Free Radical Research, February 2005; 39(2): 163–173

Primary hepatocyte apoptosis is unlikely to relate to caspase-3 activity under sustained endogenous oxidative stress

YASUHIRO ISHIHARA, DAI SHIBA, & NORIO SHIMAMOTO

Department of Biology, Graduate School of Science, Osaka University, Osaka 532-8686, Japan

Accepted by Professor E. Niki

(Received 27 September 2004; in revised form 27 December 2004)

Abstract

We previously showed that inhibition of catalase and glutathione peroxidase activities in rat primary hepatocytes by 3-amino-1,2,4-triazole (ATZ) and mercaptosuccinic acid (MS) results in endogenous oxidative stress and apoptosis. For the present study, we determined whether this apoptosis involved activation of caspase-3, which is known to execute apoptosis in many cell types. ATZ and MS increased levels of reactive oxygen species (ROS) from 3–9 h, just before the onset of chromatin condensation (apoptosis) and decreases in protein thiols. Pretreatment with either SKF, a cytochrome P450 inhibitor, or L-ascorbic acid, an antioxidant, completely suppressed the increase in ROS levels and apoptosis, suggesting that the sustained ROS increases may cause the apoptosis. SKF also abolished the decrease in protein thiol content, further supporting the contribution of the P450 system to increased ROS levels. DEVD-CHO, a caspase-3 inhibitor, even at 1 mM had no effect on apoptosis. Caspase-3 activity remained unchanged and pro-caspase-3 processing was not detected during 18 h incubation with ATZ and MS. Moreover, the amount of unoxidized pro-caspase-3 decreased even below the level of untreated hepatocytes. These findings suggest that the sustained oxidative stress is a major cause for the hepatocyte apoptosis, which occurs independently of the caspase-3 related pathway.

Keywords: *Hepatocyte, apoptosis, sustained oxidative stress, redox state, thiol oxidation, caspase-3*

Abbreviations: *ActD, actinomycin D; ATZ, 3-amino-1,2,4-triazole carboxy-H_2DCFDA-5-(and-6)-carboxy-2′,7′-dichloro-dihydrofluorescein diacetate; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); GPx, glutathione peroxidase; MS, mercaptosuccinic acid; ROS, reactive oxygen species; SKF, SKF-525A; TNF α, tumor necrosis factor α*

Introduction

Reactive oxygen species (ROS) are well known to be constantly generated, even under normal conditions, as a consequence of aerobic metabolism [1]. The generation and elimination of ROS are well balanced in normal cells, but this balance can be shifted in the direction of oxidative stress by increased ROS generation or decreased ROS elimination [2]. Two kinds of endogenous molecules are involved in the elimination of ROS. One kind includes the enzymes superoxide dismutase, glutathione peroxidase and catalase, and low molecular weight antioxidants such as ascorbate, α-tocopherol and reduced glutathione. These molecules are able to efficiently and specifically scavenge ROS at relatively low concentrations [3]. The other kind includes free amino acids, peptides, and proteins in general. These have a relatively low antioxidant activity per mole. Such molecules also contribute to overall ROS scavenging activity, because they are present in cells at high levels [4–6].

Oxidative stress affects quite a variety of cell functions [3]. When cells are exposed to oxidative stress, they often respond by undergoing their demise *via* apoptosis or necrosis [7–9]. Apoptosis and necrosis are two forms of cell death with clearly different morphological and biological features, and the levels of ROS are thought to determine apoptosis or necrosis. Higher concentrations of ROS induce

Correspondence: N. Shimamoto, Faculty of Pharmaceutical Sciences at Kagawa Campus, Tokushima Bunri University, 1314-1, Shido, Sanuki, Kagawa 769-2193, Japan. Tel: 81 87 894 5111. Ex. 8401. Fax: 81 87 894 0181. E-mail: n-shimamoto@kph.bunri-u.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis Ltd
DOI: 10.1080/10715760500043231

necrosis while moderate to low concentrations induce apoptosis [10,11]. Among the numerous factors affecting apoptosis, caspases, a family of cysteine proteases, are widely recognized to play fundamental roles in apoptosis [12–14], and the active-site cysteine residues of caspases are very sensitive to oxidation [15]. Recombinant caspase-3 is reversibly inactivated by hydrogen peroxide (H_2O_2); activity is restored by the addition of reducing agents [16]. This suggests that sustained oxidative stress is required to keep caspase-3 inactivated. Caspase-3, similar to other caspases, is synthesized as a proenzyme and is proteolytically processed to form an enzymatically active heterodimer. This processing is inhibited under a thiol-oxidized state [17,18]. Thus, ROS can regulate caspase activity leading to apoptosis.

We previously showed that oxidative stress and apoptosis are induced in rat primary hepatocytes when catalase and glutathione peroxidase activities are inhibited for 24 h by 3-amino-1,2,4-triazole (ATZ) and mercaptosuccinic acid (MS), respectively [19]. Since protein thiols are targets of ROS, it is important to clarify the relationship between ROS levels and caspase activity. Thus, this study was undertaken to determine whether caspase-3 was involved in primary hepatocyte apoptosis. Caspase-3 was chosen because it is a well-known executor of apoptosis in several cell types.

Materials and methods

Materials

WILLIAMS' E medium, ATZ, MS, SKF-525A (SKF), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), tumor necrosis factor α (TNF α), actinomycin D (ActD), and Hoechst 33342 were obtained from Sigma-Aldrich (St. Louis, MO). L-ascorbic acid sodium salt (vitamin C) was obtained from Wako Pure Industries, Ltd (Osaka, Japan); 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) from Molecular Probes (Eugene, OR); DEVD-CHO from BIOMOL (Plymouth Meeting, PA). Anti-caspase-3 rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (Beverly, MA); Activated Thiol Sepharose 4B from Amersham Biosciences Corp. (Piscataway, NJ). All other chemicals were obtained from Sigma-Aldrich or Wako and were of the highest quality commercially available.

Isolation and culture of rat hepatocytes

Male Wistar rats (150–200 g body weight) were obtained from Nippon CLEA (Osaka, Japan). Isolation of hepatocytes was described in a previous report [19]. Briefly, rats were anesthetized with intraperitoneal administration of sodium pentobarbital (50 mg/kg). The liver was cleared of blood by perfusion through the portal vein with Ca^{2+}-free buffer at 37°C for 5–7 min. This was followed by perfusion with a buffer containing 0.025% freshly prepared collagenase solution at 37°C for about 10 min. The liver was dissected, minced and dispersed in WILLIAMS' E medium by gentle agitation, and then filtered through two layers of gauze. The resulting cell suspension was centrifuged at 50 × *g* for 1 min at 4°C and the pellet was resuspended in WILLIAMS' E medium. This centrifugation and resuspension procedure was repeated three times. Cell viability was determined by trypan blue exclusion; about 90% of cells were viable. Cells were counted and plated at a density of 6.5×10^5 cells per 6-well collagen type I-coated plate or 1.5×10^6 cells per 60 mm collagen type I-coated dishes (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) (depending on experimental protocols) in WILLIAMS' E medium containing 10% FBS, 300 nM insulin and 100 nM dexamethasone. Cell suspensions were incubated at 37°C for 2 h under a 95% air, 5% CO_2 atmosphere. Medium was then changed. Cells were further incubated in the presence or absence of inhibitors or an antioxidant for 24 h.

