P2Z purinoreceptor ligation induces activation of caspases with distinct roles in apoptotic and necrotic alterations of cell death

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Abstract Myeloic cells express a peculiar surface receptor for extracellular ATP, called the P2Z/P2X$_7$ purinoreceptor, which is involved in cell death signalling. Here, we investigated the role of caspases, a family of proteases implicated in apoptosis and the cytokine secretion. We observed that extracellular ATP induced the activation of multiple caspases including caspase-1, -3 and -8, and subsequent cleavage of the caspase substrates PARP and lamin B. Using caspase inhibitors, it was found that caspases were specifically involved in ATP-induced apoptotic damage such as chromatin condensation and DNA fragmentation. In contrast, inhibition of caspases only marginally affected necrotic alterations and cell death proceeded normally whether or not nuclear damage was blocked. Our results therefore suggest that the activation of caspases by the P2Z receptor is required for apoptotic but not necrotic alterations of ATP-induced cell death.

Key words: Apoptosis; ATP; Caspase; Necrosis; P2Z; Purinoreceptor

1. Introduction

The P2Z/P2X$_7$ receptor for extracellular ATP belongs to the family of purinergic receptors which exert a wide range of biological activities including mitogenic stimulation, excitatory transmitter function, cytokine secretion and induction of cell death [1–4]. It contains two transmembrane domains and a large extracellular loop, structural features that are characteristic of members of the P2X family. Unlike other P2X receptors, the P2Z receptor has an unusually long C-terminal domain which does not contain known signalling motifs. P2Z receptor expression is rather restricted to the macrophage lineage, such as dendritic cells, macrophages and microglial cells. Triggering of the P2Z receptor elicits two types of cellular responses [3]. Like with all P2X receptors, the ligation of ATP results in a transient membrane current through divalent cation channels. A second and unique response is the sustained membrane depolarization and the increase in cytosolic free calcium by the opening of a transmembrane pore which is permeable to hydrophilic molecules of a molecular weight up to 900 Da. Important activities of the P2Z receptor include the release of IL-1β [5–7] and induction of cell death [8,9].

Recent studies have shown that both the release of IL-1β and the induction of apoptotic cell death are controlled by a family of intracellular proteases, called caspases [10]. In mammalian cells, at least 13 caspases exist, which are cysteine proteases that cleave their substrates after aspartate residues. Caspases are synthesized as inactive pro-enzymes and proteolytically processed to form an active complex composed of two heterodimeric subunits of about 10 and 20 kDa. A number of proteins are cleaved by caspases and for some of them an apoptotic function could be attributed. Among different substrates are enzymes involved in genome function and DNA repair, regulators of the cell cycle, structural proteins and the inhibitory subunit of a novel endonuclease, designated CAD for caspase-activated DNase [11].

In this study, we investigated the role of caspases in response to P2Z receptor activation in microglial cells. So far, the signal transduction pathways elicited by the P2Z receptor are largely unknown. While it is established that caspases play a crucial effector function in many apoptotic pathways, their role in necrosis and lytic membrane alterations is rather unclear. We found that activation of the P2Z receptor rapidly induced apoptotic DNA fragmentation, accompanied by the proteolytic processing of multiple caspases and caspase substrates. Inhibitors of caspases specifically suppressed DNA fragmentation and other morphological signs of apoptotic damage. In contrast, cytoplasmic vacuolization and cell lysis remained unaffected and cell death proceeded normally whether or not the caspase activation was blocked. Interestingly, activation of caspase-1 but not caspase-3 appeared to involve the proteasome pathway, since a specific inhibitor of the proteasome function prevented caspase-1 processing. Collectively, our results suggest that P2Z receptor ligation causes the activation of multiple caspases, which are regulated by different pathways and are required for apoptotic but not necrotic alterations during ATP-induced cell death.

2. Materials and methods

2.1. Cell culture and reagents

The mouse microglial cell line N13 [12] was provided by Dr Ricciardi-Castagnoli (University of Milan, Italy) and grown in RPMI 1640 medium supplemented with 10% FCS. ATP and other nucleotides were purchased from Boehringer-Mannheim (Mannheim, Germany). The protease inhibitors YVAD (*N*-acetyl-Tyr-Val-Ala-Asp-chloromethylketone), DEVD (*N*-acetyl-Asp-Glu-Val-Asp-aldehyde) and zVAD (benzyloxycarbonyl-Val-Ala-Asp-(OMe)fluoro-methylketone) were obtained from Bachem (Heidelberg, Germany) and Enzyme Systems (Dublin, CA, USA). The proteasome inhibitor lactacystin was obtained from Calbiochem (Bad Soden, Germany). Rabbit

