Research article

Pathological apoptosis by xanthurenic acid, a tryptophan metabolite: activation of cell caspases but not cytoskeleton breakdown

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Abstract

Background: A family of aspartate-specific cysteinyl proteases, named caspases, mediates programmed cell death, apoptosis. In this function, caspases are important for physiological processes such as development and maintenance of organ homeostasis. Caspases are, however, also engaged in aging and disease development. The factors inducing age-related caspase activation are not known. Xanthurenic acid, a product of tryptophan degradation, is present in blood and urine, and accumulates in organs with aging.

Results: Here, we report triggering of apoptotic key events by xanthurenic acid in vascular smooth muscle and retinal pigment epithelium cells. Upon exposure of these cells to xanthurenic acid a degradation of ICAD/DFF45, poly(ADP-ribose) polymerase, and gelsolin was observed, giving a pattern of protein cleavage characteristic for caspase-3 activity. Active caspase-3, -8 and caspase-9 were detected by Western blot analysis and immunofluorescence. In the presence of xanthurenic acid the amino-terminal fragment of gelsolin bound to the cytoskeleton, but did not lead to the usually observed cytoskeleton breakdown. Xanthurenic acid also caused mitochondrial migration, cytochrome C release, and destruction of mitochondria and nuclei.

Conclusions: These results indicate that xanthurenic acid is a previously not recognized endogenous cell death factor. Its accumulation in cells may lead to accelerated caspase activation related to aging and disease development.

Background

Xanthurenic acid is formed upon tryptophan degradation by indoleamine-2,3 dioxygenase (IDO). The end products of this degradation pathway are, alternatively, nicotinate and xanthurenic acid. IDO activity is stimulated by superoxide radicals, liposaccharides and interferon-γ [1]. Kynurenine aminotransferase (KAT), the enzyme directly responsible for xanthurenic acid formation from 3-hydroxykynurenine, is found in the cytoplasm and mitochondria, and is highly expressed in the retina [2,3]. Xanthurenic acid is present in blood and urine at concentrations of 0.7 and 5-10 µM, respectively [4,5]. A several - fold increase is observed in vitamin B6 deficiency and some diseases such as tuberculosis [6,7]. Xanthurenic acid's presence in the blood is linked to malaria development, and in the lenses to senile cataract

formation [8,9,10]. Xanthurenic acid binds covalently to proteins, leads to their unfolding, and to cell death [11]. Here, we report that xanthurenic acid induces cell death associated with caspase-3, -8, and -9 activation, nuclear DNA cleavage, and cytochrome C release. However, cell death is not associated with cytoskeleton breakdown, usually observed because of actin depolymerization by caspase-3-cleaved gelsolin [12].

Results and Discussion
Xanthurenic acid leads to caspase-3 activation in smooth muscle cells
Xanthurenic acid induced cell death in a concentration-dependent manner. Death was associated with caspase-3 activation (Fig. 1). Caspase-3 can be induced by two primary pathways: by activation of cell surface receptors (FAS, TRAIL, TNF), or by activation of a stress response pathway leading to cytochrome C release from mitochondria and caspase-9 activation [13,14,15]. Procaspase-3 (CPP32) is cleaved at ASP 175, leading to an autocatalytic process which liberates the active p17 fragment [16]. In this study, xanthurenic acid-dependent cleavage of CPP32 was observed by Western blotting (Fig. 1e), and formation of caspase-3 p17 was detected by immunostaining (Fig. 1f, Fig. 3a).

Xanthurenic acid provokes degradation of caspase-3 substrates DFF-45, PARP, and gelsolin
Caspase 3 is required for the degradation of DFF45/ICAD with formation of the carboxy-terminal fragment p11, which is necessary for DNA cleavage [17,18]. In cells exposed to xanthurenic acid, DFF45 was cleaved with generation of the p11 fragment, recognized with an antibody directed against full-length DFF45 (Fig. 2a). Processed DFF45 leads to internucleosomal cleavage, and indeed the DNA of cells exposed to xanthurenic acid was fragmented as shown by Hoechst 33342 staining and fluorescence microscopy (Fig. 2b). The amount of PARP protein was increased in xanthurenic acid-exposed cells, and PARP was degraded to the apoptotic p85 fragment (Fig. 2c, 3b), which was reported to be formed upon caspase-3 cleavage [19]. Also gelsolin was cleaved in cells in a xanthurenic acid concentration-dependent manner with formation of p41, the amino terminal part of gelsolin, called "N-half" (Fig. 2d. Fig. 3d,e). The latter is a product of caspase-3 activity [12, 20]. The p41 fragment was further degraded, indicating that xanthurenic acid activated additional enzyme(s) involved in gelsolin processing. It was reported that N-half leads to depolymerization of actin filaments [20].

