7-Ketocholesterol Induces Protein Ubiquitination, Myelin Figure Formation, and Light Chain 3 Processing in Vascular Smooth Muscle Cells

Wim Martinet, Martine De Bie, Dorien M. Schrijvers, Guido R.Y. De Meyer, Arnold G. Herman, Mark M. Kockx

Objective—Oxysterols such as 7-ketocholesterol (7-KC) are important mediators of cell death in atherosclerosis. Therefore, in vitro studies of human smooth muscle cell (SMC) death in response to 7-KC were undertaken to investigate the potential mechanisms.

Methods and Results—Human aortic SMCs treated with 7-KC showed enhanced immunoreactivity for the oxidative stress marker 4-hydroxy-2-nonenal and upregulated several stress genes (70-kDa heat shock protein 1, heme oxygenase 1, and growth arrest and DNA damage–inducible protein 153) at the mRNA but not at the protein level. 7-KC–treated SMCs rapidly underwent cell death as determined by neutral red, counting of adherent cells, and depolarization of the mitochondrial inner membrane. Cell death was associated with upregulation of ubiquitin mRNA and ubiquitination of cellular proteins. Inhibition of the proteasome by lactacystin potentiated considerably the toxicity of 7-KC. Transmission electron microscopy revealed formation of myelin figures, extensive vacuolization, and depletion of organelles. Formation of autophagosomes was suggested by labeling cells with LysoTracker and monitoring processing of microtubule-associated protein 1 light chain 3 (LC3). Analogous to our in vitro studies, human atherosclerotic plaques showed signs of ubiquitination in SMCs.

Conclusions—7-KC activates the ubiquitin–proteasome system and induces a complex mode of cell death associated with myelin figure formation and processing of LC3 evocating autophagic processes. (Arterioscler Thromb Vasc Biol. 2004; 24:2296-2301.)

Key Words: 7-ketocholesterol ▪ ubiquitination ▪ smooth muscle cells ▪ myelin figures ▪ LC3 ▪ autophagy ▪ atherosclerosis

Cell death is a major event in the progression of atherosclerosis. Indeed, a large body of evidence suggests that apoptosis or type I programmed cell death frequently occurs in advanced human plaques (1% to 2% TUNEL-positive nuclei). However, results from electron microscopy studies showed that the majority of dying cells have an ultrastructure typical of cells undergoing “accidental” cell death or oncosis (type III programmed cell death). Furthermore, it is important to note that there are multiple pathways leading to cell death. Cells sometimes die with a morphology that is intermediate between apoptosis and oncosis (eg, aponecrosis and paraposis), or they undergo cell death with a less clear morphology or mechanism such as lysosome-mediated cell death or autophagy (type II programmed cell death). It is presently unknown whether cells in human atherosclerotic plaques die by mechanisms distinct from apoptosis or oncosis, as shown recently for myocytes in failing human hearts.

Oxidative processes, particularly oxidation of low-density lipoprotein (LDL), are thought to play a pivotal role in atherogenesis. Oxidized LDL (oxLDL) exerts its proatherogenic effects in several ways, including stimulation of inflammatory responses, foam cell formation, and induction of cell death. The mechanism of oxLDL-induced cell death is unclear, as oncosis and apoptosis have been reported, and largely depends on the oxidation degree, exposure time, and concentration of oxLDL. In macrophages, oxLDL induces mitochondrial dysfunction and lysosomal damage, indicating that oxLDL uptake is essential for its cytotoxic properties. In addition, it has been suggested that oxysterols such as 7β-hydroxycholesterol and 7-ketocholesterol (7-KC) are the primary cytotoxins of oxLDL. Although their role in oxLDL-mediated cytotoxicity remains unclear, oxysterol-induced cell death is associated with an enhanced production of reactive oxygen species and formation of myelin figures evocative of autophagic vacuoles.

In the present study, we show that 7-KC induces a complex mode of cell death in human vascular smooth muscle cells...
Cells were treated with 100 μmol/L 7-KC for up to 24 hours. A, Cell death was examined by a neutral red viability assay, analysis of cell adherence, and disruption of the mitochondrial transmembrane potential ($\Delta W_m$), PI incorporation, and annexin V labeling. B, Western blot analysis of procaspase-3 (procasp-3) cleavage. Monocytic U937 cells treated with 50 μmol/L etoposide (−) for 5 hours served as a positive control. Results are representative of at least 3 independent experiments. **$P<0.01$; ***$P<0.001$ vs 0 hours (ANOVA followed by Dunnett test).