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Abbreviations: BrdU, 5′-bromo-2′-deoxyuridine; DEVD, *N*-acetyl-Asp-Glu-Val-Asp-aldehyde; LDH, lactate dehydrogenase; PARP, poly(ADP-ribose) polymerase; YVAD, *N*-acetyl-Tyr-Val-Ala-Asp-chloromethylketone; zVAD, benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoro-methylketone

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anti-PARP and mouse anti-lamin B were purchased from Boehringer-Mannheim and Camon (Wiesbaden, Germany), respectively. Antibodies to caspase-1 and caspase-3 were produced by immunizing rabbits with the recombinant proteins. An antibody against caspase-8 was obtained from Biomedia (Baesweiler, Germany).

2.2. Immunoblotting

Proteolytic processing of caspases and caspase substrates was detected by Western blotting as described [13]. Cell extracts were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked for 1 h with 5% non-fat dry milk powder in TBS, incubated with the respective primary and secondary antibodies and stained by enhanced chemoluminescent staining.

2.3. Gel electrophoresis of fragmented DNA

After the indicated treatments, cells were trypsinized and collected together with the floating cells in the supernatant. DNA was extracted from the cells by the salting out technique [14].

2.4. Measurement of DNA fragmentation by BrdU release

DNA fragmentation was quantified by the release of 5′-bromo-2′-deoxyuridine (BrdU) using the commercial cellular DNA fragmentation ELISA kit (Boehringer-Mannheim) according to the manufacturer's instructions.

2.5. Measurement of LDH release

The cell viability was determined by measuring the release of lactate dehydrogenase (LDH) using a cytotoxicity detection kit (Boehringer-Mannheim). The LDH activity released from dead cells was measured spectrophotometrically in the supernatants according to the manufacturer's instructions. In order to obtain the total LDH activity, cells were lysed with 1% Triton X-100. The percentage of LDH release represents the fraction of LDH activity found in the supernatants with respect to the overall enzyme activity.

2.6. Measurement of hypodiploid DNA

Fragmentation of genomic DNA to hypodiploid DNA was assessed by FACS analysis [15]. After the desired cell treatments, cells were washed with PBS, fixed for 30 min in cold acetone-methanol (1:1) and incubated for 1 h in RNase A (1 mg/ml) and propidium iodide (100 μg/ml).

2.7. Morphological detection of cell death

Cells were grown on coverslips and treated with ATP in the presence and absence of zVAD. Cells were then washed carefully in PBS, fixed for 10 min in methanol/ethanol (1:1) and stained with the DNA dye Hoechst 33342 (Sigma, Deisenhofen, Germany) in the presence of 0.1 mg/ml RNase. After 15 min, cells were mounted in Mowiol (Hoechst, Frankfurt, Germany) and inspected by phase contrast and fluorescent light microscopy.

3. Results and discussion

3.1. Extracellular ATP causes DNA fragmentation upon P2Z receptor ligation

A most striking effect of ATP is the induction of cell death which can be triggered by different purinoreceptors. We investigated ATP-induced cell death in the mouse microglial cell line N13 that expresses P2Y and P2Z purinoreceptors [9]. In initial experiments, the induction of cell death was analyzed by measuring the DNA fragmentation following cell treatment with ATP and other nucleotides for 6 h. As revealed by DNA gel electrophoresis, treatment of cells with 3 mM ATP caused the appearance of a classical DNA ladder (Fig. 1A). DNA fragmentation was not observed with lower concentrations of ATP and also other nucleotides were unable to induce DNA fragmentation or other features of cell death. In particular, UTP that activates also the P2Y receptor did not induce DNA fragmentation (Fig. 1A), indicating that cell death was specifically mediated by the P2Z receptor. This assumption was supported by other findings. ATPγS, a non-hydrolysable ATP derivative, also induced cell death, demonstrating that degradation of ATP was not required (data not shown). In contrast, oxidized ATP, which inhibits the P2Z receptor [16], prevented ATP-induced DNA fragmentation. Furthermore, neither DNA cleavage nor other cytotoxic features were induced in derivative cell clones that lack the P2Z receptor but still express P2Y purinergic receptors ([9], data not shown). Induction of cell death was also observed by measuring the formation of hypodiploid DNA. FACS analysis revealed that 3 mM ATP induced the formation of hypodiploid DNA in about 30% of the cells within 4 h (Fig. 1B). A dose-dependent increase of this apoptotic alteration is demonstrated in Fig. 1C.