Apoptosis induced by xanthurenic acid did not cause cytoskeleton depolymerization
In the presence of xanthurenic acid the caspase-3 cleaved gelsolin did not cause cytoskeleton breakdown

(Fig. 3d). To the contrary, the elongated cytoskeleton was strongly stained for N-half of gelsolin, and the staining increased with xanthurenic acid concentration (Fig. 3d), the condition which led to activation of caspase-3 (Fig. 3a) and caspase-9 (Fig. 3c). N-half contains two polyphosphoinositide (PIP) binding domains. Phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4-bisphosphate form a stable complex with gelsolin, which prevents caspase-3 and -9 activation [21]. We prepared a part of N-half containing amino acid residues 162-187 (human sequence of gelsolin), which contains a PIP binding domain in position 162-169, and raised a polyclonal antibody against the peptide, called GPIP1. This antibody stained the cytoskeleton of xanthurenic acid-exposed cells but not of the control cells (Fig. 3e). This indicates that after xanthurenic acid-dependent gelsolin cleavage the sequence containing GPIP1 binds to the cytoskeleton. The cleavage of gelsolin in the presence of xanthurenic acid did not lead to breakdown of the cytoskeleton (Fig. 3e), in contrast to experiments where gelsolin was overexpressed [12].

Xanthurenic acid induces mitochondrial damage
We observed activation of caspase-9 in cells after exposure to xanthurenic acid, indicating the triggering of a mitochondrial pathway (Fig. 3c) and release of cytochrome C in the cells exposed to xanthurenic acid (Fig. 4a,b,c). We used Mitotracker CMXRos to detect mitochondria and a possible loss of the mitochondrial membrane potential, previously seen when gelsolin had been cleaved [22]. Mitotracker CMXRos dramatically sensitized mitochondria to xanthurenic acid. Whereas in the experiments reported above cells were exposed for one week to xanthurenic acid, 3 hours of exposure to xanthurenic acid together with Mitotracker CMXRos sufficed to cause relocalisation of mitochondria from the nuclear region (Fig. 4d) to the cytoskeleton (Fig. 4e). At higher xanthurenic acid concentration mitochondria lost their membrane potential (Fig. 4f,g,h,i,j), released cytochrome C (Fig. 4f) and strong staining for N-half of gelsolin was observed (Fig. 4f). Under these conditions cells died within 24 hours.