(SMCs) associated with myelin figure formation and light chain 3 (LC3) processing, evocating autophagic processes. We also demonstrate that exposure of SMCs to 7-KC is associated with accumulation of ubiquitinated proteins in the cytoplasm. Furthermore, we provide evidence that ubiquitination also occurs in SMCs of advanced human atherosclerotic plaques.

Methods
The Methods sections is available online at http://atvb.ahajournals.org.

Results
Induction of Complex Mode of Cell Death in 7-KC–Treated SMCs
Treatment of human aortic SMCs with 100 μmol/L 7-KC disturbed the typical cell spreading of SMCs (acquisition of an elongated shape followed by rounding up of cells) and rapidly induced cell death as assessed by neutral red and counting of adherent cells (Figure 1A). The majority of cells did not contain cleaved caspase-3 (Figure 1B) and were negative for annexin V labeling and propidium iodide (PI) incorporation (Figure 1A), albeit a small but significant increase in annexin V and PI labeling was noticed after 12 hours of treatment (Figure 1A). Cells treated with 7-KC showed enhanced immunoreactivity for 4-hydroxy-2-nonenal (Figure I, available online at http://atvb.ahajournals.org), a major product of endogenous lipid peroxidation and an important marker of oxidative stress. We also observed a significant depolarization of the mitochondrial inner membrane potential as early as 2 hours after 7-KC administration (Figure 1A). However, internucleosomal DNA fragmentation typical of apoptosis could not be detected, and labeling of cells with Hoechst revealed neither a significant rise in chromatin condensation nor fragmentation of the nucleus (Figure II, available online at http://atvb.ahajournals.org). Transmission electron microscopic analysis of cells treated with 7-KC for 12 hours revealed formation of membranous whorls (also called myelin figures) in different stages of development, extensive vacuolization, and depletion of organelles (Figure 2C through 2F). Several of these features, such as myelin figure formation and vacuolization, initiated within 2 hours after 7-KC administration (Figure III, available online at http://atvb.ahajournals.org). Of note, untreated controls also showed presence of small vesicles, especially in older cells (passage >5), suggesting that these vesicles are formed spontaneously to remove damaged intracellular components or protein aggregates (Figure 2A and 2B). To exclude the possibility that the inclusions in the vacuoles of dying cells represent heterophagocytized remnants of dead cells rather than autophagocytized intracellular components, SMCs were treated with 7-KC in the presence of fluorescent beads. After 12 hours, a small group of cells (2% to 3%) contained one single bead (Figure IV, available online at http://atvb.ahajournals.org). However, uptake of multiple beads, as shown in control J774 macrophages incubated with beads for 1 hour, could not be demonstrated (Figure IV). Accumulation of acidic organelles in 7-KC–treated cells was confirmed by labeling cells with LysoTracker (Figure 3A and 3B). Moreover, initiation of 7-KC–induced cell death was associated with the conversion of the cleaved 18-kDa protein microtubule-associated protein 1 LC3 (LC3-I) into the 16-
kDa protein LC3-II (Figure 3C), which is considered a reliable marker of autophagosome formation.15,16

To further characterize 7-KC–induced cell death, transcript levels of 205 apoptosis-related and 234 stress-related genes were analyzed using cDNA expression arrays. Twelve hours after exposure to 7-KC, 4 stress-related genes with a differential steady-state expression level of >5-fold could be identified: 70-kDa heat shock protein 1 (HSP-70; 25.3-fold), heme oxygenase 1 (HO1; 7-fold), and growth arrest and DNA damage-inducible protein 153 (GADD153; 5.9-fold) were upregulated in 7-KC–treated SMCs versus untreated cells, whereas proliferating cyclic nuclear antigen (PCNA; 5.2-fold) was downregulated. Furthermore, it is noteworthy that the housekeeping gene ubiquitin was moderately upregulated (2.5-fold). Neither ubiquitin-like protein Nedd8 nor typical apoptosis-related genes such as Bcl-2 family members, caspases, or death receptors showed differential gene expression. Array results could be confirmed by real-time RT-PCR (HSP-70, P<0.05; 28.3±2.8 [GADD153], P<0.01; 6.6±3.3 [PCNA], P=0.23; and 1.8±0.1 [ubiquitin], P<0.01; n=3). However, differential expression of HSP-70 and PCNA at the protein level could not be observed at the different time points studied (0 to 24 hours; data not shown). Expression of HO1 protein did not increase but significantly decreased after 2 hours of 7-KC stimulation (data not shown), possibly as a result of protein degradation.