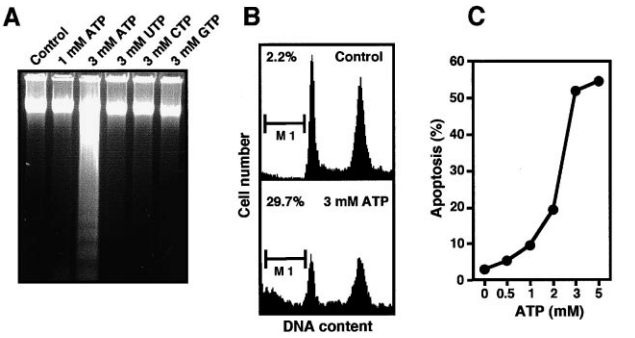

Fig. 1. Extracellular ATP but not other nucleotides induce apoptotic DNA fragmentation in N13 cells. (A) Gel electrophoresis of genomic DNA. Cells were incubated with the indicated concentrations of ATP and other nucleotides. DNA was isolated after 6 h. (B) Detection of hypodiploid DNA. Typical histograms of control cells (upper panel) and cells treated for 4 h with 3 mM ATP (lower panel) are shown. (C) Dose-dependent effect of ATP on the formation of hypodiploid DNA. Cells were incubated for 6 h with the indicated concentrations of ATP.

3.2. Involvement of caspases in ATP-induced cell death

Since caspases have been implicated as the principal mediators of apoptosis, we first investigated the cleavage of known caspase substrates during ATP-induced cell death. Poly(ADP-ribose)polymerase (PARP), a DNA repair enzyme, has been shown to serve as a substrate for caspase-3 [17]. Fig. 2A demonstrates that PARP, a 116 kDa protein, was cleaved into the characteristic 89 kDa fragment in the course of ATP treatment. Another substrate comprises lamin B, a component of the nuclear envelope, which can be cleaved by caspase-6 [18]. A marked cleavage of lamin B, a 66 kDa protein, into its 46 kDa fragment occurred within 4 h following ATP treatment (Fig. 2A).

Caspases are synthesized as inactive zymogens and converted to an active tetrameric complex composed of two heterodimeric subunits. As shown in Fig. 2B, ATP treatment of N13 cells resulted in the conversion of the inactive caspase-1 precursor to the proteolytically cleaved p20 subunit. Processing was maximal after 3 h and thereafter declined. In contrast, proteolytic processing of caspase-3 from the 32 kDa zymogen to the 17 kDa subunit occurred with a slightly different kinetic and was most pronounced after 5 h (Fig. 2B). We also investigated the processing of caspase-8, the most proximal caspase during CD95-mediated apoptosis. Caspase-8 is synthesized as a precursor of 54 kDa and, following formation of a 43 kDa intermediate cleavage product, processed to a p18 and p10 heterodimer [19]. In untreated N13 cells, an antibody against

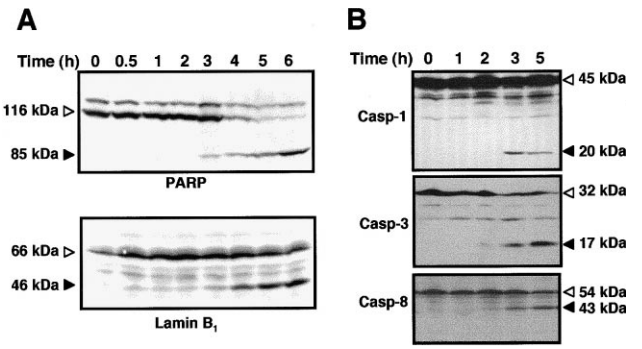

Fig. 2. P2Z receptor ligation triggers the activation of caspase proteolytic activity. N13 cells were treated with 3 mM ATP and after the indicated times, cellular lysates were prepared and subjected to immunoblotting using antibodies against the caspase substrates PARP and lamin B (A) and individual caspases. Open arrowheads indicate the full length and closed arrowheads the cleaved forms of the proteins.

the p18 subunit detected a doublet protein band of about 54 kDa representing procaspase-8a and -8b [20]. ATP treatment of N13 cells resulted in the conversion of the protein doublet to proteins of about 43 kDa corresponding to the intermediate cleavage products (Fig. 2B). So far, activation of caspase-8 appeared to be restricted to apoptosis triggered by death receptors [21]. However, since N13 cells are not CD95-sensitive, caspase-8 is presumably processed in cell death pathways other than CD95-mediated apoptosis. Consistently, we have observed that caspase-8 is specifically cleaved by a CD95-independent mechanism during chemotherapeutic drug-induced apoptosis [13].