Effector caspases in demolition phase of xanthurenic acid-induced apoptosis
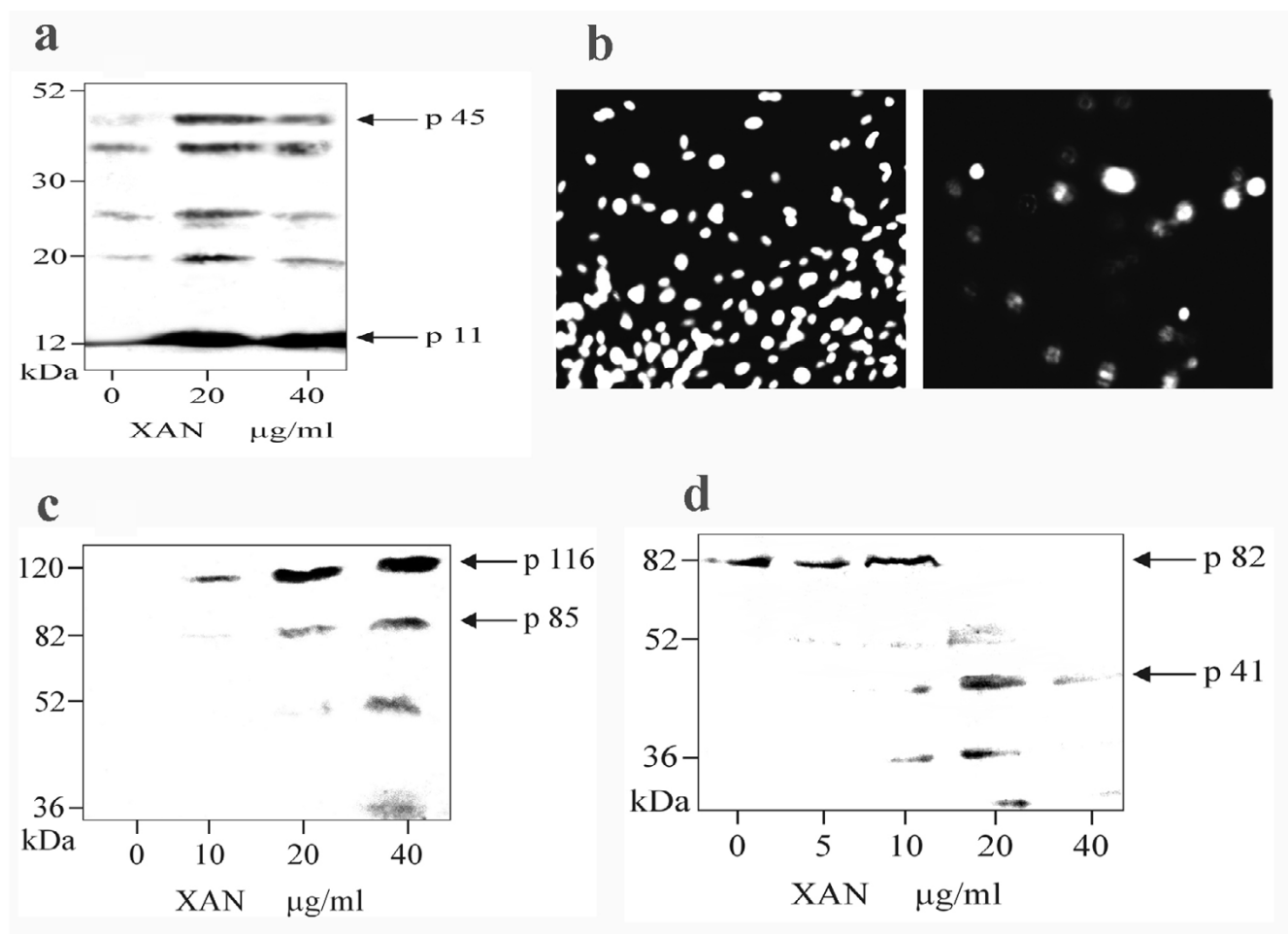In the presence of xanthurenic acid the cell death was associated with DFF p11 formation, which is characteristic for caspase-3 activity. Recently, it was reported that executioner caspases-3, 6, and 7 play non-redundant roles during the demolition phase of apoptosis. Caspase-6 and caspase-7 are not involved in DNA degradation but in lamin A degradation, and caspase-7 activation provokes PARP cleavage [23]. Our Western blot analysis showed that in the presence of xanthurenic acid lamin A cleavage does not occur in VSMC suggesting that these caspases
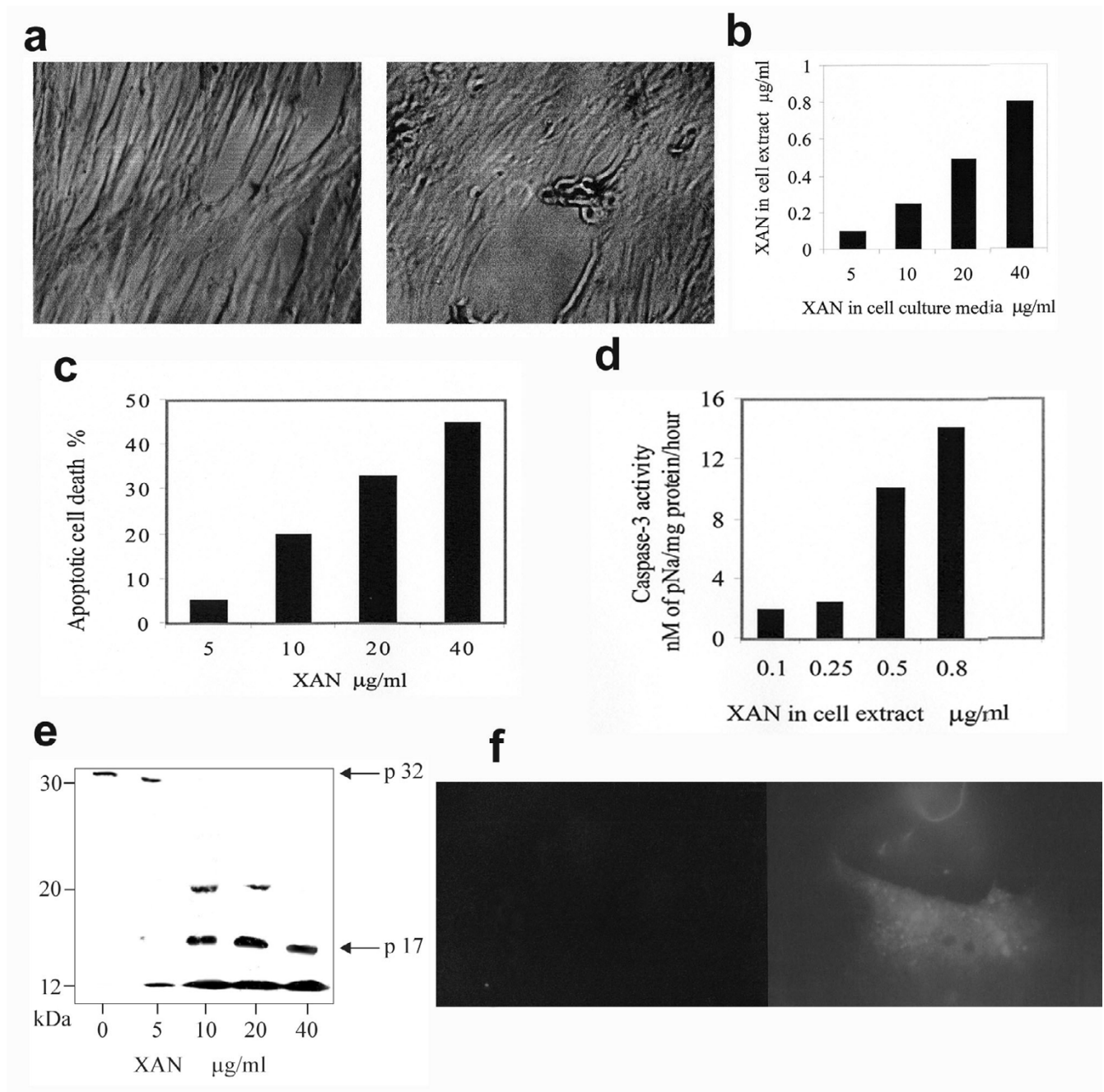
Figure 1
Caspase-3 activation and apoptosis upon xanthurenic acid (XAN) accumulation in vascular smooth muscle cells (VSMC). **a,** VSMC after one week of growth in MEM without XAN (left panel), and in the presence of XAN at a concentration of 5 μg/ml (right panel). The nuclear DNA fragmentation at 40 μg/ml, see Fig. 2b. **b,** Concentration of xanthurenic acid in the cell extract used for Western blot analysis. XAN was determined in the cell extracts as described previously [11]. **c,** Apoptotic cell death measured as a ratio of the cells with condensed or fragmented nuclei to total nuclei. **d,** Caspase-3 activity measured by caspase-3 substrate Ac-DEVD-pNa cleavage, **e,** Western blot of active caspase-3 in VSMC after exposure to XAN. Procaspase-3 (CPP32) was cleaved in the presence of XAN with the formation of pl7 and pl2. **f,** Immunofluorescence staining for caspase 3 pl7 in control cells (left panel) and in VSMC exposed to 40 μg/ml of XAN for one week (right panel).

