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7-Ketocholesterol induces reversible cytochrome *c* release in smooth muscle cells in absence of mitochondrial swelling

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Abstract

Objective: 7-Ketocholesterol, a major oxysterol in oxidized low-density lipoproteins in advanced atherosclerotic plaques, induces vascular smooth muscle cell (SMC) death. We investigated whether cytochrome *c* release participated in SMC death induced by 7-ketocholesterol and whether the processes were reversible. **Methods:** SMC cultures derived from the rabbit aorta were exposed to 25 μM 7-ketocholesterol. Cytochrome *c* and Bax were studied by means of immunofluorescence and immunoblotting, apoptosis by the TUNEL technique and mitochondrial structure by transmission electron microscopy. **Results:** 7-Ketocholesterol induced rapid upregulation of the proapoptotic protein Bax and its translocation from cytosol into the mitochondria (4 h). This was followed by mitochondrial cytochrome *c* release (65% at 8 h) into the cytosol, which was almost complete at 16 h. The mitochondria became spherical and ultracondensed, without showing signs of lysis. They clustered around the nucleus and were wrapped by wide cisternae of the rough endoplasmic reticulum. Cytochrome *c* release was not blocked by the pan-caspase inhibitor zVAD-fmk, in contrast to DNA fragmentation and SMC loss. Interestingly, upon removal of 7-ketocholesterol after 16 h and re-exposure to serum for 24 h, the mitochondrial cytochrome *c* content, their transmembrane potential and TUNEL labelling normalised and SMC loss decreased. However, none of these cell death markers was rescued when the SMCs had been exposed to the oxysterol for 24 h. **Conclusion:** The results indicate that cytochrome *c* release during oxysterol-induced SMC apoptosis is not caspase-dependent and occurs as a result of a reversible mitochondrial conformational change rather than swelling and rupture of the outer membrane. The reversibility of these events suggests that the apoptotic cascade could be arrested before a point of no return.

Keywords: Rabbit smooth muscle; Mitochondria; Cholesterol; Apoptosis; Atherosclerosis

1. Introduction

Oxidized low-density lipoproteins (LDL) play an important role in the development of atherosclerosis. Oxysterols are associated with the cytotoxicity of oxidized LDL and are found in high concentrations in advanced atherosclerotic plaques [1], especially in foam cells around the necrotic core [2]. Previously, we have demonstrated that these regions showed increased loss of smooth muscle cells (SMCs) through apoptosis [3]. Since SMCs are involved in the biosynthesis and maintenance of the fibrous cap, their disappearance could increase the vulnerability of the plaque to rupture. Interestingly, aggressive lipid lowering in cholesterol-fed rabbits was associated with a drastic reduction of SMC apoptosis and an increased deposition of fibrillar collagen in the plaque [4].

In vitro experiments have shown that 7-ketocholesterol, a major oxysterol in plaques, induces vascular SMC death with features of apoptosis, such as nuclear condensation and internucleosomal DNA fragmentation [5] through caspase 3 activation [6]. Caspases participate in the extrinsic activation of apoptosis triggered by ligands of death

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receptors at the cell surface, and in the executioner pathway [7]. More recently, a critical role of mitochondria in mediating intrinsic procaspase activation, starting inside the cell, has been demonstrated [8,9]. Biochemical changes of mitochondria precede the execution phase of apoptosis, including the release of apoptosis-inducing factor (AIF) [10,11], cytochrome *c* [12,13] and a subsequent reduction of the mitochondrial membrane potential ($\Delta\Psi_m$) [14,15]. Cytochrome *c* is a soluble protein of the intermembrane space, which participates in the electron transfer between complex III and complex IV of the respiratory chain [16]. The pro-apoptotic Bcl-2 family members (tBid, Bax) promote the translocation of cytochrome *c* from mitochondria to cytosol [17–19], an event that is prevented by anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-x_L) [8,14,20]. Mitochondrial cytochrome *c* release occurs in response to many intrinsic stimuli, e.g. growth factor deprivation, cytotoxic drugs or irradiation [8,9] as well as extrinsic signals (tumour necrosis factor α, Fas ligand) [21], when it is mediated by caspase 8-cleaved Bid (tBid) [17]. In the cytosol cytochrome *c* triggers the activation of procaspase 9 through binding of Apaf-1. Caspase 9 in turn activates the executioner procaspase 3.

The precise details of the cytochrome *c* release remain controversial and several models have been proposed [8]. Some involve mitochondrial swelling and rupture of the outer membrane due to the opening of the permeability transition pore (PTP) [20] or closure of the voltage-dependent anion channel (VDAC) [15]. Other models envision selective loss of outer membrane barrier function without irreversible damage to the mitochondria [8], either through direct channel formation by proapoptotic members of the Bcl-2 family [22] or through their interaction with VDAC, thereby increasing its pore size [23].

Therefore, the aim of this study was to examine whether cytochrome *c* release occurred during SMC apoptosis induced by 7-ketocholesterol, whether caspases were involved, whether it was the result of mitochondrial swelling and rupture, and whether these events were reversible.

