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Apoptosis-triggered redistribution of caspase-9 from cytoplasm to mitochondria

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Abstract **Caspase-9 is an apoptosis initiator protease activated as a response to the mitochondrial damage in the cytoplasmic complex apoptosome. By fluorescence labelling of proteins, confocal microscopy and subcellular fractionations we demonstrate that caspase-9 is in the cytoplasm of non-apoptotic pituitary cells. The activation of apoptosis with rotenone triggers the redistribution of caspase-9 to mitochondria. Experiments using the general caspase inhibitor z-VAD.fmk and the specific caspase-9 inhibitor z-LEHD.fmk show that the caspase-9 redistribution is a regulated process and requires the activity of a caspase other than the caspase-9. We propose that this spatial regulation is required to control the activity of caspase-9.**

Key words: Caspase-9; Apoptosis; Activation; Intracellular distribution; Mitochondrion; Rotenone

1. Introduction

Apoptosis is essential for controlling the quality and the number of cells [1]. It has a crucial role in tissue development and remodelling and, when deregulated, can result in cancer and in neurodegenerative diseases [2]. An ideal tissue in which to study these processes is the anterior pituitary, which is subject to tissue remodelling under different physiological conditions, such as physiological and psychological stresses, pregnancy and weaning [3]. The anterior pituitary responds by changing the secretory capacity of existing cells and by changes in apoptosis and in cell division [4]. Intense regulation of cell numbers may account also for the relatively high prevalence of pituitary tumors. Cell lines derived from pituitary adenomas were used as the model to study apoptosis before, e.g. AtT20 [5], GH3B6 [6] and MMQ [7]. Also, the heterozygous mice with disrupted retinoblastoma gene – the best characterized tumor suppressor gene – have pituitary tumors and are extensively used for studying cancerogenesis [8–10]. We studied differentiated pituitary somatotrophs, which synthesize and secrete growth hormone. Like in the other pituitary cells, the amount of growth hormone discharged from somatotrophs is regulated by numerous cellular signalling mechanisms [11] and also by the number of secretory cells available by apoptosis [12–14].

The central effectors of apoptotic process are intracellular cysteine proteases, the members of the caspase family [15–18]. Caspases are divided into initiator and effector caspases. The initiator caspases start the process of apoptosis by activating the downstream effector caspases. Caspase-9 is a central initiator caspase, triggered in a response to stimuli that damage mitochondria, either directly, or by signals originating in the other parts of the cells [19–22]. The irreversible activation of caspase-9, which involves the cleavage of its zymogen procaspase-9, occurs in the cytoplasm through its binding to Apaf-1 and cytochrome *c* in the presence of dATP [23–25]. In some cases, caspase-9 is also activated through cleavage by the other activated caspases, such as caspase-3 [26], caspase-8 [27] and caspase-12 [20]. The activation of caspase-9 can thus initiate the apoptotic process, or amplify it if it has started already.

There is disagreement about the intracellular localization of caspase-9 in the literature. It was reported to be in the mitochondria [28–30], in the nucleus [26,31] or in the cytoplasm [30–33]. Since the main route of caspase-9 activation is thought to be through the cytoplasmic Apaf-1 complex [23–25], one would expect that procaspase-9 is transported to this location in order to be activated.

Using immunocytochemistry, confocal microscopy and subcellular fractionations we found that procaspase-9 is in the cytoplasm in the non-apoptotic pituitary cells. We show that after the induction of apoptosis by the application of rotenone, caspase-9 becomes co-localized with mitochondria. The redistribution of caspase-9 is inhibited in the presence of z-VAD.fmk, a wide-range caspase inhibitor, whereas the specific inhibition of caspase-9 by z-LEHD.fmk does not affect the shift of caspase-9 from cytoplasm to mitochondria. An activated caspase, other than caspase-9, is therefore required for triggering the procaspase-9 redistribution to mitochondria in rotenone triggered apoptosis. Even the procaspase-9 was reported to be proteolytically active [34,35]. We propose that the spatial regulation described here controls the activity of caspase-9, since it affects the local concentration, possibly dimerization, of the procaspase-9 or limits the contacts of procaspase-9 with its targets in non-apoptotic cells.