7-KC–Treated SMCs Accumulate Ubiquitinated Proteins

In addition to upregulation of ubiquitin mRNA, Western blots for ubiquitin demonstrated that SMCs accumulated various ubiquitinated proteins or protein aggregates of high molecular weight (>100 kDa) after 6 to 12 hours of exposure to 7-KC (Figure 4A). Protein ubiquitination was accompanied by a progressive increase in the expression of E2 ubiquitin–conjugating enzymes UbcH6, UbcH7, and Ubc9, and the E3 ubiquitin ligase Itch (Figure 4A). Immunostaining of 7-KC–treated SMCs for ubiquitin showed an intense granular signal in the cytoplasm (Figure 4C and 4D), whereas in untreated SMCs, ubiquitin was difficult to detect (Figure 4B). Accumulation of ubiquitinated proteins could already be detected in cells treated with 6.25 μmol/L 7-KC for 12 hours (Figure 4E), but significant induction of cell death was only observed within 24 hours if cells were exposed to 7-KC concentrations ≥50 μmol/L. Besides 7-KC–treated SMCs, SMCs treated with the peroxynitrite-donor SIN1A (1 mmol/L) showed accumulation of ubiquitinated protein (Figure 4F), but this was not the case with SMCs undergoing amino acid deprivation (data not shown), suggesting that acute oxidative stress rather than autophagy is responsible for protein ubiquitination. Because oxidative damage induced by SIN1A occurs almost instantly, maximal levels of ubiquitinated protein were obtained after 1 to 2 hours of treatment (Figure 4F). Because polyubiquitination leads to protein degradation via the 26S proteasome, we investigated whether the proteasome
inhibitor lactacystin could affect 7-KC–induced cell death. As shown in Figure V (available online at http://atvb.ahajournals.org), ubiquitinated proteins accumulated much faster in the cytoplasm (≤2 hours) compared with 7-KC–treated cells (Figure 4A). This resulted in a strong accumulation of the proapoptotic proteins p53, Bax, and Bid, as well as the nuclear factor κB (NF-κB) inhibitor IκBα (Figure V) and a more pronounced decrease in cell viability (15±2% cell viability versus 29±2% \( P<0.01; n=3 \) in 7-KC–treated cells 12 hours after treatment) without significant induction of apoptosis or necrosis. None of these proteins accumulated in 7-KC–treated SMCs (data not shown). Treatment of SMCs with lactacystin alone stimulated accumulation of ubiquitinated proteins (Figure V) but did not induce cell death.

Advanced Human Atherosclerotic Plaques Show Signs of Ubiquitination
To extend our in vitro observations described above to advanced human atherosclerotic plaques, we examined ubiquitin expression by immunohistochemistry in advanced plaques (thin fibrous cap atheromas) from carotid endarterectomy specimens. The majority of α-SMC actin–positive cells in the plaque showed a strong nuclear staining for ubiquitin, whereas SMCs in adjacent margins of normal media were negative (Figure 5A through 5C). CD68-positive macrophages showed an occasional nuclear staining (Figure 5D). Because cells in advanced stages of atherosclerosis often lose specific markers such as CD68 and α-SMC actin, double immunostainings could not always be used to establish the identity of ubiquitin-immunoreactive cells. Therefore, we also combined immunodetection of ubiquitin with a periodic acid Schiff (PAS) stain. SMCs, particularly those present in the fibrous cap, but not macrophages were surrounded by a cage of PAS-positive basal lamina. By using this technique, we found macrophages (PAS-negative) around the necrotic core that stained negative for CD68 and positive for ubiquitin (only nuclear staining). Moreover, we could detect immunoreactivity for ubiquitin in the cytoplasm of α-SMC actin–negative SMCs that were located in the fibrous cap and surrounded by a prominent cage of PAS-positive material (Figure 5E). Immunoreactivity for ubiquitin colocalized with Ubc9 expression (Figure 5F) but not with HSP-70 and HO1 expression (Figure VI, available online at http://atvb.ahajournals.org). HSP-70 and HO1 were detected predominantly in macrophages of the plaque showing weak nuclear staining for ubiquitin. HO1 expression was located specifically around microvessels in the plaque. Interestingly, 1% to 4% ubiquitin-immunoreactive SMCs located in the fibrous cap and enclosed by PAS-positive thickened basal laminae did not contain an intact nucleus and completely disintegrated into myriad vesicles. To distinguish apoptotic SMCs from SMCs undergoing nonapoptotic death, we combined ubiquitin immunostainings with TUNEL. Although 1% to 2% TUNEL-positive SMCs with numerous vesicles and surrounded by cages of basal laminae could be found, these cells were always ubiquitin negative (Figure 5G and 5H), indicating that ubiquitin-positive disintegrated SMCs may die an apoptosis-unrelated death. Importantly, this type of death coexists with apoptosis of SMCs in the same specimen.