2. Materials and methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Smooth muscle cells

Cultures of rabbit aortic SMCs obtained by enzymatic digestion as described [24] were used between passages 2 and 4. SMCs were seeded in 8-well slides (Falcon), cultured for 2 days in HamF'10 medium supplemented with 5% foetal bovine serum (FBS), and then incubated with PBS (untreated), 0.5 % ethanol (control) or 12.5, 25 or 50 μM 7-ketocholesterol (Sigma) in the presence of 2% FBS for 4, 8, 16 and 24 h. To determine viability SMCs were cultured in 12-well plates (Becton Dickinson) and neutral red (1%) was added 2 h prior to the end of the incubation [25]. The dye was extracted with 0.05 M NaH_2PO_4 in 50% ethanol and its absorbance (540 nm) was expressed as percentage of PBS-treated SMCs. Cell protein was precipitated with 5% trichloro-acetic acid, dissolved in 1 ml 0.1 N NaOH with 0.5% SDS and quantified [26]. In experiments using peptide caspase inhibitors, the cells were incubated at 37 °C for 1 h with Cbz-Val-Ala-Asp-fluorometyl ketone (zVAD-fmk) or Ac-Asp-Glu-Val-Asp-acid aldehyde (DEVD-CHO) (Biosource) before the addition of 7-ketocholesterol. $\Delta\Psi_m$ was measured using the J aggregate forming 5,5′ ,6,6′ -tetrachloro-1,1′ ,3,3′ -tetraethylbenzimidazolocarbocyanide iodide (JC-1, Molecular Probes). Cells were incubated with 3 μM JC-1 at 37 °C in the dark for 30 min before measuring fluorescence.

2.2. Subcellular fractionation and Western blotting

At different times after addition of 7-ketocholesterol, SMCs were trypsinized, collected by centrifugation, washed in PBS and suspended in an isotonic buffer (10 mM Hepes, pH7.4, 0.2 M mannitol, 0.07 M sucrose) supplemented with protease inhibitors [phenylmethylsulfonyl fluoride (0.15 mM), leupeptin (2μM), aprotinin (0.2 μM)]. After homogenisation, the suspension was centrifuged (900 × *g*, 5 min) to pellet the nuclei, followed by centrifugation at 10,000 × g (30 min, 4 °C) to obtain the heavy membrane pellet (HM) enriched in mitochondria. The supernatant was centrifuged at 130,000 × *g* (Beckman Ti80 rotor) for 1 h to yield the light membrane fraction (not analysed) and the soluble fraction. Samples were dissolved in isotonic buffer with 0.1% Triton X-100 and protein concentrations were determined [26]. Ten micrograms (HM fraction) and 8 μg (soluble fraction) were used for SDS-PAGE (12% Tris-glycine gels; BIORAD) and transferred to a nitro-cellulose membrane (Amersham). After blocking non-specific sites for 1 h at room temperature with 10% non-fat dry milk in Tris-buffered saline supplemented with 0.2% Tween 20, the membranes were incubated overnight at 4 °C with anti-cytochrome *c* or anti-Bax monoclonal antibody (mAb, dilution 1:1000, Pharmingen). To confirm equal loading and transfer, the membranes were subsequently stripped and re-probed with mAbs against cytochrome oxidase (COX-IV), lactate dehydrogenase (LDH) or β-actin. The immunoreactive proteins were visualized using horseradish peroxidase-linked goat anti-mouse antibody (Jackson ImmunoResearch Laboratories) with enhanced chemiluminescence (Amersham Life Sciences).

2.3. Immunohistochemistry

Cells were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.2% triton X-100 in PBS,

washed and incubated for 2 h with an anti-cytochrome *c* mAb (dilution 1:200 in PBS + 5% normal goat serum, Pharmingen). The cells were washed twice, and developed with a Cy3-labelled goat anti-mouse antibody (Molecular Probes). In the final wash, 2 μM Hoechst 33258 was added to the cells. For co-localisation studies, cells were first incubated with MitoTracker Green FM (0.2 μg/ml, Molecular Probes) for 30 min at 37 °C, and then fixed in paraformaldehyde before incubation with the anti-cytochrome *c* mAb detected by Cy3 conjugated second antibody. The slides were mounted in Vectashield mounting medium and observed by fluorescence microscopy.

2.4. Detection of DNA fragmentation

In situ visualization of DNA fragmentation was performed by TdT-mediated dUTP-biotin nick-end labelling (TUNEL) as described [3]. TUNEL positive nuclei were counted (10 fields) and expressed as percentage of the total number of nuclei.

2.5. Ultrastructural study

SMCs were fixed for 20 min in 2.5% glutaraldehyde (Fluka, Germany) in 0.1 M phosphate buffer at 4 °C. After rinsing in cold buffer, cells were postfixed at room temperature in 1% osmium tetroxide (Merck, Germany), dehydrated through ethanol (70%, 95%, 100%), incubated in ethanol–epon mixture, and impregnated overnight in Epon 812 resin (TAAB, England). The samples were embedded in Epon 812 resin blocks. Ultra-thin sections of comparable thickness were cut with a Reichert OmU3 ultramicrotome and recovered on copper rhodium grids. The grids were stained with uranyl acetate and lead citrate and observed with a Philips EM201 transmission electron microscope.

