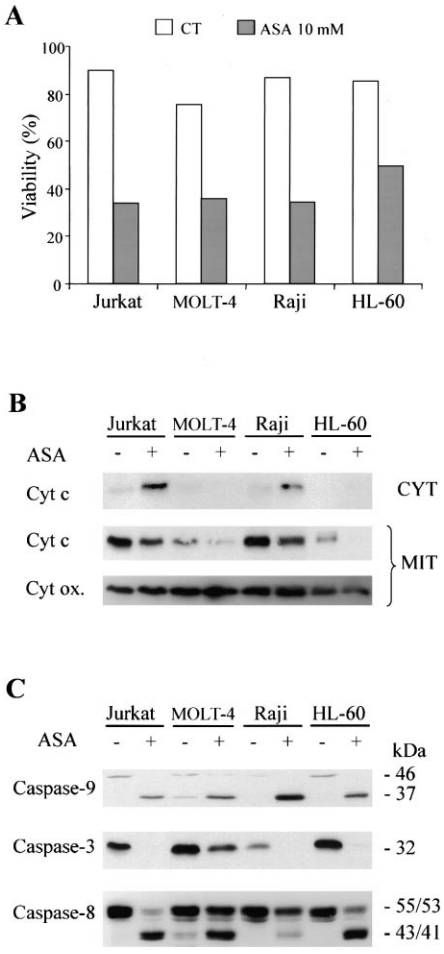

Fig. 1. Effect of aspirin on cell viability, cytochrome *c* release and caspase processing. Jurkat, MOLT-4, Raji and HL-60 cell lines were treated with 10 mM aspirin (ASA) for 12 h. A: Analysis of cell viability by phosphatidylserine exposure and PI uptake. Cell viability was measured as the percentage of annexin V- and PI-negative cell population. B: Analysis of cytochrome *c* in cytosolic (CYT) and mitochondrial (MIT) extracts, by Western blot. Cytochrome oxidase subunit II (Cyt ox.) was also analyzed in the mitochondrial extracts, as a control for mitochondrial protein loading. C: Analysis of the processing of caspases 9, 3 and 8. Whole cell extracts were analyzed by Western blot with antibodies against caspases 9, 3 and 8. The migration position of precursor forms and the cleavage products caspase 9 (37 kDa) and caspase 8 (43/41 kDa) are indicated.

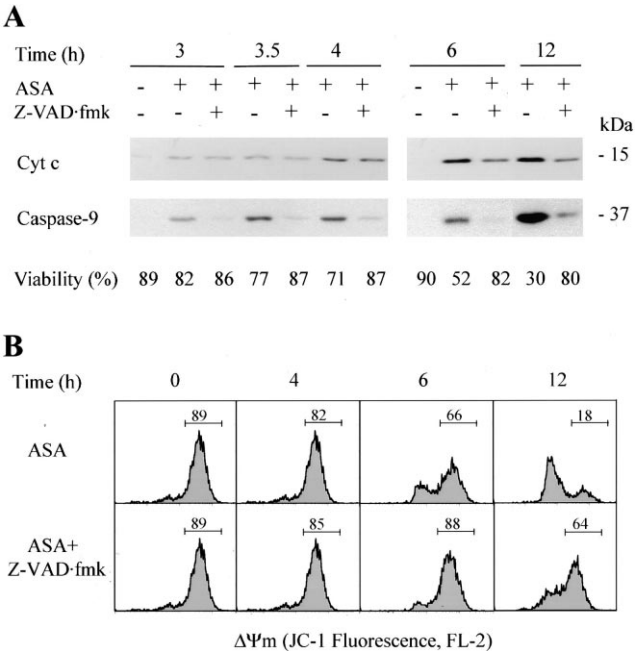

Fig. 2. Time kinetics and effect of Z-VAD·fmk on aspirin-induced cytochrome *c* release, caspase 9 processing and loss of $\Delta\Psi_m$. Jurkat cells were pre-incubated without or with 100 μM Z-VAD·fmk for 1 h and then treated with 10 mM aspirin (ASA) for the indicated times. A: Analysis of cytochrome *c* (Cyt c) and processing of caspase 9 in cytosolic and whole cell extracts, respectively, by Western blot. Cell viability at the indicated times is also shown. B: Analysis of changes in $\Delta\Psi_m$ by staining with JC-1. The loss in $\Delta\Psi_m$ is seen as a shift to lower JC-1 red fluorescence (FL-2). Results show the percentage of cells with high $\Delta\Psi_m$ and correspond to samples in A.

processing of caspase 9 and the decrease in cell viability at all times analyzed.