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Abbreviations: EGFP, enhanced green fluorescent protein; TMRM, tetramethylrhodamine methyl ester; Casp9E, a fusion protein between caspase-9 and EGFP; Sec61E, a fusion protein between Sec61 and EGFP

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2. Materials and methods

2.1. Construction of fusion protein Casp9E

A fragment containing human caspase-9 was amplified from ICE-Lap6his pcDNA3 [36] with the primer pairs N′casp9 (5′-ccc cgg tac cgc cat gga cga ag-3′) and C′casp9 (5′-ccc cgg atc ccg gtg gtg tga tgt ttt aaa gaa aag-3′) by polymerase chain reaction (PCR). The PCR fragment was introduced into *Kpn*I and *Bam*HI sites of a plasmid pEGFP-N1 (Clontech). In the resulting fluorescent fusion protein Casp9E, the caspase-9 and enhanced green fluorescent protein (EGFP) moieties are linked by the peptide: HHRDPPVAT.

2.2. Cell cultures, transfection, subcellular fractionation and immunocytochemistry

Primary cell cultures enriched with somatotrophs were isolated from the anterior lobe of pituitary glands of adult male rats (Wistar, 200–300 g) as described by Ben-Tabou et al. [37]. Cells were placed on poly-L-Lys-coated coverslips, incubated in a standard medium [37] and 24 h later the DNA was introduced using Lipofectamine PLUS™ reagent (Invitrogen), according to the manufacturer's instructions. The cells were observed under a confocal microscope or were labelled by immunocytochemistry. Mitochondria were labelled by 600 nM tetramethylrhodamine methyl ester (TMRM, Molecular Probes). Whenever necessary, 30 or 300 μM rotenone was added for 2 h in a humidified CO_2 atmosphere at 37°C. Apoptotic cells were detected by the annexin V conjugate with the dye Alexa Fluor 568 (Molecular Probes) according to the manufacturer's instructions [38]. Subcellular fractions were prepared from rat anterior pituitaries in *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES)-based buffer as is described by Werner and Neupert [39]. The samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting and were detected by the same antibodies as were used for immunocytochemistry.

The immunocytochemistry was performed using the standard protocol as described by the suppliers. The following antibodies were used: anti-caspase-9 (Cell Signalling Technology, Inc.), anti-cytochrome *c* (BD Biosciences) and the appropriate secondary antibodies conjugated to fluorescent dyes Alexa Fluor 546 (red, Molecular Probes) and Alexa Fluor 488 (green, Molecular Probes). The coverslips were mounted using Light Antifade Kit (Molecular Probes). For double staining the two primary antibodies (raised in different species) were added sequentially. Unspecific labelling by antibodies was tested by staining the cells with fluorescent secondary antibodies only.

2.3. Confocal microscopy and image analyses

The fluorescent images were collected using a Zeiss LSM 510 confocal microscope with an oil immersion objective (40× and the numerical aperture 1.3). EGFP and the conjugate Alexa Fluor 488 were excited by argon laser (at 488 nm). The fluorescence was collected through the band pass filter (505–530 nm). For excitation of the conjugate Alexa Fluor 546 the He/Ne laser with bandwidth of 543 nm was used in combination with a long pass filter, which cut off the light below 560 nm.

The images obtained were analyzed by the program written for Matlab software (unpublished), which counted the numbers of green, red and co-localized pixels for each image. We calculated the percentages of co-localized pixels regarding to all pixels above the threshold. Data were subjected to statistical analyses using unpaired, two-tailed Student's *t*-test for equal variances.

3. Results

3.1. Casp9E mimicks the intracellular distribution of the endogenous caspase-9

We constructed a fusion protein between the caspase-9 and the EGFP (Casp9E), then introduced it into pituitary somatotrophs, which produce growth hormone. Using an antibody against the growth hormone, the cell cultures were checked to contain about 80% of somatotrophs (not shown) [37].

The subcellular distribution of Casp9E was compared with that of the endogenous caspase-9 by using fluorescently labelled antibody against caspase-9 (α-Casp9), which recognized