3. Results

3.1. Selection of 7-ketocholesterol concentrations

From 8 h onwards 7-ketocholesterol (12.5–50 μM) induced a concentration- and time-dependent loss of SMC viability subsequently followed by the disappearance of adherent cell protein (Fig. 1). The loss of protein was not seen with 12.5 μM 7-ketocholesterol. Since viability loss was about 50% when the SMCs were exposed to 25 μM 7-ketocholesterol for 16 h, these conditions were selected for further experiments.

3.2. Redistribution of cytochrome c and Bax

Untreated control SMCs displayed a punctuate cytochrome *c* distribution consistent with its mitochondrial

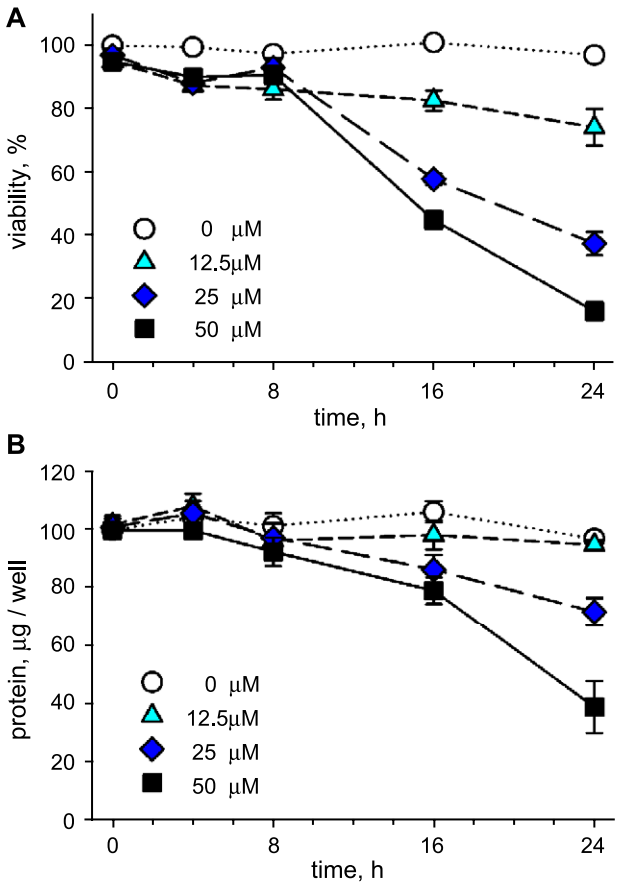

Fig. 1. Concentration- and time-dependent decrease of viability (% uptake of neutral red (A) and of adherent cell protein (B) induced by 7-ketocholesterol in rabbit SMCs (mean ± S.E.M., $n = 4$).

location, as confirmed by the co-localization with MitoTracker Green (Fig. 2A–C). In cultures exposed to 25 μM 7-ketocholesterol for 16 h most cells (about 95%) displayed cell condensation and a diffuse cytochrome *c* staining throughout the cytoplasm (Fig. 2D–F). Mitochondrial cytochrome *c* release was not observed after 4 h, but became evident at 8 h after addition of oxysterol (not shown). Western blot analysis confirmed the significant decrease of cytochrome *c* from the heavy membrane fraction at 8 h (−65%) and 16 h (−93%) of oxysterol-treated SMCs and its concomitant increase in the cytosol (Fig. 3A–B). Addition of the caspase inhibitors zVAD-fmk 100 μM or DEVD-CHO 50 μM before the oxysterol treatment did not affect mitochondrial cytochrome *c* release (Fig. 3A).

To test whether the cytochrome *c* release was reversible, 7-ketocholesterol was removed after 16 h and the SMCs were re-exposed to 5% FBS for 24 h. Immunohistochemistry revealed the reappearance of a punctuate mitochondrial cytochrome *c* staining in most cells (Fig. 2G–I). The effect of re-exposure to serum was blocked by 2 μM cycloheximide, which neither induced cytochrome release