3.3. Activation and regulation of different caspase members by ATP

Some forms of apoptosis have been shown to involve the proteasome pathway [22,23]. Thus, inhibitors of the proteasome function prevented cell death and activation of caspase-1 in sympathetic neurons following NGF withdrawal [23]. Using lactacystin, a highly specific inhibitor of the 20S-proteasome [24], we investigated whether proteasome activity was required for ATP-induced caspase activation. As shown in Fig. 3A, lactacystin at a concentration of 2.5 μM almost completely prevented ATP-induced caspase-1 activation. Interestingly, under the same conditions, processing of caspase-3 (Fig. 3A) and ATP-induced cytotoxicity (Fig. 3B) were not affected. These results suggest that the proteasome pathway is involved in the caspase-1 activation but not required for the activation of caspase-3 and cell death.

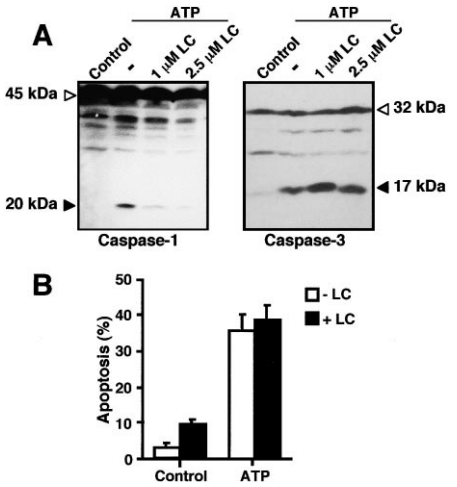

Fig. 3. Proteasome activity is involved in processing of caspase-1 but not caspase-3. (A) Cells were either left untreated (control) or incubated for 4 h with ATP in the presence and absence of the indicated concentrations of the proteasome inhibitor lactacystin (LC). Following SDS-PAGE of cellular lysates, processing of caspase-1 (left panel) and caspase-3 (right panel) was analyzed. (B) The effect of lactacystin on ATP-induced cytotoxicity. Cells were pretrated with or without 2.5 μM lactacystin and then exposed to ATP for 4 h. Cell death was measured as described in Fig. 1B.

3.4. Caspases are involved in ATP-induced apoptotic nuclear alterations but not cytolytic and membrane damage

To explore the functional involvement of caspases in ATP-induced cell death, we used peptide inhibitors more specific to certain caspases. Pretreatment of N13 cells with the broad spectrum inhibitor zVAD almost completely abrogated ATP-induced DNA fragmentation as measured by the release of incorporated BrdU into culture supernatants (Fig. 4A). YVAD inhibited DNA damage only when applied at high concentrations (Fig. 4B). In contrast, the BrdU release was markedly suppressed by DEVD, an inhibitor more selective for caspase-3 (Fig. 4C). This indicates that presumably caspase-3 rather than caspase-1 was functionally involved in the ATP-induced DNA fragmentation.

Since P2Z receptor ligation also induces characteristic fea-

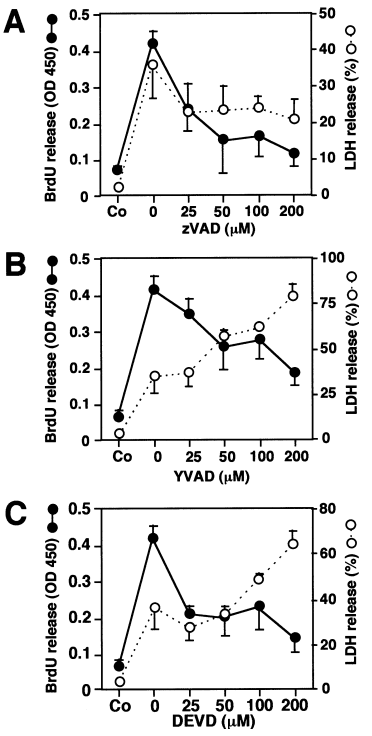

Fig. 4. The different effect of caspase inhibitors on ATP-induced DNA fragmentation and cell viability. N13 cells were either left untreated (Co) or treated with 3 mM ATP in the presence of the indicated concentrations of caspase inhibitors zVAD (A), YVAD (B) or DEVD (C) for 5 h. DNA fragmentation was assessed by released BrdU using an ELISA (closed symbols). The cell viability was determined by the release of LDH into supernatants and calculated as percentage of the total cellular LDH activity (open symbols). Data represent mean ± S.D. from three experiments.