Treatment with inhibitors or antioxidant

ATZ and MS at final concentrations of 20 mM and 7 mM, respectively, were added to the well at time 0 (when the medium was changed after seeding) and the incubation continued for another 24 h. SKF (30 μM), vitamin C (1 mM) or DEVD-CHO (100, 300 μM or 1 mM) were added 20 min before time 0. ATZ, MS, vitamin C and DEVD-CHO were dissolved in distilled water; SKF in 0.1% dimethyl sulfoxide (DMSO). This DMSO concentration did not have significant effects on the results.

Determination of cell survival/apoptosis

Viable cells were evaluated by trypan blue exclusion at 0, 9, 12, 15, 18, 21 and 24 h after the medium was changed. Trypan blue dye was added to each well at a final concentration of 0.1% and cells were incubated for 2–3 min at room temperature. More than 300 cells were examined using an inverted phase contrast microscope and the percentage of viable cells was calculated. Chromatin condensation and DNA fragmentation were used as indices of apoptosis. To assess chromatin condensation, DNA binding fluorochrome Hoechst 33342 was assessed at the indicated times. Hoechst 33342 was added to each well to a final concentration of 5 μg/ml and cells were incubated for 5 min at room temperature.

Chromatin condensation was determined (at identical times that were used for the assessment of cell viability) from fluorographs using an excitation wavelength at 365 nm of at least 300 nuclei. Data were

captured by a digital CCD camera (Hamamatsu C4742-95-10, Hamamatsu Photonics, Hamamatsu, Japan) associated with a computer-assisted inverted phase contrast fluoromicroscope. For the detection of DNA fragmentation, hepatocytes were scraped and pelleted after 24 h incubation. Cellular DNA was extracted and was purified using an Apoptosis DNA ladder kit (Wako). DNA was dissolved in Tris-EDTA buffer and electrophoresed in a 1.5% agarose gel, followed by visualization under UV light after staining with SYBR Green I. Fluorographs of the DNA ladder were stored in the computer-assisted image analyzer (Fluor-S Multilmager, Bio-Rad, Hercules, CA).

Evaluation of ROS levels by flow cytometry

ROS levels were determined using carboxy-H_2DCFDA as a fluorescent probe. Carboxy-H_2DCFDA, which is cell membrane-permeable, is hydrolyzed to Carboxy-H_2DCF by esterases inside the cells. Fluorescence from the intracellular oxidation of carboxy-H_2DCF in the presence of ROS, mainly H_2O_2, was used as a general and reliable index of intracellular oxidative stress [20–22]. At the indicated times, hepatocytes were incubated with 10 μM carboxy-H_2DCFDA for 10 min under the above culture conditions. Cells were then washed twice with ice-cold phosphate-buffered saline (PBS) and detached from the plates by trypsinization. Fluorescence was measured by flow cytometry using a FACSCalibur instrument (BD Bioscience, San Jose, CA) with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Generally, 10,000 events were monitored and data analysis was performed using the Cell Quest software program (BD Bioscience).

Measurement of protein thiol content

The number of thiol groups was estimated by the reaction between thiols and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) [23,24]. Following incubation, hepatocytes were collected at 0, 6, 12, 18 and 24 h by centrifugation and the supernatant was discarded. The cell pellet was washed twice with 6.5% trichloroacetic acid: this entailed centrifugation at 13,000 × *g* for 5 min at 4°C, and resuspension in 0.5 M Tris-HCl buffer at pH 7.6 containing 100 μM DTNB. After 20 min incubation in the dark at room temperature, the absorbance at 412 nm was measured by a spectrophotometer (U-3300, HITACHI). Data were expressed as nmol SH per mg protein, calculated from a calibration curve using L-cysteine as a standard.

Caspase-3 enzymatic assay

Caspase-3 activity was determined by a Caspase-3 Fluorometric Assay kit (R&D Systems Inc., Minneapolis, MN). Hepatocytes were collected at the indicated times and washed with ice-cold PBS. Cells were lysed by the addition of Cell Lysis Buffer and incubated on ice for 10 min. The enzymatic reaction of caspase-3 was started with 50 μM of DEVD-AFC (a caspase-3 substrate) and the lysate was then incubated for 30 min at 37°C. The fluorescence from 7-amino-4-(trifluoromethyl) coumarin (AFC) released by cleavage of the substrate was continuously monitored using an excitation wavelength of 390 nm and an emission wavelength of 538 nm using a Fluoroskan Ascent FL instrument (Labsystems, Mountain View, CA). The fluorescence from cell lysates without a fluorescent substrate was used as a blank. Caspase-3-like activity in cells treated with ATZ plus MS, or TNF α plus ActD, were compared to those of untreated cells and expressed as a fold change.

Immunoblotting analysis

After 6, 12 and 18 h of incubation, hepatocytes were collected by scraping and washed by dilution with ice-cold PBS and centrifugation. The cells were resuspended in hypotonic buffer (20 mM HEPES; pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EGTA, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 20 μM APMSF) and homogenized. After three freeze-thaw cycles, cell debris was removed by centrifugation at 13,000 × *g* for 5 min at 4°C, and the resulting supernatant was used for immunoblotting. The supernatant proteins were examined by SDS-PAGE using a 7.5–15% (w/v) gradient polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane in a wet-blotting apparatus. The membrane was then washed with TBS (20 mM Tris-HCl; pH 7.4, 150 mM NaCl), and incubated with Block Ace (Dainippon Pharmaceutical, Osaka, Japan) for 60 min at room temperature. The blocked membranes were incubated over night at 4°C with an anti-caspase-3 rabbit polyclonal antibody diluted 1:200 in TTBS (TBS containing 0.05% Tween 20). After washing with TTBS, the membrane was incubated for 60 min with alkaline phosphatase-conjugated anti-rabbit goat-polyclonal antibody diluted 1:1000 in TTBS. Finally, after washing with TTBS, the membrane was incubated with BCIP and NBT as alkaline phosphatase substrates and the visualized bands were stored in the computer-assisted image analyzer (Fluor-S Multilmager, Bio-Rad).

Estimation of intracellular caspase-3 redox state

Separation of unoxidized from oxidized thiol proteins was performed by covalent chromatography using Activated Thiol Sepharose 4B basically according to the manufacturer's instructions. Hepatocytes were scraped and washed with ice-cold

PBS at 6, 12 and 18 h after treatment with ATZ and MS. The cells were lysed on ice in cell lysis buffer (50 mM Tris; pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% (v/v) TritonX-100, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 20 μM APMSF). After disrupting the cell membranes by sonication, cell debris was removed by centrifugation at 13,000 × *g* for 5 min at 4°C and the supernatant was used as the cell lysate. Cell lysates were mixed with Activated Thiol Sepharose 4B (80 μl drained gel/mg protein) pre-equilibrated with cell lysis buffer, and kept over night at 4°C to allow protein thiols to bind covalently to the resin. After centrifugation at 10,000 × *g* for 1 min at 4°C, the precipitated resin was washed twice with cell lysis buffer and then protein thiols bound to the resin were eluted with cell lysis buffer containing 10 mM dithiothreitol (DTT). Oxidized pro-caspase-3 in the supernatant and unoxidized pro-caspase-3 in the eluents were analyzed by immunoblotting as described in "Immunoblotting Analysis".