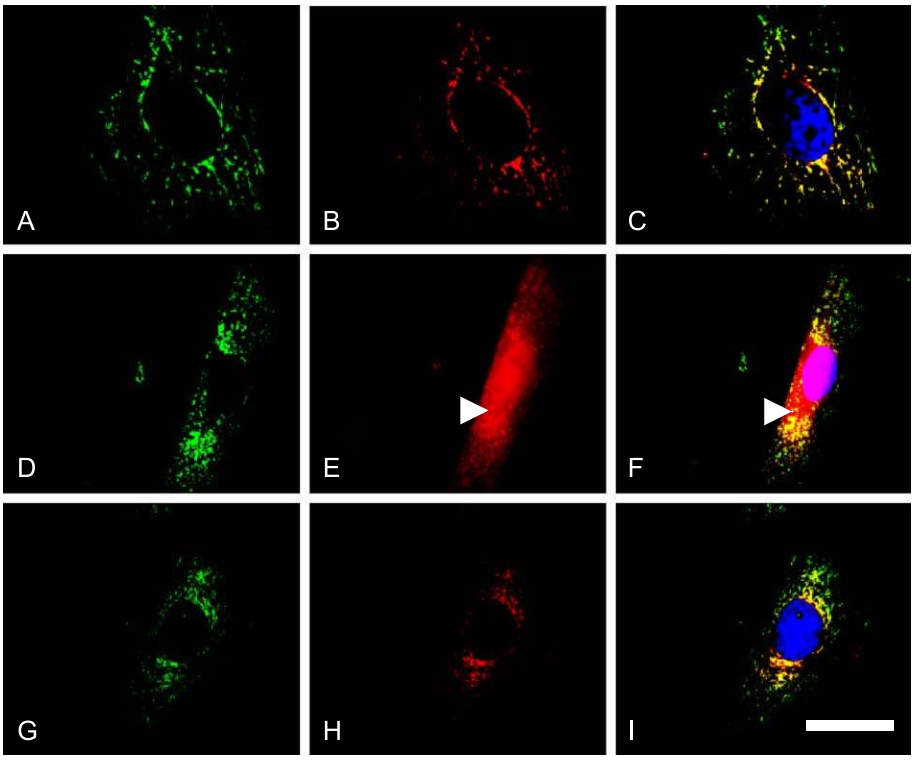

Fig. 2. 7-Ketocholesterol induces reversible cytochrome *c* release from mitochondria. In control SMCs immunofluorescence of MitoTracker Green (A) and cytochrome *c* (B) showed an orange color in the overlay (C), demonstrating co-localization of cytochrome *c* and the mitochondrial marker. SMCs treated with 25 μM 7-ketocholesterol for 16 h showed shrinking, increased peri-nuclear clustering of the mitochondria (D) and diffuse cytoplasmic cytochrome *c* staining (E) outside the mitochondria (E–F, arrow heads). Upon removal of 7-ketocholesterol after 16 h the SMCs showed a normalisation of cell shape, mitochondrial distribution (G) and recovery of the granular cytochrome *c* staining (H) that co-localized with MitoTracker Green (I) in the subsequent 24 h. Bar, 20 μm.

nor cell death by itself (data not shown). The mitochondrial cytochrome *c* recovery was confirmed by Western blot (Fig. 3C).

Immunoblot analysis showed Bax upregulation as early as 4 h after treatment with 7-ketocholesterol (Fig. 4A). In untreated cells Bax was mainly found in the cytosol (Fig.

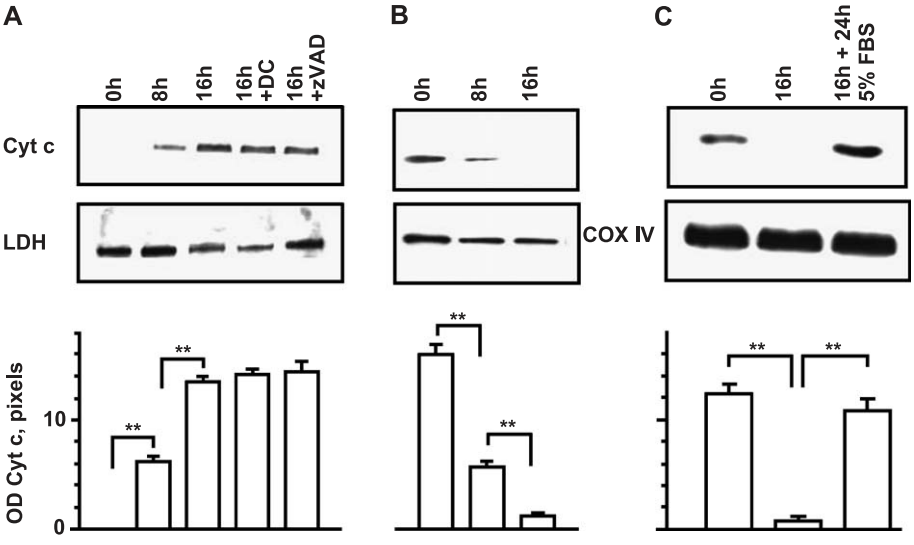

Fig. 3. Western blot analysis of cytochrome *c* redistribution in SMCs. After treatment with 25 μM 7-ketocholesterol for 8 or 16 h cytochrome *c* appeared in the soluble fraction (A), a process not inhibited by the caspase inhibitors DEVD-CHO (DC, 50 μM) or zVAD-fmk (zVAD, 100 μM) and cytochrome *c* disappeared from the heavy membrane fraction (B). In (C) SMCs were treated with 7-ketocholesterol or re-exposed to serum after oxysterol treatment and the heavy membrane fractions containing mitochondria were analysed for cytochrome *c*. Lactate dehydrogenase (LDH) and cytochrome *c* oxidase subunit IV (COX IV) were used as gel loading controls. The bottom panels show the statistical summary of the densitometric scanning of the cytochrome *c* bands (mean ± S.E.M., $n = 3$, $^{**}P < 0.01$).