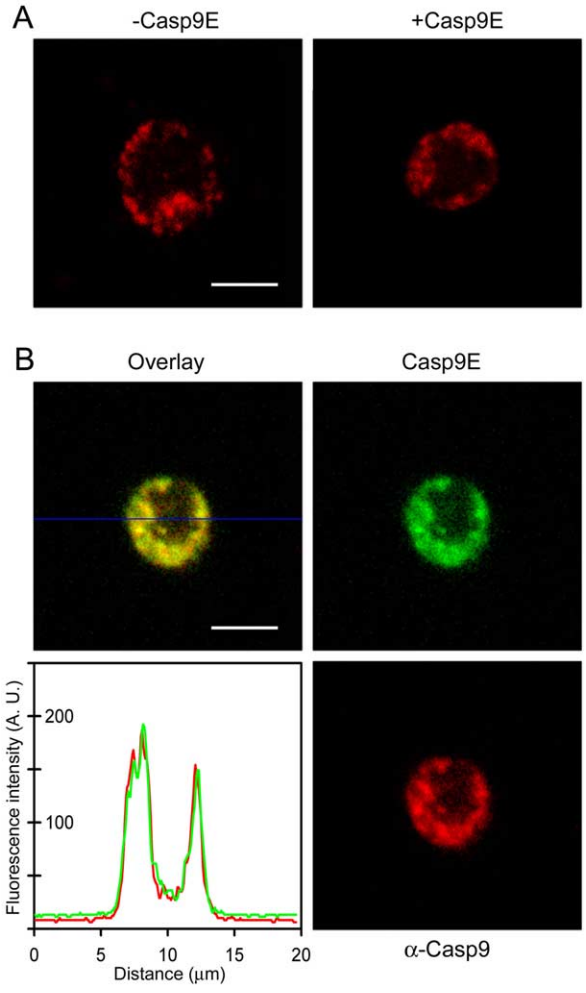

Fig. 1. Casp9E mimicks the intracellular distribution of caspase-9. Bar: 5 μm. A: Localization of endogenous caspase-9 (−Casp9E) and of the endogenous caspase-9 with Casp9E (+Casp9E) stained with rabbit antibody against the caspase-9 moiety (α-Casp9). B: The representation of complete co-localization of the two fluorescent markers; green: Casp9E and red: α-Casp9. The level of co-localization was in this cell 64%. The intensity profiles of the two fluorescence channels were measured along the straight line through the center of the cell image parallel to the plane of the coverslip (blue line).

both the wild-type caspase-9 and its fluorescent counterpart Casp9E. The images of the cells expressing the endogenous caspase-9 were compared to the images of the cells expressing both the endogenous caspase-9 and the fluorescent Casp9E (Fig. 1A). The red fluorescence signals of the labelled α-Casp9 antibody were similarly distributed in both types of the cells. Thus, the intracellular distribution of Casp9E mimicks that of its wild-type counterpart within the cells.

The comparison of the emissions of endogenous caspase-9 and Casp9E was taken as an example of completely co-localized markers (Fig. 1B). The green and the red fluorescence signals were sequentially acquired, in order to prevent the cross talk between the two channels. Co-localization of the two markers appears yellow in confocal images. Appearance of some green and red pixels in otherwise yellow overlaid image is likely due to unequal intensities of the two fluorescent markers, inherent in fluorescent microscopy. The steric hindrance of antibodies with attached labels was previously

observed to contribute to the reduction of the antibody binding [40] and also may contribute to the small scatter of fluorescence intensities.

The distribution of the two fluorescent markers was compared by plotting their intensity profiles (Fig. 1B) and showed that the intensities of both signals overlapped. The fluorescence intensities of the two channels were measured along the straight line through the center of the cell images parallel to the plane of the coverslips. The intensities of the two fluorescent signals were normalized so that the intensity of each covered the entire range of shades (0–255). The two adjacent pixels were averaged to filter out the noise.

We determined the degree of co-localization of the fluorescent signals of α-Casp9 and Casp9E on 20 cells from three experiments. To avoid the bias due to subjective analyses, the procedure was automated using a computer program (unpublished), which counts the number of red, green and yellow, i.e. co-localized, pixels for each cell. The intensities of the two fluorescent signals were normalized for each cell in order to compare the images. To be counted, the pixel intensity had to be above the threshold, which was arbitrarily taken to be 20% of the intensity of the brightest pixel of each image. The number of co-localized pixels was expressed as the ratio between the co-localized pixels (yellow) and the number of all fluorescent pixels of the cell image. For example, if half of the pixels of each channel were co-localized in a cell, then the percentage of co-localization equalled 33%. The average percentage of co-localized pixels from 20 fluorescent cells in the case of perfectly co-localized markers Casp9E and α-Casp9 was 68 ± 3%.

In conclusion, the red fluorescence signals of the labelled α-Casp9 antibody are equally distributed in the cells expressing only the wild-type caspase-9 and the cells expressing both caspase-9 and Casp9E. Therefore, Casp9E is a suitable marker for tracing the subcellular localization of the endogenous caspase-9 in the rat pituitary cells.