Discussion
An increasing body of evidence suggests that SMC death and the progressive thinning of the fibrous cap are major events in the progression of atherosclerosis. Infiltration of inflammatory cells and intracellular accumulation of oxidized lipids, particularly oxysterols, are considered the main triggers responsible for initiation of cell death. Recently, Zahm et al reported that 7-KC is a potent inducer of SMC death characterized by loss of cell adhesion, modification of actin organization, decrease in mitochondrial transmembrane potential, and condensation or
fragmentation of the nucleus. According to previous reports from several groups, 7-KC cytotoxicity in SMCs is mediated by apoptosis.\textsuperscript{10,18,19} In this study, we present evidence that human SMCs treated with 7-KC undergo a complex mode of cell death with some characteristics of autophagy (type II cell death). However, we do not rule out the possibility that apoptosis and necrosis occurred in a small subgroup of treated cells because a small increase in annexin V labeling and PI incorporation was noticed. Therefore, it is reasonable to assume that 7-KC may induce simultaneously various types of cell death but at different rates. Although this hypothesis may seem contradictory to published literature, it should be noted that apoptosis can be easily detected with various techniques, whereas at present, autophagy requires electron microscopy for unambiguous detection. On the other hand, it is of note that the 7-KC concentration used in different studies varies from 12.5 to 200 $\mu$mol/L. This might have an important impact on the type of death and the incubation period before cell death becomes imminent. We used a concentration of 100 $\mu$mol/L 7-KC, which is $\approx$1000-fold higher than the 7-KC levels in human plasma\textsuperscript{20} and $\approx$25-fold higher than the plasma 7-KC levels after a fat-rich meal.\textsuperscript{21}

Autophagy is a general manifestation of injury that involves the sequestration of intracellular components and their subsequent degradation in secondary lysosomes.\textsuperscript{16,22} This process occurs in at least 2 steps: first, organelles or portions of cytosol are sequestered by an enveloping membrane, resulting in the formation of an autophagosome. The latter subsequently fuses with a primary lysosome to form an autolysosome where the cell content is digested by lysosomal enzymes. We could demonstrate that 7-KC–treated SMCs contain myelin figures evocative of autphagic vacuoles, as shown previously for 7-KC–treated monocytes and endothelial cells.\textsuperscript{13,14} Moreover, other markers of autophagy such as extensive vacuolization and depletion of organelles were present. Formation of acidic vacuoles was confirmed by labeling cells with LysoTracker and by demonstrating LC3 processing. These features were never observed in apoptosis or oncosis but are considered characteristic of autophagy.\textsuperscript{16,22} In addition, 7-KC–induced cell death was characterized by a rapid depolarization of the mitochondrial inner membrane potential, which is a common mechanism in apoptotic, oncosic, and autophagic cell death.\textsuperscript{23} If cellular injury is severe, a major proportion of the cytosol and organelles (most noticeably the mitochondria and endoplasmic reticulum) will be destroyed, and this eventually leads to autophagic cell death. Importantly, autophagy does not directly destroy the plasma membrane (typical of necrosis) or the nucleus (absence of nuclear or internucleosomal DNA fragmentation), as shown in 7-KC–treated SMCs, perhaps because the nucleus is too large to be engulfed.