Determination of protein content

Protein content was determined according to the method of Bradford [25] using bovine serum albumin as a standard.

Statistical analysis

Data for each variable are expressed as mean ± SE. Data obtained from the two groups were compared using Student's *t*-test with Holm's correction for multiple comparisons. *P* values of less than 0.05 were considered significant.

Results

Inhibition of hepatocyte apoptosis by treatment with cytochrome P450 inhibitor or antioxidant

Treatment with ATZ and MS for 9 h had no effect on cell viability. However, treatment periods longer than 12 h induced a time-dependent

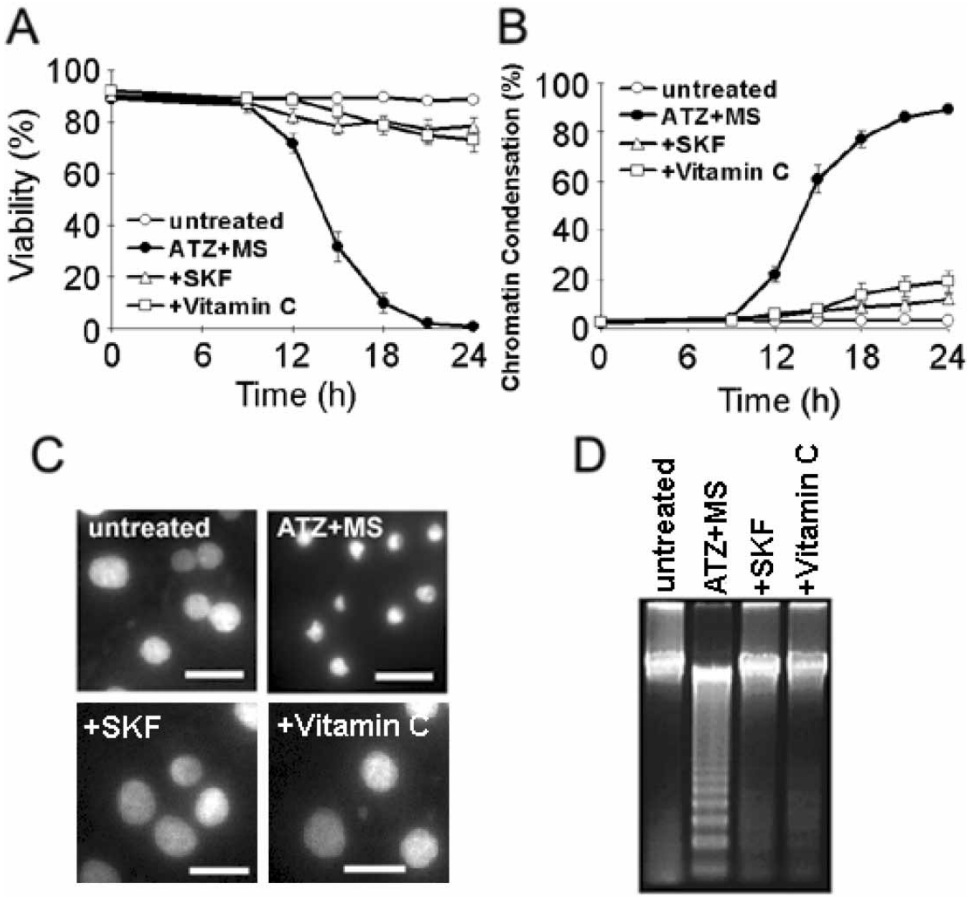

Figure 1. Inhibitory effects of cytochrome P450 inhibitor, SKF or antioxidant, vitamin C, on ATZ and MS-induced hepatocyte apoptosis. Primary rat hepatocytes were pretreated with SKF (30 μM) or vitamin C (1 mM) for 20 min and then incubated for 24 h in the presence or absence of ATZ (20 mM) and MS (7 mM). Panel A and B show time-courses for percent viability and chromatin condensation, respectively. The values are given as the mean ± S.E. of seven separate experiments. Panel C shows fluorescence micrographs of Hoechst 33342 stained nuclei of untreated hepatocytes (upper left), treated hepatocytes with ATZ and MS in the absence (upper right) or presence of SKF (bottom left) or vitamin C (bottom right), respectively. Bars in the micrographs represent 25 μm. Panel D shows agarose gel electrophoresis results. After hepatocytes were scraped and collected, cellular DNA was extracted and then electrophoresed.

decrease in cell viability (Figure 1A). Chromatin condensation occurred at 12 h and increased thereafter in a time-dependent manner (Figure 1B, C). The onset and time course for the decrease in cell viability was very similar to that for the increase in chromatin condensation. The addition of SKF, a cytochrome P450 inhibitor, or vitamin C, an antioxidant, inhibited the decrease in cell viability and the increase in chromatin condensation to almost the same extent (Figure 1A, B, C). DNA fragmentation was not detected after treatment with SKF or vitamin C (Figure 1D).

Inhibitory effects of SKF or Vitamin C on sustained and increased ROS levels

We next examined the levels of ROS by flow cytometry. Typical examples and results from seven separate experiments of flow cytometric analyses of carboxy-H_2DCFDA-stained hepatocytes are shown in Figure 2A and B, respectively, following exposure to