It has been described that cytochrome *c* release can be induced by mitochondrial permeability transition (PT) characterized by loss of $\Delta\Psi_m$ [15,21,22]. As salicylates induce mitochondrial PT and loss of $\Delta\Psi_m$ in isolated mitochondria [23,24] and hepatocytes [25], we analyzed the effect of aspirin on $\Delta\Psi_m$ in Jurkat cells using the JC-1 dye. As shown in Fig. 2B, aspirin had no effect on $\Delta\Psi_m$ at 4 h and induced loss of $\Delta\Psi_m$ at 6 and 12 h, later than mitochondrial cytochrome *c* release and caspase 9 processing. Furthermore, Z-VAD·fmk blocked loss of $\Delta\Psi_m$ induced by aspirin at 6 h and had a marked inhibitory effect at 12 h, indicating that loss of $\Delta\Psi_m$ is caspase-dependent. Altogether, these results demonstrate that aspirin-induced cytochrome *c* release precedes loss of $\Delta\Psi_m$.

4. Discussion

The mechanism underlying the induction of apoptosis by aspirin, salicylate and other NSAIDs is unknown and currently under research. So far, several pathways have been implicated including cyclooxygenase inhibition [26,27], p38 mitogen-activated protein kinase activation [6], nuclear factor-κB inhibition [28,29], and peroxisome proliferator-activated receptor δ inhibition [30]. Although several groups have demonstrated the involvement of caspases [4,7,8,31], the pathways leading to caspase activation remain unknown. The results reported in this paper show that aspirin induces caspase activation through cytochrome *c* release from mitochondria preceding loss of $\Delta\Psi_m$.

Release of cytochrome *c* from mitochondria is a central event in apoptosis [32,33]. Our results demonstrate that cytochrome *c* release from mitochondria is an early event in aspirin-induced apoptosis. The fact that early aspirin-induced cytochrome *c* release is not inhibited by Z-VAD·fmk demonstrates that this event is caspase-independent. Once cytochrome *c* is released, a sequential ordering of caspase activation has been reported [20]. Our results demonstrate that aspirin induces activation of caspases 9, 3, and 8. Thus, the early aspirin-induced cytochrome *c* release could be sufficient to trigger caspase activation.

Salicylates induce mitochondrial PT and loss of $\Delta\Psi_m$ in isolated mitochondria [23,24] and hepatocytes [25]. However, our results indicate that aspirin-induced cytochrome *c* release precedes the decrease in $\Delta\Psi_m$ in intact cells. Furthermore, pretreatment of Jurkat cells with Z-VAD·fmk inhibited loss of $\Delta\Psi_m$ whereas no effect was detected on early cytochrome *c* release. These facts indicate that the decrease in $\Delta\Psi_m$ may be a consequence of caspase activity rather than the effector mechanism of aspirin-induced cytochrome *c* efflux. Thus, loss of $\Delta\Psi_m$ is not required for aspirin-induced release of cytochrome *c* and apoptosis, as described for many apoptotic stimuli including staurosporine, hydrogen peroxide, Fas, UVB irradiation, DNA-damaging agents and ionizing radiation [19,34–40]. The caspase-dependent drop in the $\Delta\Psi_m$ and late cytochrome *c* release may have a role as a potentiating mechanism by a feedback amplification loop linking caspase activation and mitochondrial dysfunction.

In conclusion, the results presented in this report demonstrate that aspirin-induced apoptosis involves release of cytochrome *c* from mitochondria preceding caspase activation and loss of $\Delta\Psi_m$. The elucidation of the mechanisms involved in the induction of cytochrome *c* release by aspirin warrants further investigation.

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