3.2. *Caspase-9 is localized in the cytoplasm of non-apoptotic cells*

The intracellular distribution of Casp9E appears punctate (Fig. 1A), which may be a sign of its association with organelles. We therefore compared the localization of caspase-9 to that of Sec61, an integral protein of endoplasmic reticulum [41], and to the integral protein of the mitochondrial inner membrane Tim23 [42]. When the intracellular distribution of the wild-type caspase-9 is compared to the localization of EGFP-labelled Sec61 (Sec61E, Fig. 2A), the localization of each marker is distinct. Likewise, the two fluorescence signals do not overlap on the fluorescence intensity profiles. The degree of co-localization of Sec61E and α-Casp9 is 38 ± 4% from the analysis of 18 cells, which is considerably lower than in the case of complete co-localization of the two markers (Fig. 1). Therefore, the lack of association of caspase-9 with the ER is reflected also in this low degree of numerical co-localization; this is a quantitative example of intracellular distribution of the two unrelated fluorescent markers in the cell.

We then investigated the intracellular distribution of caspase-9 in relation to the mitochondrial marker Tim23 by comparing the fluorescence signals of Casp9E and labelled antibodies against Tim23 (α-Tim23, Fig. 2B). The two markers do not appear co-localized, either in the whole cell images or when the intensities of the two channels are plotted. The per-

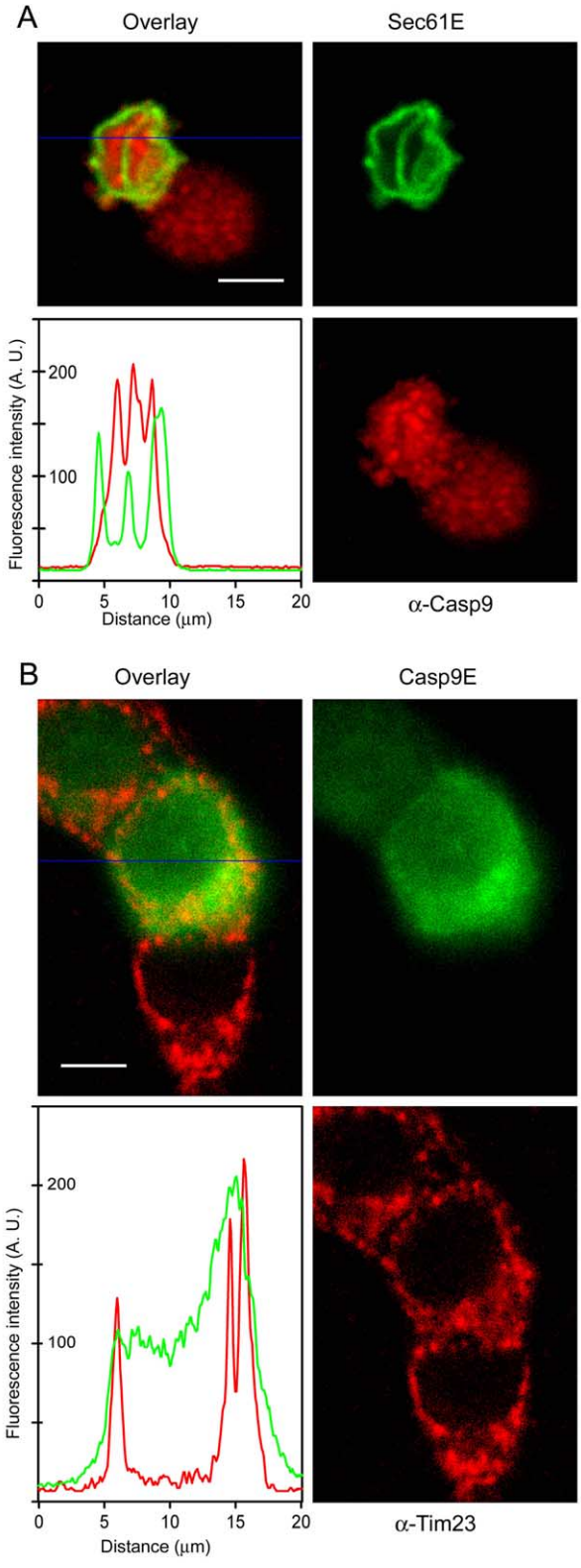

Fig. 2. Caspase-9 is localized in the cytoplasm of non-apoptotic cells. Bar: 5 μm. A: Co-localization of the endogenous caspase-9 with the endoplasmic reticulum marker, Sec61 fused to EGFP (Sec61E). There are two cells in the image, one of them labelled with Sec61E (green). B: Co-localization of Casp9E with the endogenous mitochondrial protein Tim23, labelled with specific antibodies (α-Tim23) was in the cell shown 22%. Two of the three cells in the image are labelled with Casp9E.