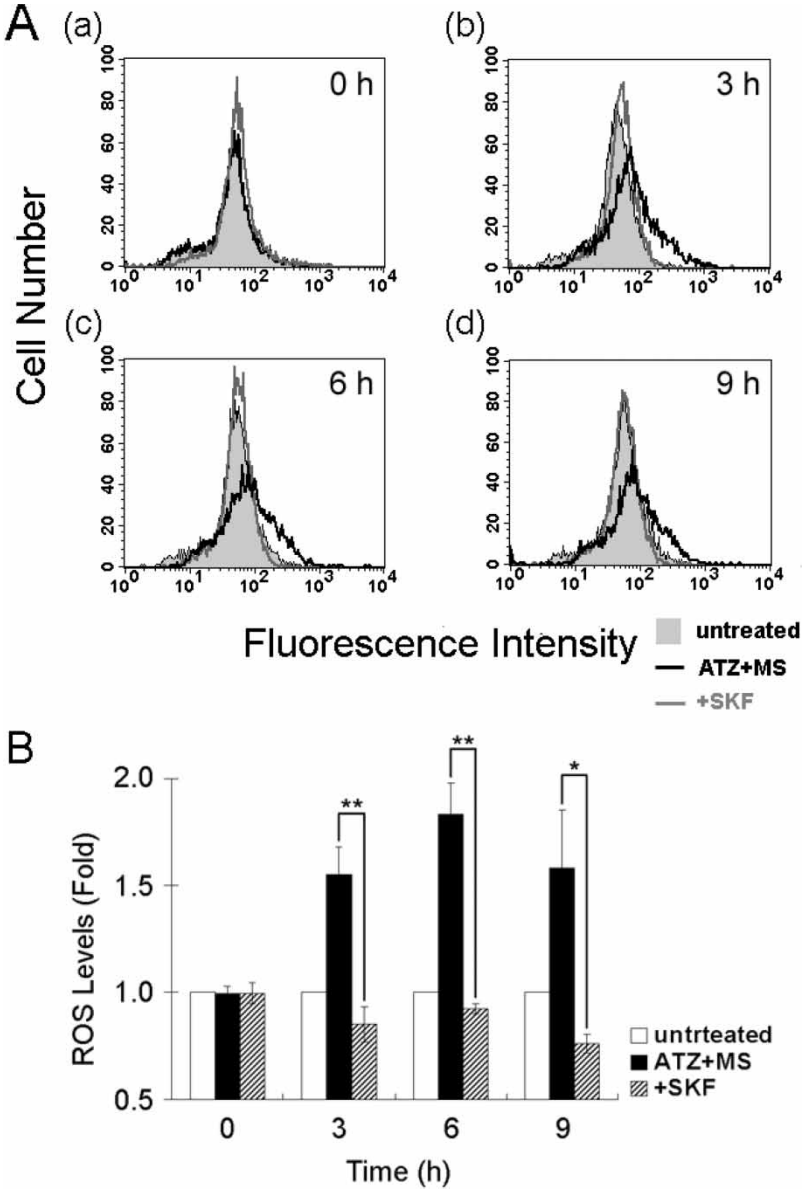

Figure 2. Flow cytometric analysis of intracellular ROS levels. Primary rat hepatocytes were pretreated with SKF (30 μM) for 20 min and then incubated for 9 h in the presence or absence of ATZ (20 mM) and MS (7 mM). Cells were treated with carboxy-H_2DCFDA and fluorescence intensity derived from carboxy-H_2DCFDA oxidation was measured by a flow cytometer. Panel A shows the overlay of three representative fluorescence flow cytometry analyses at 0 h (a), 3 h (b), 6 h (c), and 9 h (d) incubation. Panel B shows the mean fluorescent intensity in treated hepatocytes divided by that in untreated cells at the indicated time. The ROS levels in untreated cells at the indicated times are almost the same (0 h, 100 ± 13; 3 h, 85 ± 11; 15 h, 87 ± 8; 18 h, 104 ± 12 arbitrary unit, respectively). Values are given as mean ± S.E. of seven separate experiments. Data were analyzed using Student's *t*-test with Holm's correction for multiple comparisons. *P* values less than 0.05 were considered significant. **; $p < 0.01$, *; $p < 0.05$.

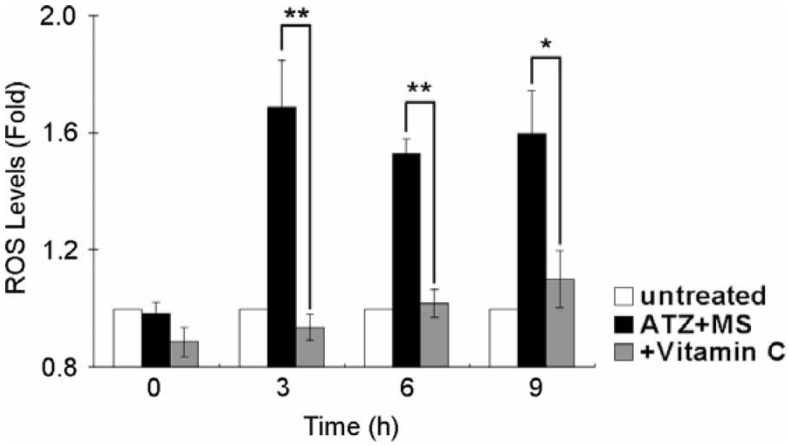

Figure 3. Suppression of increased and sustained ROS levels by treatment with antioxidant. Primary rat hepatocytes were pretreated with vitamin C (1 mM) for 20 min and then incubated for 9 h in the presence or absence of ATZ (20 mM) and MS (7 mM). The fluorescence intensity of carboxy-DCF was measured by a flow cytometer. The mean value of the fluorescent intensity of treated cells was divided by that of untreated ones at the indicated times, and are shown as fold changes. The data represent the mean ± S.E. of six separate experiments. Data were analyzed using Student's *t*-test with Holm's correction for multiple comparisons. *P* values less than 0.05 were considered significant. *; $p < 0.05$.

ATZ and MS at 3 h intervals up to 9 h. An increase in ROS by ATZ and MS treatment was noted at 3 h, as evidenced by a significant shift in carboxy-DCF intensity to the right (Figure 2A). The shift was observed for up to 9 h, showing that the hepatocytes were exposed to excess levels of ROS. The ROS levels in untreated cells remained unchanged up to 9 h (0 h, 100 ± 13; 3 h, 85 ± 11; 6 h, 87 ± 8; 9 h, 104 ± 12 arbitrary unit). Increased ROS levels in ATZ and MS treated cells were 1.5, 1.8 and 1.6 fold at 3, 6, and 9 h, respectively, compared to ROS levels in untreated cells (Figure 2B). These increased and sustained levels of ROS were totally suppressed to the ROS levels of untreated cells as early as 3 h after exposure of cells to SKF (Figure 2A, B) or vitamin C (Figure 3). The inhibitory effects of SKF or vitamin C persisted for up to 9 h (Figures 2A, B and 3).

As described above, a decrease in cell viability and an increase in chromatin condensation were observed after 12 h of treatment with ATZ and MS. These results indicate that drastic changes in the living cell population and cell size occurred after 12 h incubation, showing that flow cytometry is not applicable in such situations. Thus, the levels of ROS induced by treatment with ATZ and MS were estimated only up to 9 h of incubation.

Intracellular protein thiol contents

As shown in Table I, the protein thiol contents in untreated cells were 67.9 ± 11.9 and 67.5 ± 12.9 nmol SH/mg protein at 0 h and 24 h, respectively, although only slight changes were noted throughout the 24 h incubation. Thus, the protein thiols can be regarded as constant during the experimental period. Incubation of cells with ATZ and MS had no effect on intracellular thiol contents up to 6 h. However, after 12 h incubation, ATZ and MS treatment resulted in a decrease in protein thiols, which reached a plateau at 18 h. The protein thiol contents in ATZ and MS treated cells were significantly lower than those of untreated cells at 18 and 24 h. The decrease in protein thiols was completely inhibited by pretreatment with SKF.

Hepatocyte apoptosis and caspase-3

The addition of the caspase-3 inhibitor, DEVD-CHO, at a concentration of 100, 300 μM or 1 mM did not have any effect on decreases in cell viability and increases in chromatin condensation caused by treatment with ATZ and MS (Figure 4A, B, C). The time course for the decrease in cell viability or the increase in chromatin condensation in DEVD-CHO treated cells was the same as that for ATZ and MS treated cells (Figure 4A, B). Furthermore, DEVD-CHO had no effect on DNA fragmentation at 24 h (Figure 4D). The above findings clearly show that a caspase-3 inhibitor has no effect on hepatocyte apoptosis induced by treatment with ATZ and MS. To corroborate this finding, the issue of whether ATZ and MS treatments affect caspase-3-like activity was investigated as a function of time. The caspase-3-like activity in untreated cells remained unchanged throughout the experiment (0 h, 0.79 ± 0.16; 6 h, 0.79 ± 0.14; 12 h, 0.83 ± 0.09; 18 h, 0.83 ± 0.12 arbitrary unit/mg protein). As shown in Figure 5, ATZ and MS treatments did not affect caspase-3-like activity. In fact, caspase-3-like activity in the ATZ and MS treated cells was 0.8 ± 0.1, 1.1 ± 0.1 and

Table I. Inhibition of the decrease in protein thiol contents by SKF.