are not activated in the presence of this compound. This suggests that the cleavage of PARP occurred due to caspase-3 activation. Plectin, a cytolinker responsible for the mechanical stability of the cytoskeleton, is cleaved by
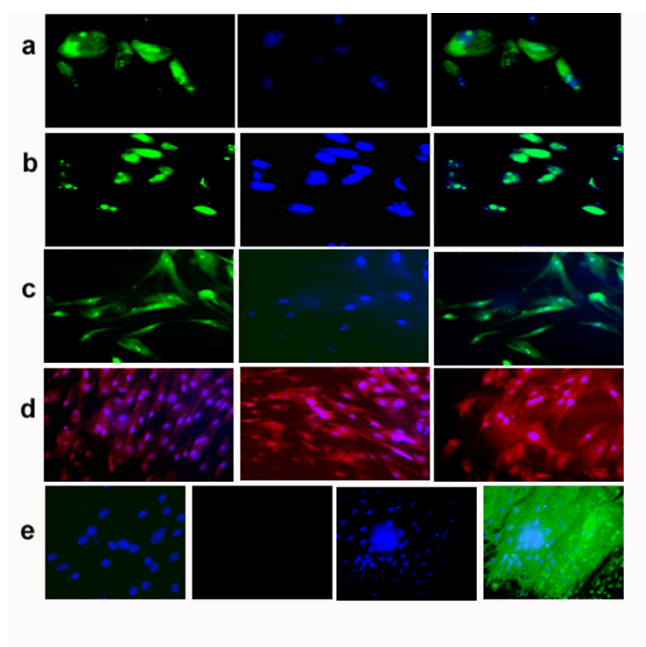
Figure 2
Cleavage of caspase-3 substrates PARP, DFF45/ICAD, and gelsolin in VSMC exposed to xanthurenic acid (XAN) for one week. DFF45 and PARP were slightly upregulated in the presence of XAN. **a,** Western blot of DFF45/ICAD. **b,** Nuclear fragmentation in control cells (left panel) and VSMC grown in the presence of XAN at 40 μg/ml for one week (right panel). **c,** Western blot of PARP. **d,** Western blot of gelsolin.