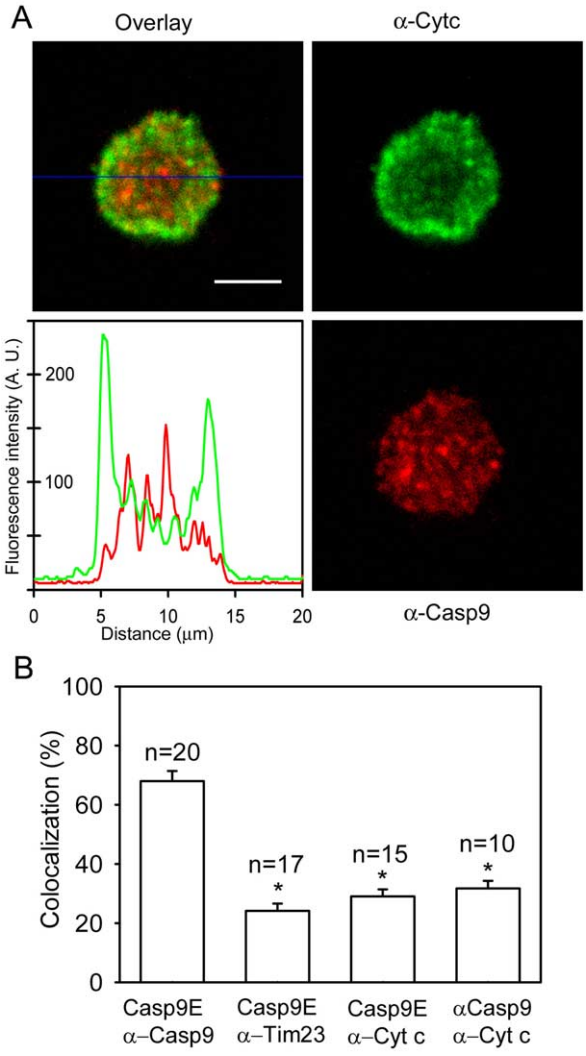

Fig. 3. Caspase-9 is not co-localized with mitochondria in non-apoptotic cells. Bar: 5 μm. A: The localizations of the endogenous caspase-9 (α-Casp9) and of the endogenous cytochrome *c* (α-Cytc) was in the cell shown 27%. B: The representation of the degree of co-localization of caspase-9 with several mitochondrial markers. (*n*: the number of analyzed fluorescent images; *: $P \ll 0.01$).

centage of co-localized pixels from 17 cells equals to 24 ± 3%. These results indicate that at least a major part, if not all of caspase-9 is in the cytoplasm of pituitary cells.

Caspase-9 was detected in the intermembrane space of mitochondria in previous studies [28,29]. We therefore decided to double check the intracellular localization of caspase-9 with that of cytochrome *c*, the protein of the mitochondrial inter-