	Protein thiol contents (nmol/mg protein)		
Time (h)	untreated	ATZ + MS	+SKF
0	67.9 ± 11.9	69.6 ± 11.6	78.5 ± 13.1
6	88.0 ± 15.1	73.8 ± 10.4	68.5 ± 12.8
12	77.4 ± 10.7	47.0 ± 10.1	78.2 ± 10.5
18	76.8 ± 14.2	33.2 ± 4.1†	68.1 ± 9.6*
24	67.5 ± 12.9	26.8 ± 5.2†	68.4 ± 12.5*

Primary rat hepatocytes were pretreated with SKF (30 μM) for 20 min and then incubated for 24 h in the presence or absence of ATZ (20 mM) and MS (7 mM). Protein thiol levels were determined by spectrophotometry using DTNB, which specifically reacts with thiol groups. The values are given as the mean ± S.E. of five separate experiments. Data were analyzed using the Student's *t*-test with Holm's correction for multiple comparisons. *P* values less than 0.05 were considered significant. †; $p < 0.05$ vs. untreated hepatocytes. *; $p < 0.05$ vs. ATZ and MS treated hepatocytes.

0.8 ± 0.1 fold at 6, 12 and 18 h, respectively, compared to that of untreated cells. Treatment with TNF α and ActD, at final concentrations of 50 and 50 ng/ml, respectively, induced cell death in primary rat hepatocytes (data not shown). TNF α and ActD were added to the well at time 0, and incubated for a further 24 h. Cell death induced by TNF α and ActD treatments was accompanied by chromatin condensation and DNA fragmentation; DEVD-CHO clearly inhibited this apoptosis (data not shown). Caspase-3-like activity was dramatically increased in a time-dependent manner in hepatocytes that had been treated with TNF α and ActD compared to untreated hepatocytes (Figure 5).

The processing of pro-caspase-3 occurred in a time-dependent manner in hepatocytes treated with TNF α and ActD, as evidenced by the time-dependent increases in the p17 large subunit showing the cleavage

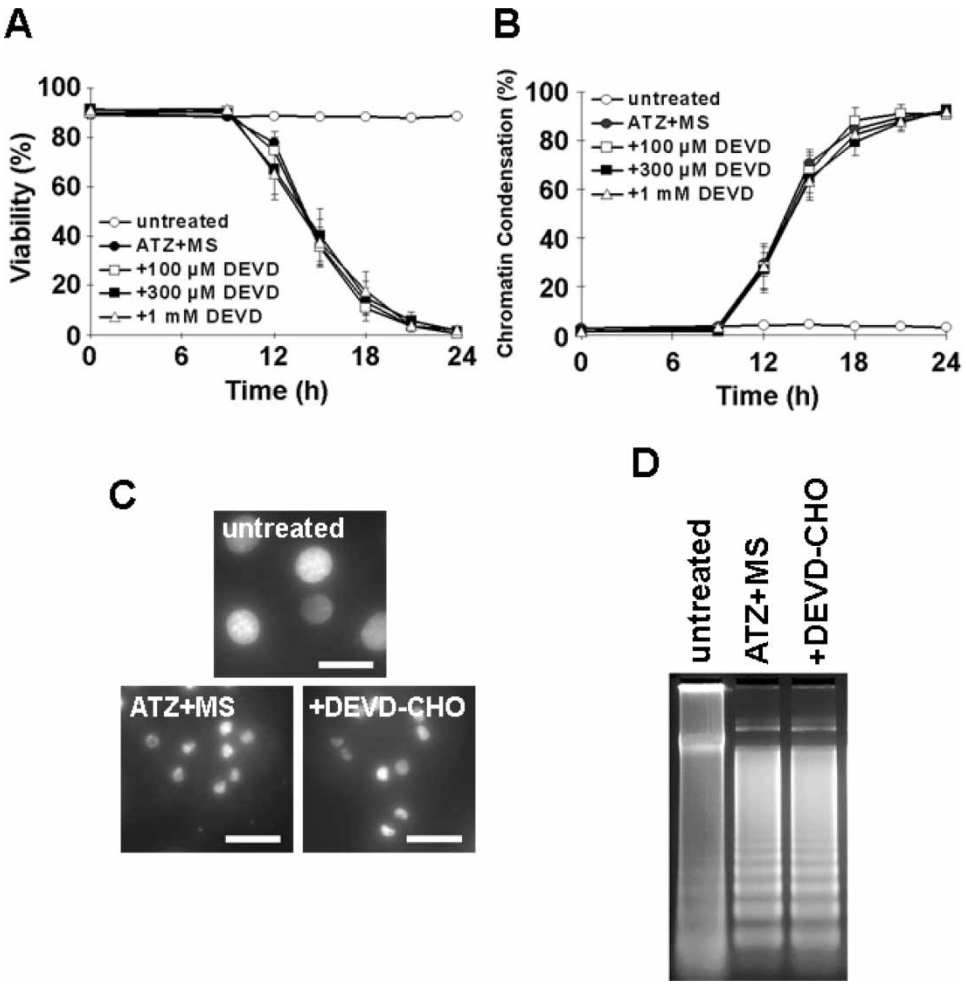

Figure 4. Failure of the caspase-3 inhibitor DEVD-CHO to prevent ATZ and MS-induced apoptosis Primary rat hepatocytes were pretreated with DEVD-CHO (100, 300 μM or1 mM) for 20 min and then incubated for 24 h in the presence or absence of ATZ (20 mM) and MS (7 mM). Panel A and B show time-courses for percent viability and chromatin condensation, respectively. Values are given as the mean ± S.E. of seven separate experiments. Panel C shows fluorescence micrographs of Hoechst 33342 stained nuclei of untreated hepatocytes (upper panel), treated hepatocytes with ATZ and MS in the absence (bottom left) or presence of 100 μM DEVD-CHO (bottom right), respectively. Bars in the micrographs represent 25 μm. Panel D shows agarose gel electrophoresis results. Untreated hepatocytes and treated hepatocytes with ATZ and MS in the absence or presence of 100 μM DEVD-CHO were collected at 24 h. Cellular DNA was extracted and then electrophoresed.