caspase-8 at ASP 2395. The cytoskeleton lost intermediary fibers due to plectin cleavage, and cells lost their integrity. Full-length caspase-8 co-localises with mitochondria and active caspase-8 is translocated to plectin [24].

In the presence of xanthurenic acid caspase-8 was activated in VSMC (Fig. 5a, b). In the control cells caspase-8 was observed in the perinuclear region (Fig. 5a, left panel), and in the presence of the xanthurenic acid the antibody directed against caspase-8 stained the whole cytoskeleton (Fig. 5b, right panel). In the presence of xanthurenic acid plectin was translocated from the mitochondrial region to the cytoskeleton (Fig. 5c, left and right panel, respectively). Western blot analysis using an antibody specific for the amino terminus of plectin showed the formation of the fragment of about 130 kD

characteristic for caspase-8 cleavage. However, the p130 fragment was further degraded, indicating that xanthurenic acid activated additional enzyme(s) involved in plectin processing (not shown). Caspase-8 activation in the presence of xanthurenic acid is an upstream event for the cell pathology in the presence of xanthurenic acid. Downstream caspase-3 can be directly activated by caspase-8 or *via* the mitochondrial pathway consisting of the translocation to mitochondria of truncated BID (t-BID) [25, 26]. In our study, t-BID was translocated in the presence of xanthurenic acid from the cytoplasm to mitochondria (Fig. 5d, left and right panel respectively) indicating that caspase-8 activation is an upstream event leading to the observed cytochrome C release, which in turn can activate caspase-9 by apoptosome formation [27]. Caspase-8 can be activated by death receptors, or by an amplification loop with caspase-9 [28, 29, 30]. The
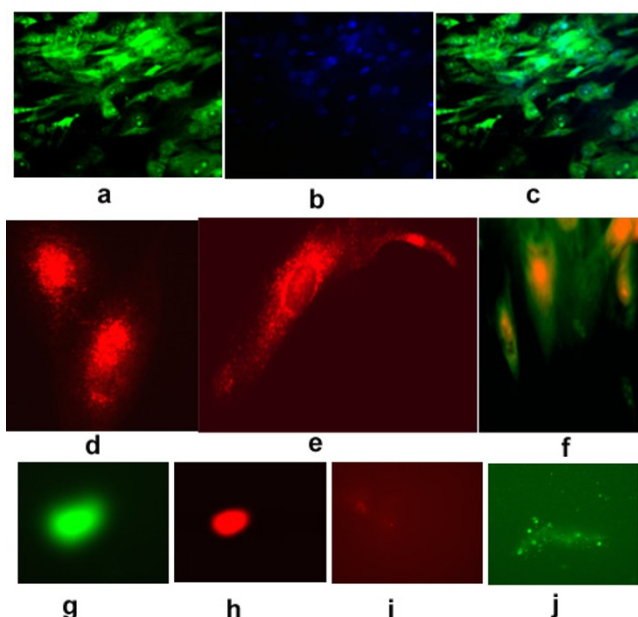
Figure 3
Detection of apoptotic proteins by immunofluorescence in human retinal pigment epithelium cells after exposure to 20 μM xanthurenic acid for one week (magnification 200-fold). Panels **a-c** from left to right represent immunodetection of apoptotic protein with respective antibody, staining of nucleus with Hoechst, and merge of both. No staining for these enzymes was observed in control cells. **a,** Detection of active caspase-3 pI7. **b,** Detection of PARP p85. **c,** Detection of active caspase-9. Panel **d,** Detection of N-half of gelsolin bound to the cytoskeleton. From left to right: without xanthurenic acid, with 10 μM xanthurenic acid, with 20 μM xanthurenic acid. **e,** Detection of cleaved gelsolin containing polyphosphoinositide binding domain by antibody directed against GPIP1 in apoptotic cells. From left to right: first two panels, control cells; Hoechst staining, no staining with antibody directed against GPIP1. Next two panels: cells incubated for one week with 20 μM xanthurenic acid; Hoechst staining, staining with antibody against GPIP1.

pathways of caspase-8 activation and cytochrome C release in the presence of xanthurenic acid are currently under study in our laboratory.

Conclusions
Our results indicate that an accumulation of the tryptophan metabolite, xanthurenic acid, leads to cleavage of caspases substrates and apoptosis. Unexpectedly, xanthurenic acid-induced apoptosis is associated with an abnormal function of cleaved gelsolin. The results indicate that xanthurenic acid is an important factor involved in aging and disease development.
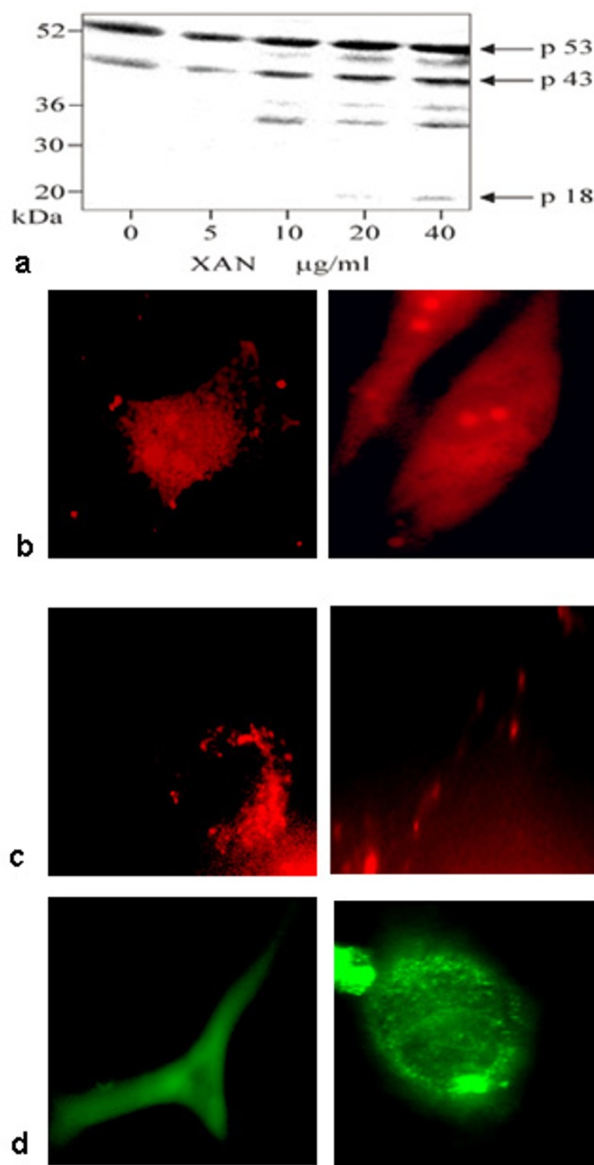