7-KC–induced SMC death was associated with protein ubiquitination, as described previously for cells treated with aggregated LDL\textsuperscript{24} or oxLDL.\textsuperscript{25,26} Ubiquitin targets proteins for degradation by a multisubunit, ATP-dependent protease termed the proteasome, and thus fulfills an important function in elimination of damaged or unneeded proteins.\textsuperscript{27} Indeed, SMCs treated with the peroxynitrite donor SIN1A showed increased ubiquitination, most likely to remove oxidatively modified proteins, but this was not the case with autophagic SMCs undergoing starvation. Because 7-KC–treated cells showed enhanced immunoreactivity for the lipid peroxidation marker 4-hydroxy-2-nonenal, it is tempting to speculate that oxidative stress is the major cause of protein ubiquitination during 7-KC stimulation. Furthermore, ubiquitination is one of the major mechanisms that regulate apoptotic cell death.\textsuperscript{28} Inhibition of the proteasome function by lactacystin as shown in this study leads to accumulation of several proapoptotic proteins including p53, Bax, and Bid, as well as the NF-$\kappa$B inhibitor IkB$\alpha$. Accumulation of ubiquitin/protein conjugates potentiated considerably the toxicity of 7-KC, which confirms previous studies with ox-LDL.\textsuperscript{25} However, accumulation of ubiquitin-conjugated proapoptotic proteins did not give rise to a higher incidence of apoptotic cell death. There is a possibility that certain proapoptotic proteins have a function in apoptosis and autophagic cell death, as proposed recently for Bax by Camougrand et al.\textsuperscript{29} Interestingly, the core protein machinery necessary to drive formation of autophagosomes includes a ubiquitin-like protein conjugation system.\textsuperscript{25,22} Moreover, Kostin et al\textsuperscript{8} reported that autophagic vacuoles are positive for ubiquitin immunolabeling. Therefore, it is reasonable to assume that ubiquitination of proteins and autophagic cell death are closely interacting and self-supporting phenomena during 7-KC–mediated cell death.

In analogy to our in vitro studies, SMCs in advanced human atherosclerotic plaques showed signs of ubiquitination, apoptosis, and nonapoptotic cell death. Similar findings were reported for the cellular degeneration of myocytes in failing human hearts.\textsuperscript{8} Moreover, ubiquitin immunoreactivity of SMCs is enhanced in unstable, infarct-related coronary plaques, predominantly in plaque regions of tissue degeneration,\textsuperscript{20} indicating that the ubiquitin–proteasome system might play an essential role in the destabilization and rupture of atherosclerotic plaques. In contrast to medial SMCs, most SMCs in the plaque showed nuclear staining for ubiquitin, which could indicate changes in nuclear functioning such as regulation of transcription, DNA repair, and replication.\textsuperscript{31} However, a significant number of SMCs in the fibrous cap showed cytoplasmic immunoreactivity for ubiquitin. These SMCs are surrounded by a cage of thickened basai laminae and often disintegrate into numerous cytoplasmic fragments. Previous work in our group revealed that this form of cell death has several characteristics of apoptotic cell death (DNA fragmentation and upregulation of Bax).\textsuperscript{2} However, it is important to note that we could detect disintegrated SMCs enclosed by thickened basal laminae, which were immunoreactive for ubiquitin in their cytoplasm and TUNEL negative, suggesting that they died a nonapoptotic death. Interestingly, lipid-laden SMCs in human plaques but not macrophages upregulate death-associated protein (DAP) kinase,\textsuperscript{32} a positive mediator of apoptotic cell death that also mediates membrane blebbing and the formation of autophagic vesicles.\textsuperscript{33} Because autophagy and proteasome-mediated degradation are the major cellular pathways for turnover of damaged protein and organelles,\textsuperscript{16,22} there is a possibility that ubiquitination of cytoplasmic proteins in SMCs of atherosclerotic plaques together with enhanced levels of DAP kinase and cellular disintegration into TUNEL-negative remnants point to autophagic cell death. If autophagy would occur in SMCs of advanced human plaques, its significance remains to be elucidated. Indeed, several lines of evidence support the hypothesis that autophagy is not a death pathway but a survival mechanism activated during cellular distress.\textsuperscript{34}
In summary, treatment of SMCs with 7-KC may induce oxidative damage, activation of the ubiquitin–proteasome system, and induction of cell death. Although we do not exclude apoptosis or oncosis, our data indicate that the majority of 7-KC–treated SMCs preferentially undergo a complex mode of cell death associated with the formation of myelin figure and LC3 processing, evocating autophagy. Because the ubiquitin–proteasome system could be a unique target for therapeutic intervention, additional work may reveal the precise role of ubiquitin in plaque destabilization.

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References
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Lactacystin
Lactacystin + 7-ketocholesterol

Ubiquitin
p53
Bax
Bid
IκBα
β-actin
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Online figure legends

**Figure I.** Immunocytochemical staining for 4-hydroxy-2-nonenal in human aortic SMCs before (A) and after treatment with 100 µmol/L 7-KC for 12 hours (B). Photomicrographs only show adherent cells. Scale bar=100 µm.