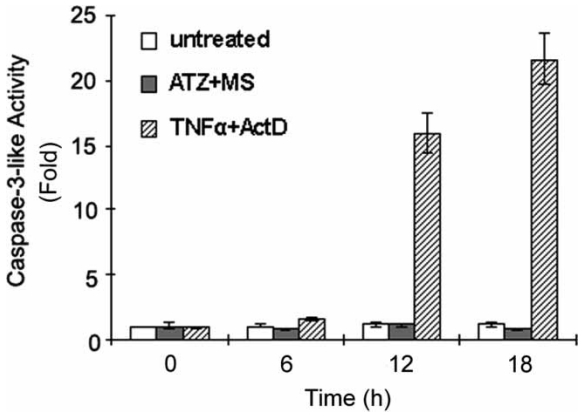

Figure 5. Effects of ATZ and MS treatment on caspase-3-like activity. Primary rat hepatocytes were treated with ATZ (20 mM) plus MS (7 mM) or TNF α (50 ng/ml) plus ActD (50 ng/ml) at time 0 and then incubated for 18 h. Caspase-3-like protease activity in cell lysates was measured using an AFC-conjugated substrate specific for caspase-3-like enzymes. "Caspase-3-like" refers to all DEVDase activities. The values are given as the mean ± S.E. of five separate experiments.

of pro-caspase-3 (Figure 6). Pro-caspase-3 was not processed by ATZ and MS treatments at 6, 12 and 18 h.

Intracellular caspase-3 redox state

Activated Thiol Sepharose 4B can separate thiol-containing proteins from other proteins [26–28]. Thus, this sepharose can be used for a separation of unoxidized thiol proteins from oxidized ones and allows us to measure unoxidized and oxidized pro-caspase-3, using the anti-caspase-3 antibody to estimate the intracellular caspase-3 redox state. The amount of unoxidized pro-caspase-3 in ATZ and MS treated hepatocytes declined in a time-dependent manner, and was barely detectable at 18 h. In contrast, the amount of oxidized pro-caspase-3 increased time-dependently (Figure 7). Both decreases in unoxidized pro-caspase-3 and increases in oxidized pro-caspase-3 were suppressed by pretreatment with SKF. These results indicate that the intracellular caspase-3 redox

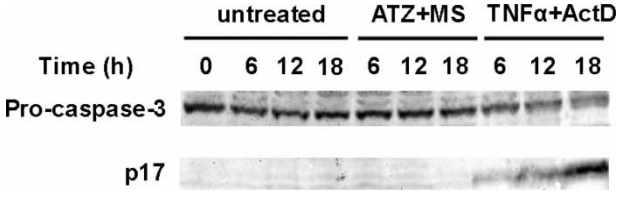

Figure 6. Effects of ATZ and MS treatments on pro-caspase-3 processing. Primary rat hepatocytes were treated with ATZ (20 mM) and MS (7 mM) or TNF α (50 ng/ml) and ActD (50 ng/ml) at time 0 and then incubated for 18 h. At the indicated times, cells were collected and lysed. The caspase-3 precursor, pro-caspase-3 and its cleaved large subunit, p17 were detected in the lysates by immunoblotting. Details of the procedure are described under "Materials and methods".

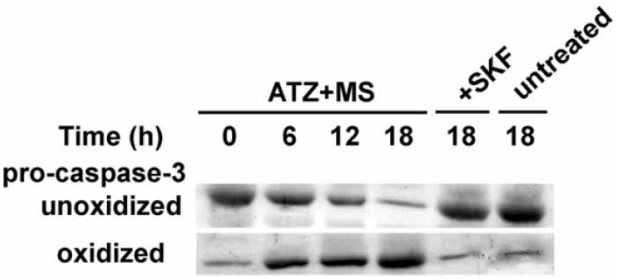

Figure 7. Time-dependent changes in amounts of unoxidized or oxidized pro-caspase-3. Primary rat hepatocytes were pretreated with SKF (30 μM) for 20 min and then incubated for 18 h in the presence or absence of ATZ (20 mM) and MS (7 mM). At the indicated times, cells were collected and lysed. Activated Thiol Sepharose 4B was added to cell lysates to bind protein thiols to the resin. Pro-caspase-3 bound to the resin was termed "unoxidized" and unbound pro-caspase-3 was termed "oxidized". Oxidized pro-caspase-3 in the supernatants and unoxidized pro-caspase-3 in the eluents were analyzed by immunoblotting. Details of the procedure are described under "Materials and methods".

state was shifted to the oxidized state as a function of time. The amount of both unoxidized and oxidized procaspase-3 in untreated cells did not change.

Discussion

Treatment of rat primary hepatocytes with ATZ and MS induced sustained increases in ROS levels, followed by typical features of apoptosis. Caspase-3 activity remained unchanged and the processing of pro-caspase-3 was not detected during apoptosis.

We previously reported that increases in lipid peroxidation were detected at 24 h, but not at 6 h [19]. Generally, lipid peroxidation is considered to be the ultimate consequence of cells being overwhelmed by oxidative stress. In the present study, protein thiol levels, which are potent targets of ROS, were gradually decreased after 12 h incubation in ATZ and MS treated hepatocytes. Decreases in cell viability and increases in chromatin condensation were detected only at 12 h and later. However, no evidence for cell abnormalities was found for periods of up to 9 h, strongly indicating that the hepatocytes were able to tolerate the increased and sustained ROS levels and were functioning normally up to 9 h after treatment with ATZ and MS. Unfortunately, we could not perform a flow cytometric analysis after 9 h incubation because apoptosis reduced the number of hepatocytes required for the analysis. However, it is reasonable to conclude that the hepatocytes were exposed to unmanageable oxidative stress after 12 h incubation, judging from the decrease in protein thiols and the increase in lipid peroxidation at 24 h [19].

Cytochrome P450 is a drug-metabolizing enzyme and forms a super family. On the other hand, cytochrome P450 has long been known to be involved in oxidative stress. Almost all isoforms of cytochrome

P450 produce ROS *via* catalytic cycle in the presence or absence of substrates [29–31]. SKF potently inhibits reduction of a wide variety of P450 isoforms in a non-competitive manner [32] and therefore, inhibits ROS production. In fact, in rat hepatocyte microsomes, ROS production *via* cytochrome P450 systems is reported to be suppressed by SKF [33]. Thus, the finding that SKF as early as 3 h completely suppressed the increased and sustained ROS levels caused by the ATZ and MS treatment confirms the major source of ROS being directly related to the cytochrome P450 enzyme [19]. Furthermore, SKF inhibited apoptosis, indicating that sustained and increased ROS levels are closely related to apoptosis. Treatment with vitamin C attenuated not only the sustained and increased ROS levels but also the decreases in cell viability, increases in chromatin condensation and DNA fragmentation. Vitamin C is a water-soluble antioxidant that has the capacity to scavenge ROS such as hydroxyl radical or superoxide anion. Thus the suppressive effect of vitamin C on increased levels of ROS is probably due to its ability to scavenge excess amounts of ROS produced by the inhibition of catalase and GPx. Thus, in the presence of vitamin C, the antioxidative capacity of the hepatocytes treated with ATZ and MS is considered to be similar to that of untreated cells. Therefore, apoptosis was not triggered. We had assessed that apoptosis was not induced when hepatocytes were treated with ATZ and MS for 3 h followed by washing them out and cultured for further 21 h (data not shown). Collectively, that the inhibition of ROS production or the replenishment of anti oxidative capacity suppressed hepatocyte apoptosis, strongly indicates that sustained and increased ROS levels are responsible for hepatocyte apoptosis after 12 h incubation under these experimental conditions.