Figure 4
Analysis of mitochondria in human pigment retinal epithelial cells exposed to xanthurenic acid. Panels **a-c** (magnification 200-fold), Cytochrome C release in the presence of 20 μM xanthurenic acid for one week. **a,** Staining for cytochrome C. **b,** Hoechst staining. **c,** Merge of both. Panels **d-f** (magnification 800-fold), Mitochondria were stained with Mitotracker CMXRos, and cells were stained with antibody against gelsolin (N-half). **d,** Control cells, **e,** Cells exposed to 5 μM xanthurenic acid (see Methods), **f,** Cells exposed to 20 μM xanthurenic acid. Lack of staining with Mitotracker CMXRos, co-staining for cytochrome C (orange), and N-half of gelsolin (green). Panels **g-h** (magnification 4000-fold), **g,** Single control mitochondrion stained for cytochrome C. **h,** Single mitochondrion stained with Mitotracker CMXRos. **i,** Same as in j, but staining with Mitotracker CMXRos. **j,** Staining for cytochrome C in cells exposed to 20 μM xanthurenic acid.

Materials and Methods
Reagents
We used the following polyclonal antibodies from Santa Cruz Biotechnology Inc. CA, USA: antibodies against full length caspase-3, caspase-8, DFF 45/ICAD, and PARP, amino terminus of gelsolin, plectin, cytochrome C, and carboxy terminus of BID. Immunocytochemistry was performed using primary antibodies against active caspase-3 pI7 (BD PharMingen, San Diego, CA, USA, and Promega, Madison, USA), active caspase-9 (BioLabs Inc., New England, UK), and anti-PARP p85 (Promega). Secondary IgG-Texas Red, fluoresceine (FITC)-conjugated antibodies and Mitotracker CMXRos were from Molecular Probes, Leiden, The Netherlands. Other reagents were from Sigma if not specified.

Figure 5
Analysis of caspase-8 and caspase-8 substrate-proteins, plectin and BID, in pig VSMC. **a,** Western blot analysis of caspase-8 in the cells after exposure to xanthurenic acid (XAN) for one week. Procaspase-8 was cleaved in the presence of XAN with the formation of pl8. Panels **b-d,** (magnification 800-fold) immunodetection of caspase-8 and plectin and BID in control cells (left panels), and after exposure to 40 μM of xanthurenic acid for 96 hours (right panels), **b,** detection of caspase-8. **c,** detection of plectin. **d,** detection of BID.

Preparation of polyclonal antibody directed against GPIP1
GPIP1, a peptide comprising 25 aminoacids corresponding to residue 162-187 (NH2-KSGLKYKKGGVA-SGFKHVVPNEVVV-COOH) in human gelsolin sequence (Swiss-Prot P06396) was synthetized (95% purity) by

MWG AG Biotechnology, Ebersberg, Germany. 200 μg of the peptide were injected 3 times into rabbits in 3 weeks intervals. Sera were used for Western blots and immunoflourescence in a 1:100 dilution.