**Figure II.** Effect of 7-KC on genomic DNA or nuclear morphology in human aortic SMCs. A, Agarose gelelectrophoresis of genomic DNA isolated from 7-KC treated SMCs (0-12 hours) and etoposide treated U937 cells (positive control for oligonucleosomal DNA fragmentation). B, DNA content as determined by flow cytometry in SMCs treated with 100 µmol/L 7-KC for 12 hours (upper graph, open histograms) or in U937 treated with 50 µmol/L etoposide for 5 hours (lower graph, open histograms). Shaded histograms represent untreated controls. The subG1 peak correlates with DNA fragmentation as shown for etoposide treated U937 cells. C-E, Evaluation of chromatin condensation and/or nuclear fragmentation after nuclei staining with Hoechst 33342. Although spontaneous chromatin condensation (arrowheads) occurred in untreated control cells (C), a significant increase in the number of condensed nuclei after treatment with 7-KC for 12 hours could not be observed, neither in adherent cells (D) nor in nonadherent cells (E). Nuclear fragmentation was an extremely rare event (<0.1%) in both treated and untreated SMCs. Results are representative of three independent experiments.

**Figure III.** Ultrastructural features of SMCs treated with 100 µmol/L 7-ketocholesterol (7-KC) for 2 hours. The majority of cells showed several myelin figures (arrows) in the cytosol and increased vacuolization. Vacuoles contained remnants of digested intracellular components.
Panel B and D represent boxed area of panel A and C, respectively. N=nucleus. Scale bar=1 µm.

**Figure IV.** Phagocytosis of fluorescent beads by human aortic SMCs during 7-KC treatment measured by flow cytometry (shaded histogram). SMCs were treated with 100 µmol/L 7-KC for 12 hours in the presence of fluorescent beads (20 beads/cell). Approximately 2-3% of SMCs ingested one single bead. However, uptake of multiple beads could not be detected. J774 mouse macrophages that were incubated with an equal amount of beads for 1 hour served as a positive control for phagocytosis (open histogram). The different peaks represent the number of ingested beads. Results are representative of three independent experiments.

**Figure V.** Western blot analysis of ubiquitin and proapoptotic proteins in SMCs treated with the proteasome inhibitor lactacystin (10 µmol/L) or with a mixture of 7-ketocholesterol (100 µmol/L) and lactacystin (10 µmol/L) for up to 12 hours. The proapoptotic proteins p53, Bax and Bid as well as the NF-κB inhibitor IκBα are well known targets for ubiquitin conjugation and accumulated significantly during treatment, with maximal levels after 2-6 hours. The results are representative of 3 independent experiments.

**Figure VI.** Immunohistochemical detection of ubiquitin, HSP-70 and HO1 in atherosclerotic plaques of human carotid endarterectomy specimens. A, Low-power photomicrograph of a liver biopsy of a patient with alcoholic liver disease immunohistochemically stained for ubiquitin (positive control for ubiquitin staining). The hepatocytes demonstrate steatosis and cytoplasmic ubiquitin positive aggregates (brown-red inclusion bodies). The ubiquitin positive aggregates correspond to Mallory bodies which are cytoplasmic aggregates of cytokeratin and are a marker for alcoholic liver disease. B, High-power photomicrograph of the boxed area in panel A showing ubiquitin positive cytoplasmic aggregates in the hepatocytes. C-E, ubiquitin (C), HSP-70 (D) and HO1 (E) immunolabeling in the plaque. Immunoreactivity for
ubiquitin does not colocalize with HSP-70 and HO1 expression. Two-five sections of each specimen were stained. Scale bar=200 μm (panel A, C-E) and 20 μm (panel B).
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Methods

Antibodies

The following mouse monoclonal antibodies were used: anti-UbcH6 (clone 42), anti-UbcH7 (clone 20), anti-Ubc9 (clone 50), anti-Itch (clone 32), anti-caspase-3 (clone 19), anti-\( \kappa B \alpha \) (clone 25) and anti-Bid (clone 7) from BD Transduction Laboratories, anti-SMC actin (clone 1A4) from Sigma, anti-Bax (clone 6A7) from BD PharMingen and anti-p53 (clone DO-7) and anti-CD68 (clone PG-M1) from DAKO.

The following rabbit polyclonal antibodies were used: anti-LC3 (gift from Dr T. Yoshimori, National Institute of Genetics, Mishima, Japan),\(^1\) anti-ubiquitin from Sigma, anti-cleaved caspase-3 from Cell Signaling Technology and anti-4-hydroxy-2-nonenal (HNE) from Alpha Diagnostic. Rabbit anti-mouse and swine anti-rabbit peroxidase-conjugated secondary antibodies were purchased from DAKO.