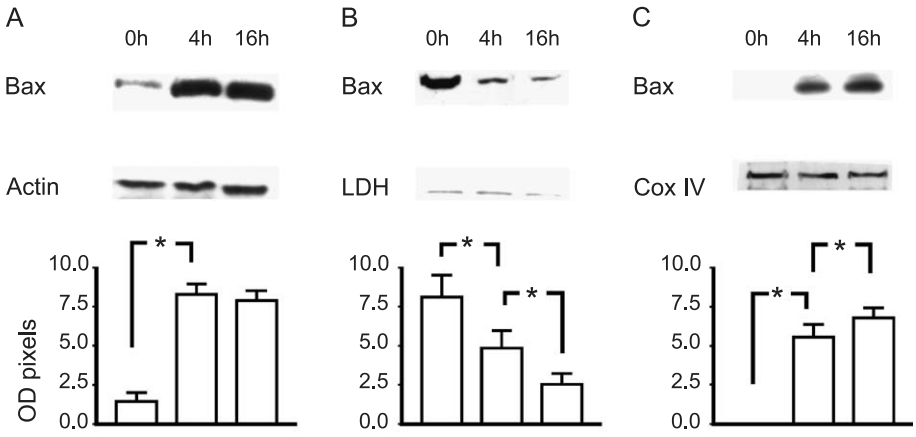

Fig. 4. Upregulation of Bax (A) and translocation from cytosol (B) into mitochondria (C) in SMCs exposed to 25 μM 7-ketocholesterol for 0, 4 and 16 h. Total cell homogenate (A), cytosolic (B) and heavy membrane fraction (C) were analysed by immunoblot with an anti-Bax mAb, and then re-probed with anti-β-actin, anti-lactate dehydrogenase (LDH), or anti-cytochrome *c* oxidase (COX IV) antibodies. The bottom panels show the statistical summary (mean ± S.E.M., $n=4$, $*P<0.05$) of the densitometric quantification of Bax.

4B). Already 4 h after treatment with 7-ketocholesterol, Bax decreased in the cytosol and became strongly associated with the mitochondria-enriched fraction as documented by the mitochondrial marker cytochrome *c* oxidase (Fig. 4C).

3.3. Mitochondrial membrane potential

Mitochondrial function was examined by using JC-1, a cationic dye that exhibits potential-dependent accumulation in mitochondria. Untreated SMCs showed the formation of orange fluorescent aggregates indicative of mitochondrial hyperpolarization (Fig. 5A). In contrast cells exposed to 7-ketocholesterol for 16 h were unable to accumulate orange fluorescent aggregates in mitochondria (Fig. 5B), which pointed to a loss of membrane potential. Cells treated with oxysterol for 16 h and re-exposed to serum for 24 h again demonstrated accumulation of orange fluorescent aggregates indicative of energized mitochondria (Fig. 5C).

3.4. Ultrastructure of mitochondria

Mitochondria in control SMCs appeared elongated-shaped with a fine granular matrix (Fig. 6A–B). The outer mitochondrial membrane was distinct and continuous. Lamellar cristae were visualized and their membranous layers were seen to be continuous with the inner membrane of the mitochondrial envelope. Mitochondria were occasionally adjacent to endoplasmic reticulum cisternae. After 16 h incubation with 7-ketocholesterol (Fig. 6C–D), nuclear condensation was not yet observed. However, a major change was the clustering of mitochondria around the nucleus. Although the mitochondrial structure was preserved, the mitochondria appeared ultracondensed with a hyper-dense matrix that obscured the internal structures. Wide cisternae of rough endoplasmic reticulum were often wrapped around the mitochondria. Only few mitochondria showed a modest degree of damage limited to a ballooning of the cristae referred to as "intracristal swelling". Swollen or ruptured mitochondria were not detected.

3.5. Tunel labelling

To prove that 7-ketocholesterol indeed induced apoptosis, DNA fragmentation was measured by means of the TUNEL-technique. After 16 h incubation with oxysterol the proportion of TUNEL-positive nuclei had increased (Fig. 7). The TUNEL frequency did not rise further at 24 h, but at that time SMC loss (−38%) became statistically significant. When the 7-ketocholesterol was removed after 16 h the percentage of TUNEL positive nuclei decreased drastically and the rate of SMC loss (−22%) slowed down upon 24 h incubation with 5% FBS. However, when 7-ketocholesterol was replaced with 5% FBS after 24 h, the percentage of TUNEL positive nuclei even tended to increase in the subsequent 24 h and the rate of SMC detachment (−42% in 24 h) remained equally high. Pre-treatment with zVAD-fmk almost abrogated TUNEL labelling and strongly (75%) suppressed SMC loss at 24 h (Fig. 7B–D).

4. Discussion

The present study shows that cytochrome *c* co-localized with mitochondrial markers in normal vascular SMCs and that 7-ketocholesterol induced a time-dependent release into the cytoplasm. The 7-ketocholesterol concentration employed in the present study (25 μM) was in the lower range (10–200 μM) used by others to induce SMC apoptosis [5,6]. Cytochrome *c* release has been reported for human leukaemia cells [27], but novel findings were that it was initially reversible and did not involve mitochondrial swelling or caspase activation, since broad-spectrum caspase inhibitors did not inhibit it. The latter finding indicates that