Protein thiols gradually decreased over time after 12 h treatment with ATZ and MS. Considering that hepatocytes treated with ATZ and MS are thought to be under unmanageable oxidative stress, and that thiol compounds are potent targets of ROS, protein thiols would likely be oxidized. This conclusion is supported by our finding that the decrease in protein thiol levels in ATZ and MS treated cells parallels the increase in the ratio of chromatin condensation. In addition, SKF inhibited apoptosis as well as the gradual decrease in protein thiol contents after 12 h incubation. This further supports the possibility that the P450 system contributes to increased ROS levels which causes apoptosis.

The caspase family play a fundamental role in apoptosis. Above all, caspase-3 is one of the key executors of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many essential proteins, such as the nuclear enzyme, poly(ADP-ribose) polymerase [34]. Pro-caspase-3 is proteolytically cleaved and activated to form caspase-3. This is the case for TNFα and ActD-induced apoptosis in primary rat hepatocytes [35–37]. As expected, treatment of hepatocytes with TNFα and ActD elicited apoptosis as evidenced by a representative morphological feature of apoptosis accompanied by a vast increase in caspase-3 activity, indicating that caspase-3 enzyme systems are functional. However, apoptosis induced by ATZ and MS does not appear to be related to caspase-3. The evidence for this is as follows: (1) the caspase-3 inhibitor, DEVD-CHO, did not inhibit this apoptosis, (2) caspase-3-like activity in hepatocytes treated with ATZ and MS was almost the same as that of untreated hepatocytes, (3) processing of pro-caspase-3 to the activated form (caspase-3) was not detected. (4) unoxidized procaspase-3 decreased during apoptosis. Furthermore, total amounts of oxidized and unoxidized pro-caspase-3 were not changed. As already established, caspases other than caspase-3 are responsible for apoptosis in many types of cells including rat hepatocytes. The pan-caspase inhibitor, z-VAD-fmk, failed to inhibit this apoptosis at concentrations ranging from 50 to 300 μM and no change in caspase-2, -6, -7, -8 and -9 activity was found under the same conditions as this study (data not shown). These findings suggest that other caspases were also in oxidized states in this study. Further studies to clarify the relationship between these caspases and this apoptosis will be required.

As mentioned above, thiol-containing compounds or enzymes are targets of ROS. Caspase-3 contains several thiol groups and cysteine residue Cys-163 is thought to be an active site. Basic amino acids surrounding the active site cysteine are considered to lower its pKa and thus, shift the thiol-thiolate anion equilibrium toward the thiolate anion [38,39]. H_2O_2 is known to react with the thiolate anion to initially produce the sulfenic acid and then the disulfide [39,40], and therefore, lower pKa is postulated to enhance its reactivity with the active site cysteine. Consequently, the active site cysteine is the most likely target of H_2O_2 and would be sensitive to oxidation [41,42]. In this study, the observation that pro-caspase-3 thiol levels decreased prior to the reduction of total protein thiol levels supports the above mechanism. In fact, caspase-3 is reported to be inactivated by oxidants such as H_2O_2 [16] and the processing of pro-caspase-3 appears to be suppressed under a thiol-oxidized state [17] showing that the oxidation of thiol groups either of pro-caspase-3 or caspase-3 causes a loss of caspase-3 proteolytic activity. However, the oxidation of thiol groups of caspase-3 has been reported to be reversible, and inactivated caspase-3 activity can be recovered by treatment with thiol-reducing agents, including DTT and thioredoxin [16,18]. Thus, this shows that sustained oxidative stress is required to keep pro- or caspase-3 in an oxidized form, as was the case in our

study. In support of this, treatment with ATZ and MS elicited time-dependent decreases in the levels of thiol groups of pro-caspase-3. Collectively, sustained and increased ROS produced by treatment with ATZ and MS continuously oxidize cysteine residues in the vicinity of the active site in both pro- and caspase-3 keeping them in the oxidized state for the period of the experiment.

An increasing number of reports regarding caspase-independent apoptosis induced by many stimuli or treatments has accumulated to date [43–45]. In recent years, caspase-independent apoptosis in relation to increased levels of ROS was reported in some cells, including thymocytes [46], retinal cells [47], MRC-5 fibroblasts [48] and PC12 cells [49]. However, the molecular mechanisms of caspase-independent apoptosis are not well characterized. Nucleosomal DNA fragmentation was not detected in these reports [46–49], although chromatin condensation and large scale DNA fragmentation were noted, suggesting the involvement of apoptosis-inducing factor, which has been reported to induce large-scale DNA fragmentation [50]. Our results clearly revealed nucleosomal DNA fragmentation. Further study is needed to clarify molecular mechanisms for the hepatocyte apoptosis induced by treatment with ATZ and MS.

In conclusion, treatment of primary rat hepatocyte with a catalase inhibitor and a GPx inhibitor elicits sustained and increased ROS levels, which are probably responsible for both apoptosis and preventing caspase-3 activation. Thus, caspase-3 related pathways do not appear to be involved in hepatocyte apoptosis under these experimental conditions.

Acknowledgements

We thank Drs S. Asahi and M. Iwasaki for helpful discussion, and Dr N. Yamazaki for statistical analysis. We are also grateful to Drs M. Fujino, T. Soda and T. Miwa for their encouragement throughout this study.