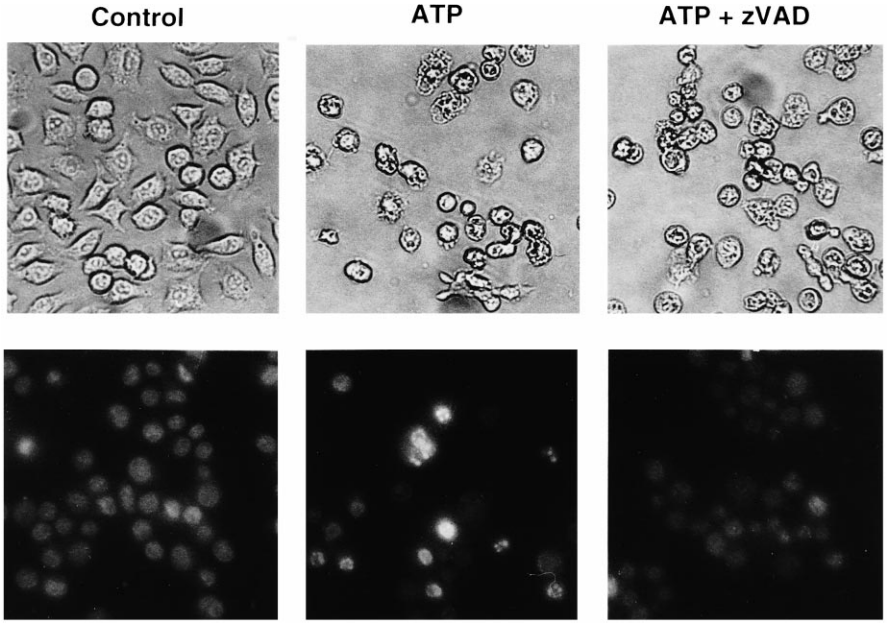

Fig. 5. Inhibition of caspases prevents nuclear but not cytoplasmic changes during ATP-induced cell death. Cells were either left untreated or incubated for 4 h with ATP in the presence and absence of 100 μM zVAD. Morphological alterations were analyzed by phase contrast microscopy (upper panel) and nuclear apoptotic changes by staining with the DNA dye Hoechst 33342 (lower panel).

tures of necrosis, we investigated the effect of the caspase inhibitors on cell lysis by measuring the cytoplasmic release of LDH. Within 5 h, ATP induced the release of about 35% of the maximal LDH activity (Fig. 4A). Pretreatment of cells with zVAD partially inhibited the LDH release. Surprisingly, when cells were pretreated with YVAD (Fig. 4B) or DEVD (Fig. 4C) prior to ATP stimulation, a dose-dependent increase in the release of LDH was observed. Control experiments revealed that this increase was not caused by a stimulation of the LDH enzymatic activity in vitro. In addition, we observed that in contrast to ATP-stimulated cells, the spontaneous release of LDH in untreated cells was dose-dependently inhibited by all caspase inhibitors (data not shown), indicating that YVAD and DEVD were not toxic on their own.

The role of caspases was further examined by microscopical evaluation and Hoechst staining of N13 cells. Morphologically, ATP treatment caused typical necrotic alterations such as cell swelling and vacuolization of the cytoplasm (Fig. 5). The nuclei of ATP-treated cells, however, revealed characteristic apoptotic alterations such as chromatin condensation and margination, visible by the brightly fluorescent cells. Inhibition of the caspase activity by zVAD had no significant effect on the cytoplasmic morphology and neither cell swelling nor vacuolization of the cytoplasm were affected. In contrast, the ATP-induced appearance of nuclear apoptotic morphology was completely prevented by the caspase inhibitor.

In summary, our experiments reveal several interesting findings. We demonstrate that stimulation of cells by extracellular ATP activated caspases selectively through the P2Z purinoreceptor. By the use of lactacystin, it is shown that activation of caspase-1 but not caspase-3 involves the proteasome pathway. However, caspase-1 activity is probably not required for cell death but rather involved IL-1β release [11], which is consistent with the observation that ATP-induced cell death is comprized neither by lactacystin nor in caspase-1 deficient mice [25]. Finally, we demonstrate that caspases are not required for all elements of P2Z receptor-mediated cell death. Although caspase inhibitors prevented apoptotic damage including chromatin condensation and DNA fragmentation, they did inhibit necrotic membrane and lytic alterations. This suggests that a single death stimulus can simultaneously initiate pathways leading to both apoptosis and necrosis. The form of cell death might be determined by the prevalence of a necrotic or apoptotic signal, but after blockade of one of the pathways, cells may still die by the other. Interestingly, similar to our data, inhibition of caspases has been observed to lead to increased oxidative stress and necrotic damage during TNF-mediated cytotoxicity in fibrosarcoma cells [26]. The potentiation of necrotic cell death may therefore constrain a potential therapeutic use of caspase inhibitors.

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