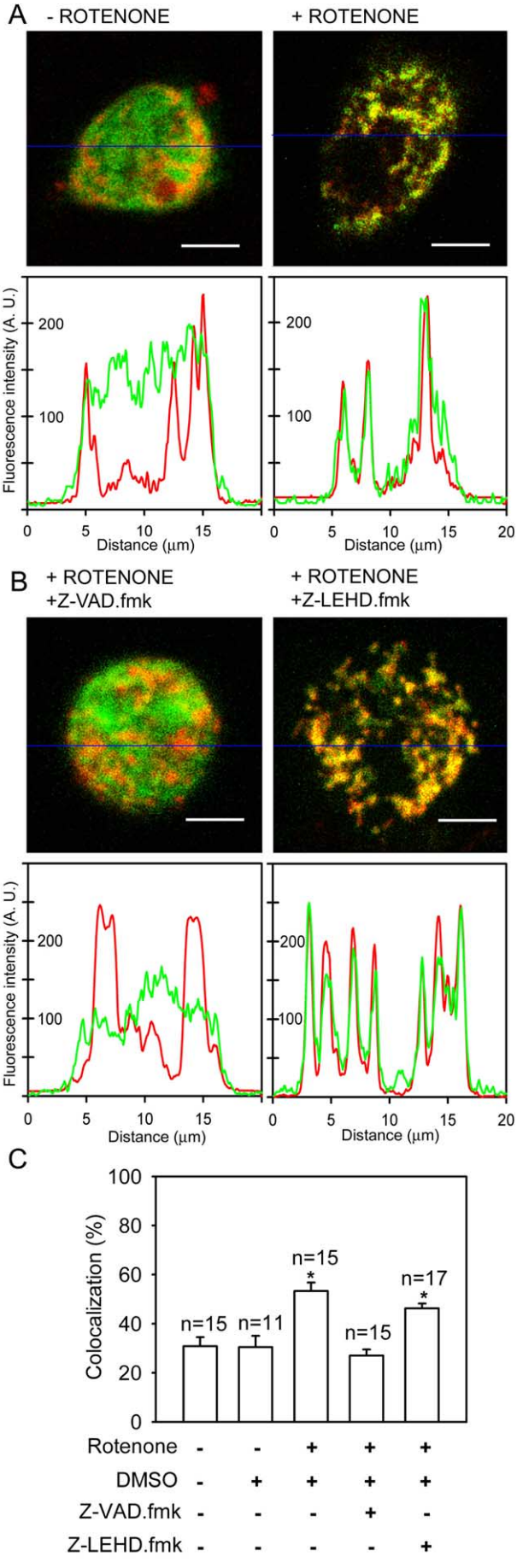

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Fig. 4. The redistribution of caspase-9 from cytoplasm to mitochondria after rotenone-induced apoptosis. The images of living cells labelled with Casp9E and the mitochondrial dye TMRM. 300 μM rotenone was added for 2 h, although comparable results were obtained by 30 μM rotenone incubated for 8 h. Bar: 5 μm. A: Images of co-localization of caspase-9 and of mitochondria before and after the addition of rotenone. The level of co-localization in the presented cell was before rotenone application 28% and after 53%. B: Images of co-localization of caspase-9 and of mitochondria after the addition of rotenone with caspase inhibitors z-VAD.fmk and z-LEHD.fmk. C: The percentage of co-localization (mean and standard error) of Casp9E and TMRM (*: $P \ll 0.01$).

membrane space. The endogenous caspase-9 and the endogenous cytochrome *c* were fluorescently labelled by secondary antibodies: red for the caspase-9 and green for the cytochrome *c* (Fig. 3A). Although both signals were of punctate appearance, they did not overlap. The alternating peaks of the red and the green fluorescence measured along the central plane of optical slices confirmed distinct distributions of caspase-9 and cytochrome *c*. The level of co-localized pixels from the analysis of 10 cells was 29 ± 2%, similar to the co-localized pixels of Casp9E with Tim23, Sec61 and cytochrome *c* (Fig. 3B). Hence caspase-9 was not localized within mitochondria under our conditions. We thought that the differing results regarding the intracellular localization of caspase-9 may be a consequence of different physiological states of the cells. So we investigated whether the activation of apoptosis influenced the subcellular distribution of caspase-9.

3.3. Apoptosis-associated redistribution of caspase-9

At least some components of apoptosome, in which caspase-9 is activated, are in different subcellular compartments. These have to be delivered to the site of assembly of the apoptosome complex upon the induction of apoptosis. To study whether the activation of apoptosis is associated with the redistribution of caspase-9, we monitored the subcellular localization of Casp9E before and after triggering of apoptosis by the application of rotenone. Mitochondria were labelled by TMRM, which accumulates in the mitochondria, depending on the mitochondrial membrane potential [43]. All fluorescent cells exhibited TMRM-labelled mitochondria.

2 h after the cells were exposed to rotenone they had no apparent morphological signs of apoptosis. Nevertheless, about 75% of the cells labelled with Casp9E were labelled also with the fluorescent conjugate of annexin V, an early marker of apoptosis [38]. In contrast, about 25% of the cells expressing Casp9E without added rotenone were labelled with annexin V (not shown). The number of necrotic cells, determined by staining the cells with the membrane impermeant nucleic acid stain Sytox Green, accounted for less than 1% of annexin V-labelled cells in either group of cells, with or without added rotenone. Therefore, practically all of the annexin V-labelled cells were apoptotic in our conditions. In conclusion, the treatment with rotenone has induced apoptosis in these conditions.