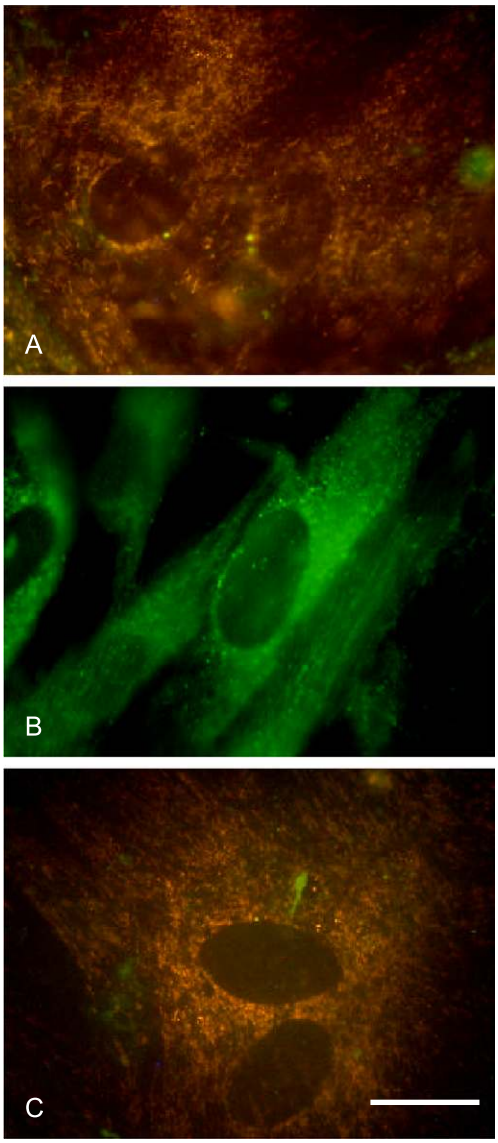

Fig. 5. Analysis of mitochondrial membrane potential. Untreated SMCs (A), cells treated with 25 μM 7-ketocholesterol for 16 h (B) or cells first exposed to oxysterol (16 h) and then to serum (24 h) (C) were incubated with JC-1 for 30 min. Polarized mitochondria are indicated by punctuate orange fluorescence (A), which is replaced by diffuse green fluorescence on depolarisation (B). Upon removal of 7-ketocholesterol the orange fluorescence re-appears, pointing to mitochondrial polarisation (C). Bar, 20 μm.

caspase-independent intrinsic pathways [28,29] rather than caspase-dependent [17,28] mechanisms were involved in the 7-ketocholesterol-induced cytochrome *c* release. Caspase 3 activation has been documented in 7-ketocholesterol-treated SMCs [6], and may amplify mitochondrial cytochrome *c* release at late stages by cleaving Bid [30] or Bcl-2 [31]. Since the late (16 h) cytochrome *c* release was not influenced by zVAD-fmk, it is further concluded that such a feedback amplification by caspase 3-cleaved proteins did not contribute substantially to the 7-ketocholesterol-induced cytochrome *c* release in SMCs.

In contrast, zVAD-fmk prevented 7-ketocholesterol-induced DNA fragmentation and inhibited SMC death by 75%. The former finding confirms the activation of downstream caspases in 7-ketocholesterol-induced SMC death [5,6]. The latter finding indicates that apoptosis was the predominant, but not the only cell death pathway. Indeed, depending on exposure time, concentration and cell type, oxysterol-induced cell death may also display hallmarks of autophagocytosis [32,33] or necrosis [32,34]. SMC loss slowed down considerably upon removal of 7-ketocholesterol after 16 h. A complete arrest of cell death was not anticipated, since SMCs showing DNA fragmentation and those that had entered the early execution phase without detectable TUNEL labelling, were expected to disappear. Interestingly, the remaining SMCs showed complete recovery of morphology, mitochondrial cytochrome *c* content and membrane potential, and DNA fragmentation had returned to control levels. In addition to cytochrome *c*, mitochondria may also release AIF, an oxidoreductase that translocates to the nucleus where it induces caspase-independent DNA fragmentation and chromatin condensation [10,11]. Co-release of cytochrome *c* and AIF has been demonstrated in SMCs, when reactive oxygen species were generated in the mitochondria [35]. The importance of AIF during oxysterol-induced SMC apoptosis remains to be determined, but it should be noted that DNA fragmentation was almost completely caspase-dependent in our setting.

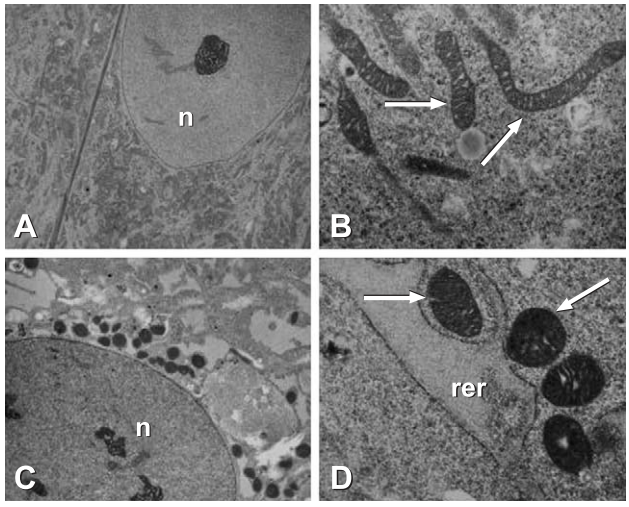

Fig. 6. Transmission electron microscopy of SMCs incubated for 16 h in the absence (A–B) or presence of 25 μM 7-ketocholesterol (C–D). Untreated cells (A, 6000 ×) showed a normal appearance with an intact nucleus (n). Mitochondria (arrows) appeared elongated-shaped and were scattered throughout the cytoplasm. At higher magnification (B, 30,000 ×), lamellar cristae are visualized and their membranous layer was continuous with the mitochondrial inner membrane. Following 16 h incubation with 7-ketocholesterol the nucleus remained normal but the mitochondria (arrow) were clustered around the nucleus (C, 6000 ×). At higher magnification (D, 30,000 ×) mitochondria appeared ultracondensed and the high electron density of the matrix obscured the internal structures. Mitochondria were often associated with wide cisternae of rough endoplasmic reticulum (rer).