Cell culture
Primary vascular smooth muscle cells (VSMCs) were prepared from porcine aorta. Retinal pigment epithelium (RPE) cells obtained from a 59 years old eye donor were provided by Dr. M. Boenke, Department of Ophthalmology, University Hospital, Bern. The cells were cultivated in Minimal Essential Medium (MEM) with Earle's salts (Life Sciences, Basel, Switzerland). Cells were grown under a humidified atmosphere of 5% CO_2 in air at 37°C in MEM supplemented with 10% fetal bovine serum, penicillin (10 U/ml), streptomycin (10 μg/ml) and fungizone (250 ng/ml). When confluent, they were incubated for one week in MEM or MEM supplemented with xanthurenic acid. A 20 mM stock solution of xanthurenic acid was prepared in 0.5 M $NaHCO_3$, and diluted in 0.05 M $NaHCO_3$.

Cytotoxicity and apoptosis assay
Cells were observed with differential interference contrast optics on a Zeiss Avionert 405 M inverted microscope. Cell viability was determined by staining the cells with Hoechst 33342 and propidium iodide (PI) (Juro, Switzerland) using 50 μg/ml of each dye. Fragmented, apoptotic, nuclei were observed with excitation at 350 nm, and necrotic nuclei at 530 nm. The extent of apoptosis was estimated by examination of 300 nuclei from each sample.

Cell lysis and immunobloting
Cell were washed twice with cold 0.01 M phosphate buffer, pH 7.4. For Western blotting, cells were lysed in buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1 %Triton X-100, and the following protease inhibitors: 1 mM phenyl-methylsulfonyl fluoride, and leupeptin, aprotinin, and pepstatin, each at 1 μg/ml. The concentration of proteins was calculated from the absorption maximum at 280 nm, as described previously [11], and concentration of xanthurenic acid from its absorptiom maximum at 342 nm (ε_M 6500). The lysate was centrifuged for 10 min at 14 000 g, and the supernatant was boiled in loading-buffer for 5 min. Proteins (50 μg per lane) were separated by SDS-PAGE containing 10 or 12.5% acrylamide. After transfer to Hybond ECL membrane (Amersham Pharmacia Biotech AB, Uppsala, Sweden) the proteins were probed with the appropriate antibodies. Chemilunimescence ECL system (Amersham Pharmacia Biotech AB, Uppsala, Sweden) was used for the detection of peroxidase-conjugated secondary antibody.

Caspase-3 activity

Caspase 3 cleavage activity is based on the spectrophotometric detection of the chromophore p-nitroaniline at 405 nm after cleavage from the substrate Ac-Asp-Glu-Val-Asp-p-nitroaniline (Ac-DEVD-pNa) (Bachem, Basel, Switzerland). Caspase activity was measured after 1 hour of incubation of 200 µM Ac-DEVD-pNa at 37°C with the cell extract containing 25 mM HEPES (pH 7.5), 300 mM NaCI, 10 mM KCl, 1.5 mM MgCl₂, 10% glycerol, 0.1 mM DTT, 1 mM phenylmethylsulfonylfluoride, and aprotinin, leupeptin, and pepstatin, each at 1 µg/ml.

Immunofluorescence

Cells grown on glass coverslips were fixed for 10 min at room temperature in 4% paraformaldehyde in 0.1 M PIPES, pH 6.8, washed in PBS and permeabilized for 5 min in PIPES containing 0.05% saponin (65 µl per coverslip), washed in PBS, incubated with cold aceton for additional fixing and permeabilisation, and again washed in PBS. Cells were incubated for 1.5 hour with the first antibody diluted in PBS containing 1% bovine serum albumine, and after washing incubated for 1.5 hour with the secondary antibody. The coverslips were then washed in PBS and incubated for 10 min with 65 µl of 4% paraformaldehyde solution containing 1 µl of Hoechst 33342 dye (1 mg/ml), washed in PBS, and incubated with Antifade Kits (Molecular Probes, Leiden, The Netherlands) according to the supplier's instruction. Staining of mitochondria was performed using Mitotracker CMXRos, as follows: confluent cells culture were pre-incubated without or with xanthurenic acid in MEM medium for 3 hours. The medium was removed and replace with medium containing 100 nM Mitotracker CMXRos. After an incubation for 45 min Mitotracker CMXRos was removed and replaced by MEM medium. The cell were cultivated for the next 20 hours, stained additionally with antibody against gelsolin (as above) and then observed by fluorescence microscopy.

Acknowledgements

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