To follow the distribution of caspase-9 in living cells we collected confocal images of the cells, which were transfected with Casp9E and stained with TMRM. The cells were either sham treated (control cells) or had had rotenone added 2 h before the images were collected. Under the control conditions, the caspase-9 was spread out throughout the cytoplasm (Fig. 4A) as before (Fig. 2B). The degree of co-localization of Casp9E with TMRM from 15 cells is 31 ± 4%, which is similar to that of caspase-9 with other mitochondrial markers shown before (Fig. 3C). The addition of dimethyl sulfoxide (DMSO) in mock treatment did not influence the localization of caspase-9, since the degree of co-localization in 11 cells was 30 ± 5%. In contrast, the mitochondria-specific dye TMRM and the fluorescent Casp9E appeared co-localized after the triggering of apoptosis with rotenone in all cells studied and was apparent also on the fluorescence intensity profiles (Fig. 4A). The percentage of co-localization between the Casp9E and TMRM significantly increased from 31 ± 4 to 53 ± 3% in comparison to the co-localization of the caspase-9 and mitochondria before and after the rotenone treatment, respectively. The shift of caspase-9 from cytoplasm to mitochondria after the rotenone-induced apoptosis was confirmed also by subcellular fractionations (Fig. 5). This shift was detected 2 h after the addition of rotenone, though all caspase was associated with mitochondria when the cells were disrupted 3 h after the rotenone addition. The induction of apoptosis program therefore resulted in redistribution of caspase-9 from cytoplasm to mitochondria.

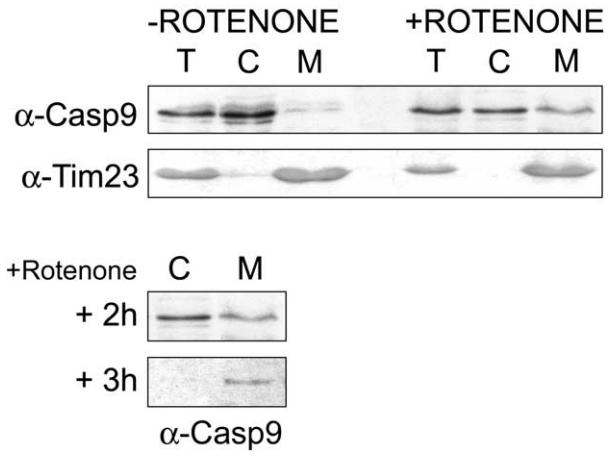

Fig. 5. Subcellular fractionations confirm the shift of caspase-9 from cytoplasm to mitochondria after the rotenone-induced apoptosis. Upper panels: The localization of caspase-9 without and with the addition of 300 μM rotenone for 2 h (−rotenone, +rotenone, respectively). Cell lysates (T), cytosolic (C) and mitochondrial fractions (M) were labelled with polyclonal antibodies against caspase-9 (α-Casp9) and against the mitochondrial marker Tim23 (α-Tim23). The lanes of the full-length caspase-9 (about 50 kDa) and of Tim23 (23 kDa) are presented. Lower panels: The shift of caspase-9 from the cytosol to the mitochondria is complete when the cells are disrupted 3 h after the addition of rotenone.

3.4. The redistribution of caspase-9 is triggered by a caspase other than caspase-9

There are two main pathways for caspase-9 activation: through an autoactivation process in the apoptosome and through activation by other types of caspases [20,23–27]. If the initiation of apoptosis by rotenone involves the activation of caspases before the activation of the caspase-9, the addition of a general caspase inhibitor should prevent the caspase-9 redistribution. Indeed, the addition of z-VAD.fmk (100 μM), a general caspase inhibitor prevents the rotenone-induced redistribution of caspase-9 (Fig. 4B). This is apparent also from the intensity profiles of the two channels and from the percentage of co-localization of 27 ± 2% from 15 cells.

We then tested whether a fraction of activated caspase-9 could trigger the redistribution of the rest of the caspase-9 in a mechanism of positive feedback loop. To investigate this possibility, we triggered the apoptosis by rotenone in the presence of z-LEHD.fmk, the specific caspase-9 inhibitor [44]. Surprisingly, the redistribution of caspase-9 was not prevented in the presence of z-LEHD.fmk (Fig. 4B). This result is also confirmed by analyses of the plotted profiles of fluorescence intensities and by the statistical analysis of the percentage of co-localization from 17 cells, which equals 46 ± 2%, which is significantly higher in comparison to the case of uninduced apoptosis (31 ± 4%) or that of rotenone-induced apoptosis in the presence of z-VAD.fmk (27 ± 2%). The neg-

ative result of z-LEHD.fmk treatment on caspase-9 redistribution might have occurred due to the inactive z-LEHD.fmk preparation, therefore we checked the number of rotenone-induced apoptotic cells in the presence of z-LEHD.fmk. The numbers of apoptotic cells were expressed as the percentage of the numbers of annexin V-labelled cells in the presence and in the absence of inhibitor, taking the value of apoptotic cells in the presence of rotenone only as 100%. In the presence of z-LEHD.fmk the percentage of apoptotic cells was significantly reduced to 46 ± 3%, which indicates that z-LEHD.fmk was active in blocking apoptosis although it did not affect the redistribution of caspase-9. We conclude that the rotenone-mediated shift of caspase-9 in the early steps of apoptosis requires the activation of a caspase other than the caspase-9.