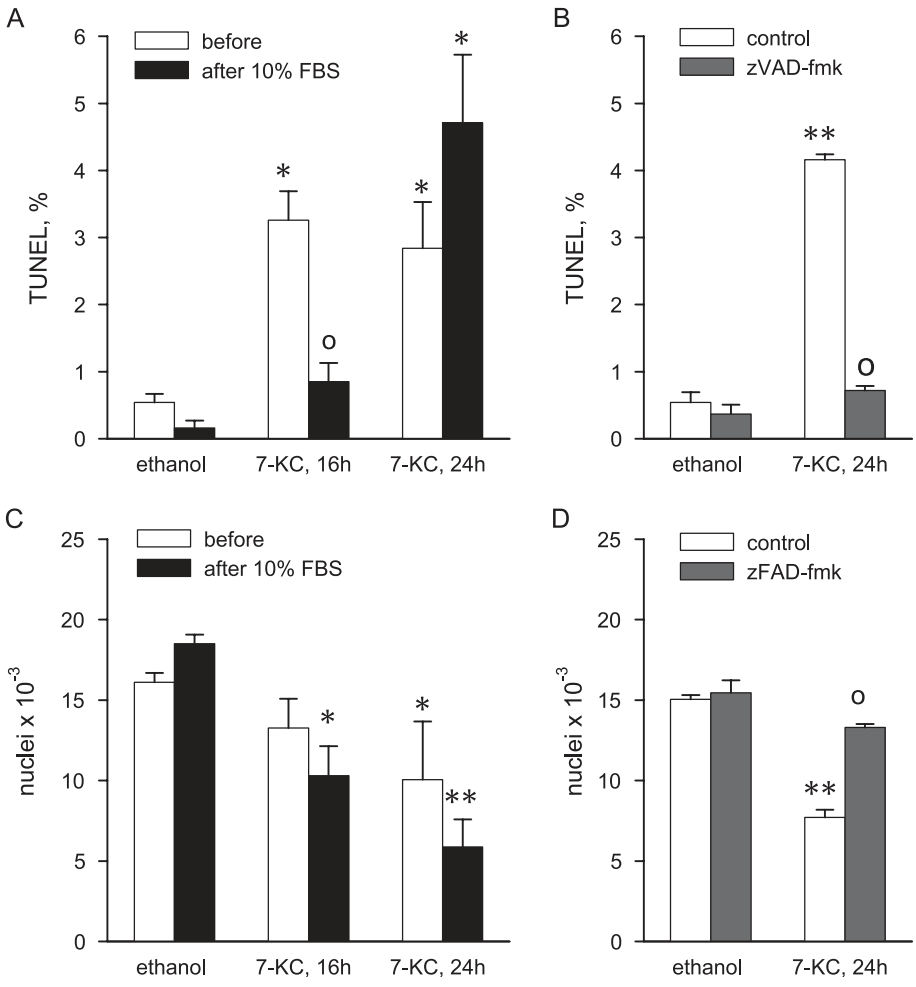

Fig. 7. 7-Ketocholesterol (25 μM) enhanced in situ TUNEL labelling (A) at 16 and 24 h, and decreased adherent SMC numbers after 24 h (C). TUNEL labelling was reversed when 7-ketocholesterol was replaced by 5% FBS after 16 h, but not after 24 h (A). The rate of SMC loss also decreased when 7-ketocholesterol was removed after 16 h, but not after 24 h exposure to oxysterol (C). Both TUNEL frequency (B) and SMC loss (D) were significantly suppressed by 100 μM ZVAD-fmk. Data are mean ± SEM of three experiments in triplicate. *$P<0.05$, **$P<0.01$, different from 0.5% ethanol; $^{\circ}P<0.05$ different from control (analysis of variance, Bonferroni post-hoc test).

The caspase-independent cytochrome *c* release was preceded by a marked upregulation and translocation of Bax into the mitochondria. Though this does not directly prove that Bax translocation was involved in cytochrome *c* release, there exists ample evidence that translocation and integration of Bax into mitochondria induces cytochrome *c* release in vivo and in vitro [19,36–39]. Indeed, a clone of murine macrophage-like cells with a highly reduced Bax expression has recently been shown to be strongly resistant to oxysterol-induced apoptosis [40]. Conversely, down-regulation of Bcl-2 has been documented in 7-ketocholesterol-treated SMCs [6] and over-expressing Bcl-2 protein suppresses oxysterol-induced apoptosis in murine macrophage-like cells [41]. However, participation of pathways not related to the Bcl-2 family cannot be excluded. Cytochrome *c* release can be triggered by the unrelated transcription factor TR3, which induces apoptotic cell death upon translocation from the nucleus to the mitochondria [9,42]. Also increased oxidative stress may induce mitochondrial cytochrome *c* release as well [9]. In oxysterol-treated pro-monocytic leukaemia cells enhanced generation of superoxide anion occurs before and after the loss of mitochondrial potential. Vitamin E counteracted mitochondrial dysfunction and cell death, suggesting that superoxide anion is a messenger of the cell death pathways triggered by 7-ketocholesterol [33]. Yet, other anti-oxidants suppressed oxysterol-evoked superoxide levels as well, but failed to rescue the cells. Therefore, superoxide anion generation could be a consequence of cell death rather than a second messenger of oxysterol triggered cell death pathways [27].