Cell culture

Human aortic SMCs (BioWhittaker) were grown in Smooth Muscle Cell Basal Medium (SmBM, Clonetics) supplemented with human recombinant epidermal growth factor (0.5 ng/ml), insulin (5 µg/ml), human recombinant fibroblast growth factor (2 ng/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml), amphotericin-B (50 ng/ml), polymyxin B (20 U/ml) and 5% fetal bovine serum. Stock solutions (25 mmol/L) of 7-ketocholesterol were prepared freshly by adding 10 mg 7-ketocholesterol per ml dimethyl sulphoxide followed by sonication of the solution. SMCs were induced to undergo cell death by treatment with 100 µmol/L 7-ketocholesterol in SmBM containing serum and supplements. Under these conditions, the DMSO concentration in the culture medium (0.25%) had no
effect on cell growth or cell viability. To exclude toxic effects of DMSO, control cells were always treated with equal amounts of DMSO. In some experiments, the medium was supplemented with 10 µmol/L lactacystin dissolved in water. In other experiments, SMCs were treated with 7-KC in the presence of fluorescent beads (1.0 µm carboxylate-modified yellow-green fluospheres, 20 beads/cell, Molecular Probes). Autophagy was induced by amino acid deprivation. For this purpose, cells were washed three times with PBS and incubated with Earle’s balanced salts solution (EBSS) at 37°C. To expose SMCs to continuous oxidative stress, 1 mmol/L of the nitric oxide/superoxide anion donor 3-morpholinosydnonimine hydrochloride (SIN1A, Cassella AG) was added to the culture medium every 20 minutes. Release of NO was assessed by measuring nitrite using the Griess reaction.2

Detection of cell death

Evaluation of cell viability was based on the incorporation of the supravital dye neutral red by viable cells.3 Cell death was detected by flow cytometry using an annexin V-propidium iodide kit (BD Biosciences). Activation of procaspase-3 was analyzed by immunohistochemistry and Western blotting. Variations in the mitochondrial membrane potential were studied by labeling cells with the mitochondrial potential sensor JC-1 (10 µg/ml; Molecular Probes) for 10 minutes at 37°C in PBS followed by flow cytometric analysis. Acidic organelles were detected by labeling cells with LysoTracker (75 nmol/L; Molecular Probes) for 30 minutes at 37°C in culture medium followed by fluorescence microscopy. DNA fragmentation into nucleosomal bands was detected by agarose gel electrophoresis as described.4 Nuclear condensation and/or fragmentation was studied by fluorescence microscopy after staining with Hoechst 33342 (Molecular Probes). Briefly, cells were fixed in PBS containing 4% paraformaldehyde for 10 minutes, washed with PBS and treated with 0.2% Triton X-100 in PBS for 5 minutes. After washing with PBS, cells were labeled with Hoechst 33342 (10 µg/ml) for 5 minutes. The cellular DNA content was determined according to Vindelov et al.5 All analyses were performed on the total pool of cells (adherent and nonadherent cells), unless it was specifically stated otherwise.
**Carotid endarterectomy specimens**

Human carotid endarterectomy specimens (n=12) were obtained from patients with a carotid stenosis of >70% as demonstrated by digital subtraction angiography and duplex ultrasonography. The specimens were opened along their longitudinal axis and fixed in 4% formalin within 2 min after surgical removal. Complete longitudinal sections of paraffin-embedded specimens contained the inner wall of the distal common carotid artery, the proximal part of the external and internal carotid artery and the carotid sinus. According to the adapted American Heart Association classification scheme as modified by Virmani et al., thin fibrous cap atheromata alternated with other stages of atherosclerosis (fibrous cap atheromata and intimal xanthomata) in the same specimen.

**cDNA expression arrays**

Total RNA was isolated from cultured SMCs using the Absolutely RNA Microprep Kit (Stratagene). Quality of RNA was verified on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano LabChip kit (Agilent Technologies). Probe mixtures were synthesized by reverse transcribing 5 µg total RNA using Superscript II reverse transcriptase (Invitrogen), cDNA synthesis primer mix (Clontech) and [α-32P] dATP. Hybridization experiments were performed with the human apoptosis array or human stress array (ClonTech). After extensive washes, the membranes were analyzed by PhosphorImaging (Molecular Dynamics). Data were analyzed using AtlasImage 2.0 software (ClonTech). Normalization of the signal intensity between two arrays was based on the overall value of all the genes on the arrays (global normalization). Weak signals were filtered out by applying a background-based signal treshold of 200%. To define differential gene induction, we used a 5-fold treshold value.