4. Discussion

We have demonstrated the redistribution of caspase-9 from the cytoplasm to mitochondria in early stages of apoptosis in rat pituitary cells. We found that the distribution of Casp9E equalled to that of the endogenous caspase-9. Both proteins appeared in the cytoplasm in a punctate manner, which may indicate that caspase-9 is in subcellular organelles. In previous studies, caspase-9 was found in mitochondria [28,29], in the cytosol [31,32], in the nucleus [26,31] or in combinations of these organelles. This and the punctate appearance of the caspase-9 signal prompted us to investigate whether caspase-9 is localized in mitochondria. The labelling of endogenous caspase-9 with endogenous cytochrome *c* by double immunocytochemistry demonstrated that both signals appeared punctate, but were intertwined. Therefore, caspase-9 is not within mitochondria of non-apoptotic pituitary cells.

We cannot exclude the possibility of nuclear localization of caspase-9. Nevertheless, unlabelled parts of the cells reminiscent of nuclei were frequently observed in confocal images (Fig. 1). We concluded that caspase-9 is not appreciably located in the nuclei of pituitary cells. Therefore, caspase-9 appears to be concentrated mainly in distinct structures within the cytoplasm. Our results on the cytoplasmic localization of caspase-9 are in agreement with those on the expression of caspase-9 in 293 and Jurkat cell lines [31] and those from cell fractionations of brain cortex [32]. In contrast, caspase-9 from rat brain was localized in mitochondria by electron microscopy [29]. Caspase-9 was associated with mitochondria also at confocal images of Rat-1 cell line and in isolated mitochondria from Balb/c mice [28]. Possibly the differences in localization of caspase-9 are due to the different cell lines. Another possibility is that the results differ because of different experimental conditions. This may explain the conflicting results of the two studies on the caspase-9 localization in rat brain [29,32].

We demonstrate here the redistribution of caspase-9 from cytoplasm to mitochondria in the early stages of apoptosis before the apoptosis-associated morphology is observed. At the same time, we can observe the early redistribution of plasma membrane phosphatidylserine by annexin V labelling in most cells, which is a general feature of apoptosis [38]. The caspase-9 redistribution does not occur in the presence of a general caspase inhibitor z-VAD.fmk, implying that the caspase activity is necessary for triggering it. Since z-VAD.fmk is a mechanism-based inactivator, it binds only to activated caspases [35]. Which caspase is active before the redistribution of caspase-9? One possibility is an initiator caspase other than caspase-9, such as caspase-12 [20]. Another possibility is that the apoptotic signal causes a small amount of already active caspase-9 to trigger the redistribution of caspase-9 in a positive feedback loop mechanism. Caspase-9 differs from other members of caspase family in that even its procaspase form can be proteolytically active [34,35].

The general caspase inhibitor z-VAD.fmk prevented the redistribution of caspase-9, although surprisingly the caspase-9-specific inhibitor z-LEHD.fmk did not. The possibility that one peptide would bind to the caspase-9, while the other would not can not be ruled out, though we think it is unlikely, since both of them were used to inhibit caspase-9 in numerous studies. Another explanation may be derived from considering the co-relation between the intracellular redistribution of caspase-9 with its activation. Since even the unprocessed monomers of caspase-9 can be proteolytically active, perhaps the most significant function of the apoptosome may be to irreversibly activate caspase-9. At least one other component of the apoptosome, cytochrome *c*, is spatially divided from other components of the apoptosome in the non-apoptotic cells. We propose that caspase-9, like cytochrome *c*, is spatially separated from the components of the apoptosome complex in non-apoptotic cells. Its punctate distribution in the cytoplasm implies that it is not freely spread around the cytosol, but it is bound to discrete structures. The rearrangement of caspase-9 is triggered as a consequence of the activity of an as yet undetermined caspase. The details of this triggering process and the connection of caspase-9 redistribution to its activation remain to be determined in further studies.

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