In view of the controversy about the mechanisms of the cytochrome *c* release, the mitochondrial ultrastructure was studied. Mitochondria in normal SMCs were elongated and randomly scattered in the cytoplasm, whereas peri-nuclear clustering occurred upon 7-ketocholesterol treatment. The intriguing clustering of mitochondria around the nucleus has also been shown in HeLa cells transfected with tBid [17], but its physiological significance remains largely unclear.

Furthermore, the mitochondria appeared to be morphologically intact, but pronounced ultracondensation was seen during 7-ketocholesterol-induced SMC apoptosis, confirming results in apoptotic nodal myocytes [43], herbimycin-treated carcinoma cells [44] and mouse embryo fibroblasts during Bax insertion into the mitochondria [39]. The ultracondensation presumably results from loss of water and ions during the transition of mitochondria from a "high energy state" to a "low energy state" [45]. In accordance with a "low energy state", the mitochondria showed a disruption of the $\Delta\Psi_m$ at 16 h. Previously the "low energy state" has been linked to upregulation of proteins involved in oxidative phosphorylation [46]. Consistent with this, we found that mitochondria of oxysterol-treated cells were closely associated with wide cisternae of the rough endoplasmic reticulum, which is an indication of increased protein synthesis and active mitochondrial import to maintain ATP generation. It is thus likely that the mitochondria of the oxysterol-exposed SMCs were still functional. This assumption is further supported by the normalisation of both mitochondrial cytochrome *c* content and $\Delta\Psi_m$ after removal of 7-ketocholesterol. The marked upregulation of manganese superoxide dismutase in mitochondria of oxysterol-exposed murine macrophage-like cells, an adaptive response to the generation of reactive oxygen species, is consistent with maintenance of their function [32].

Various mechanisms could contribute to the mitochondrial cytochrome *c* recovery after re-exposure to serum. Re-uptake of extracellular cytochrome *c* seems unlikely. Pinocytic uptake of exogenous cytochrome *c* into intact cells has been reported, but invokes caspase-dependent mitochondrial permeabilization and apoptosis [47]. Translocation of cytoplasmic cytochrome *c* into the intermembrane space constitutes another possibility. In isolated mitochondria, the early tBid-induced cytochrome *c* release and the subsequent respiratory dysfunction can indeed be rescued by re-addition of exogenous cytochrome *c* [48]. Over time, the progressive damage became irreversible, and could not be rescued by exogenous cytochrome *c*. In intact cells, however, cytochrome *c* is degraded in the cytoplasm [49] and mitochondria only import apo-cytochrome *c*, the precursor of cytochrome *c* [16]. Apo-cytochrome *c* is encoded by a nuclear gene, synthesized on cytoplasmic ribosomes and then targeted to the mitochondria. It passes through the outer mitochondrial membrane via electrostatic association with phospholipid head groups [16]. This mode of cytochrome *c* import requires an intact outer mitochondrial membrane. Consistent with this, the mitochondria of oxysterol-treated SMCs showed a condensed but well-preserved ultrastructure and were often associated with the rough endoplasmic reticulum. Hence, the recovery of cytochrome *c* could be due to mitochondrial import of newly synthesized apo-cytochrome *c*. The finding that the recovery was blocked by addition of 2 μM cycloheximide supports this assumption.

Very similar findings have been reported for nerve growth factor-deprived sympathetic neurons protected from apoptosis by caspase inhibitors: the mitochondria were depleted of cytochrome *c* and reduced in size, but retained an intact ultrastructure. After re-addition of nerve growth factor, the mitochondria recovered their morphology and cytochrome *c* content by a process requiring de novo protein synthesis as well [50]. Collectively, these data thus support models in which the efflux of cytochrome *c* is a controlled process resulting from subtle pore formation [8,22,23] and not those models envisioning mitochondrial swelling and rupture of the outer membrane, either due to opening of the PTP or closure of VDAC [15,20].

In conclusion, this study demonstrates that 7-ketocholesterol induces release of mitochondrial cytochrome *c* in vascular SMCs in a concentration that was equivalent to the 7-ketocholesterol content of advanced human carotid artery plaques [1], where evidence for apoptotic SMC death exists [3]. The cytochrome *c* release is not caspase-dependent, occurs in the absence of mitochondrial swelling and was initially reversible. The mechanisms of the reversibility remain unknown, but normalisation of oxysterol-induced oxidative stress [27,33] constitutes a possibility. The observation that SMCs can recover cytochrome *c* and survive after withdrawal of 7-ketocholesterol suggests that the apoptotic cascade can be arrested before a point of no return. This could be relevant to the observation that the frequency of apoptotic SMCs in rabbit atherosclerotic plaques drops strongly after drastic cholesterol lowering, and this was accompanied by signs of increased plaque stability [4].

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