**Real time quantitative RT-PCR**

Relative abundance of mRNA species was assessed by real time quantitative RT-PCR on an ABIPrism 7700 sequence detector system (Applied Biosystems). FAM-labeled LUX (Light
Upon eXtension) primers for HSP-70 (5'-CACCTTGAGGCCACAAAGAAGGTG-3' and 5'-GTCCTTCTCGGGAAGGTGTGAGT-3'), HO1 (5'-CAGCTTCGCGCTGACCAGGAGTAG-3' and 5'-CACCATTCTGCTAACAGACG-3'), GADD153 (5'-CTGGGAGCGGGTGTTGAGT-3' and 5'-CACCATTCTGCTAACAGACG-3') and ubiquitin (5'-CAGCTTCGCGCTGACCAGGAGTAG-3' and 5'-CACCATTCTGCTAACAGACG-3') were designed using web-based LUX Designer software at www.invitrogen.com/lux. TaqMan primers for PCNA (5'-CGGATACCTTGCCCTAGTATT-3' and 5'-AGATCTGGCATACGTGCAAA-3') and the endogenous reference gene TBP (5'-CACGAACCACGGCAGT-3' and 5'-CTTGCTGCGGACTGT-3') as well as their corresponding fluorogenic probes (5'-CAGCTGATTCTCGTCTGGAGGATGTTG-3' [PCNA] and 5'-TCTGGCTGCGGACTGT-3' [TBP]) were designed using Primer Express software (Applied Biosystems). Probes were 5'-FAM (reporter) and 3'-TAMRA (quencher) labeled. Total RNA was prepared from cultured SMCs using the Absolutely RNA Microprep Kit (Stratagene). Quantitative RT-PCR was performed in duplicate in 25 µl reaction volumes consisting of 1× Master Mix and 1× Multiscribe and RNase inhibitor Mix (TaqMan One Step PCR Master Mix Reagents Kit, Applied Biosystems). PCR cycling parameters were: reverse transcription at 48°C for 30 min, inactivation of RT at 95°C for 10 min, followed by 40 cycles consisting of incubations at 95°C for 15 sec and 60°C for 1 min. Relative expression of mRNA species was calculated using the comparative C_T method. All data were controlled for quantity of RNA input by performing measurements on TBP.

**Immunohistochemistry and DNA in situ end labeling**

The immunohistochemical reactions were carried out by an indirect peroxidase antibody conjugate method. Liver biopsies of patients with alcoholic liver disease served as a positive control for ubiquitin staining. For the detection of oligonucleosomal DNA cleavage, a stringent terminal deoxynucleotidyl transferase end labeling (TUNEL)-technique was used.

**Electron microscopy**
Samples were fixed in 0.1 mol/L sodium cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde solution for 2 hours and postfixed in 0.1 mol/L sodium cacodylate-buffered (pH 7.4) 1% OsO₄ solution for 1 hour. After dehydration in an ethanol gradient (70% ethanol [20 minutes], 96% ethanol [20 minutes], 100% ethanol [2x20 minutes]), samples were incubated with propylenoxid (2x10 minutes), impregnated with a mixture of propylenoid/LX-112 (Ladd Research Industries, 1:1) and embedded in LX-112. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined in a Jeol-100 CX II TEM at 80 kV. Photographs were made with electron microscopy film 4489 Estar Thick Base (Kodak).

**Statistical analysis**

To evaluate the effect of 7-KC on cell viability, percentage of adherent cells, propidium iodide incorporation and annexin V labeling at the different time points studied (2, 6, 12 and 24 hours) versus the control (0 hours), analysis of variance (ANOVA), followed by the Dunnett test was applied. Differences in the depolarization of the mitochondrial inner membrane at the time points studied versus the control (0 hours) were evaluated by ANOVA, followed by the Dunnett T3 test. To test whether the relative expression of HSP-70, HO1, GADD153, PCNA and ubiquitin in cultured SMCs was different from 1, the one-sample t test was used. Differences in cell viability between 7-KC treated SMCs versus 7-KC and lactacystin treated SMCs were compared with the Student t test. A value of $P<0.05$ was considered statistically significant.
References