References

[1] Droge W. Oxidative stress and aging. Adv Exp Med Biol 2003;543:191–200.
[2] Sies H. Oxidative stress: Oxidants and antioxidants. Exp Physiol 1997;82(2):291–295.
[3] Droge W. Free radicals in the physiological control of cell function. Physiol Rev 2002;82(1):47–95.
[4] Stadtman ER. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. Annu Rev Biochem 1993;62:797–821.
[5] Davies KJ, Delsignore ME. Protein damage and degradation by oxygen radicals III. Modification of secondary and tertiary structure. J Biol Chem 1987;262(20):9908–9913.
[6] Davies KJ, Lin SW, Pacifici RE. Protein damage and degradation by oxygen radicals IV. Degradation of denatured protein. J Biol Chem 1987;262(20):9914–9920.
[7] Buttke TM, Sandstrom PA. Oxidative stress as a mediator of apoptosis. Immunol Today 1994;15(1):7–10.
[8] Slater AF, Stefan C, Nobel I, van den Dobbelsteen DJ, Orrenius S. Signalling mechanisms and oxidative stress in apoptosis. Toxicol Lett 1995;82–83:149–153.
[9] Comporti M. Three models of free radical-induced cell injury. Chem Biol Interact 1989;72(1–2):1–56.
[10] Higuchi M, Honda T, Proske RJ, Yeh ET. Regulation of reactive oxygen species-induced apoptosis and necrosis by caspase 3-like proteases. Oncogene 1998;17(21):2753–2760.
[11] Gardner AM, Xu FH, Fady C, Jacoby FJ, Duffey DC, Tu Y, Lichtenstein A. Apoptotic vs. nonapoptotic cytotoxicity induced by hydrogen peroxide. Free Radic Biol Med 1997;22(1–2):73–83.
[12] Cohen GM. Caspases: The executioners of apoptosis. Biochem J 3261997;(PT 1):1–16.
[13] Thornberry NA, Lazebnik Y. Caspases: Enemies within. Science 1998;281(5381):1312–1316.
[14] Zimmermann KC, Bonzon C, Green DR. The machinery of programmed cell death. Pharmacol Ther 2001;92(1):57–70.
[15] Hampton MB, Fadeel B, Orrenius S. Redox regulation of the caspases during apoptosis. Ann NY Acad Sci 1998; 854:328–335.
[16] Borutaite V, Brown GC. Caspases are reversibly inactivated by hydrogen peroxide. FEBS Lett 2001; 500(3):114–118.
[17] Nobel CS, Burgess DH, Zhivotovsky B, Burkitt MJ, Orrenius S, Slater AF. Mechanism of dithiocarbamate inhibition of apoptosis: Thiol oxidation by dithiocarbamate disulfides directly inhibits processing of the caspase-3 proenzyme. Chem Res Toxicol 1997;10(6):636–643.
[18] Ueda S, Nakamura H, Masutani H, Sasada T, Yonehara S, Takabayashi A, Yamaoka Y, Yodoi J. Redox regulation of caspase-3(-like) protease activity: Regulatory roles of thioredoxin and cytochrome c. J Immunol 1998;161(12): 6689–6695.
[19] Shiba D, Shimamoto N. Attenuation of endogenous oxidative stress-induced cell death by cytochrome P450 inhibitors in primary cultures of rat hepatocytes. Free Radic Biol Med 1999;27(9–10):1019–1026.
[20] Negre-Salvayre A, Auge N, Duval C, Robbesyn F, Thiers JC, Nazzal D, Benoist H, Salvayre R. Detection of intracellular reactive oxygen species in cultured cells using fluorescent probes. Methods Enzymol 2002;352:62–71.
[21] Desmots F, Rissel M, Pigeon C, Loyer P, Loreal O, Guillouzo A. Differential effects of iron overload on GST isoform expression in mouse liver and kidney and correlation between GSTA4 induction and overproduction of free radicles. Free Radic Biol Med 2002;32(1):93–101.
[22] Iwai K, Kondo T, Watanabe M, Yabu T, Kitano T, Taguchi Y, Umehara H, Takahashi A, Uchiyama T, Okazaki T. Ceramide increases oxidative damage due to inhibition of catalase by caspase-3-dependent proteolysis in HL-60 cell apoptosis. J Biol Chem 2003;278(11):9813–9822.
[23] Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 1968;25(1):192–205.
[24] Di Monte D, Ross D, Bellomo G, Eklow L, Orrenius S. Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. Arch Biochem Biophys 1984;235(2):334–342.
[25] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.
[26] De Bruin SH, Joordens JJ, Rollema HS. An isolation procedure for the native α chain of bovine hemoglobin. Eur J Biochem 1977;75:211–215.

[27] Ray S, Ray M. Purification and Characterization of NAD and NADP-linked α-ketoaldehyde dehydrogenases involved in catalyzing the oxidation of methylglyoxal to pyruvate. J Biol Chem 1982;257(18):10566–10570.
[28] Mansfeld J, Vriend G, Van den Brug B, Eijsink VG, Ulbrich-Hofmann R. Probing the unfolding region in a thermolysin-like protease by site-specific immobilization. Biochemistry 1999;38(26):8240–8245.
[29] Porter TD, Coon MJ. Cytochrome P450. J Biol Chem 1991;266(21):13469–13472.
[30] Bernhardt R. Cytochrome P450: Structure, function, and generation of reactive oxygen species. Rev Physiol Biochem Pharmacol 1996;127:137–221.
[31] Zangar RC, Davydov DR, Verma S. Mechanisms that regulate production of reactive oxygen species by cytochrome P450. Toxicol Appl Pharmacol 2004;199(3):316–331.
[32] Gillette J, Sasame H, Stripp B. Mechanisms of inhibition of drug metabolic reactions. Drug Metab Dispos 1973;1(1):164–175.
[33] Bondy SC, Naderi S. Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species. Biochem Pharmacol 1994;48(1):155–159.
[34] Cryns V, Yuan J. Proteases to die for. Genes Dev 1998;12(11):1551–1570.
[35] Li J, Bombeck CA, Yang S, Kim YM, Billiar TR. Nitric oxide suppresses apoptosis via interrupting caspase activation and mitochondrial dysfunction in cultured hepatocytes. J Biol Chem 1999;274(24):17325–17333.
[36] Kim YM, de Vera ME, Watkins SC, Billiar TR. Nitric oxide protects cultured rat hepatocytes from tumor necrosis factor-alpha-induced apoptosis by inducing heat shock protein 70 expression. J Biol Chem 1997;272(2):1402–1411.
[37] Boer U, Fennekohl A, Puschel GP. Sensitization by interleukin-6 of rat hepatocytes to tumor necrosis factor alpha-induced apoptosis. J Hepatol 2003; 38(6):728–735.
[38] Rotonda J, Nicholson DW, Fazil KM, Gallant M, Gareau Y, Labelle M, Peterson EP, Rasper DM, Ruel R, Vaillancourt JP, Thornberry NA, Becker JW. The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. Nat Struct Biol 1996;3(7):619–625.
[39] Winterbourn CC, Metodiewa D. Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. Free Radic Biol Med 1999;27(3–4):322–328.
[40] Claiborne A, Yeh JI, Mallett TC, Luba J, Crane EJ, Charrier V, Parsonage D. Protein-sulfenic acids: Diverse roles for an unlikely player in enzyme catalysis and redox regulation. Biochemistry 1999;38(47):15407–15416.
[41] Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J, Elliston KO, Ayala JM, Casano FJ, Chin J, Ding GJ-F, Egger LA, Gaffney EP, Limjuco G, Palyha OC, Raju SM, Rolando AM, Salley JP, Yamin T, Lee TD, Shively JE, MacCross M, Mumford RA, Schmidt JA, Tocci MJ. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature 1992;356(6372):768–774.
[42] Thornberry NA, Molineaux SM. Interleukin-1 beta converting enzyme: A novel cysteine protease required for IL-1 beta production and implicated in programmed cell death. Protein Sci 1995;4(1):3–12.
[43] Jaattela M, Tschopp J. Caspase-independent cell death in T lymphocytes. Nat Immunol 2003;4(5):416–423.
[44] Lockshin RA, Zakeri Z. Caspase-independent cell deaths. Curr Opin Cell Biol 2002;14(6):727–733.
[45] Mathiasen IS, Jaattela M. Triggering caspase-independent cell death to combat cancer. Trends Mol Med 2002; 8(5):212–220.
[46] Jung KC, Park WS, Kim HJ, Choi EY, Kook MC, Lee HW, Bae Y. TCR-independent and caspase-independent apoptosis of murine thymocytes by CD24. cross-linking. J Immunol 2004;172(2):795–802.
[47] Carmody RJ, Cotter TG. Oxidative stress induces caspase-independent retinal apoptosis *in vitro*. Cell Death Differ 2000;7(3):282–291.
[48] Shih CM, Ko WC, Wu JS, Wei YH, Wang LF, Chang EE, Lo TY, Cheng HH, Chen CT. Mediating of caspase-independent apoptosis by cadmium through the mitochondria-ROS pathway in MRC-5 fibroblasts. J Cell Biochem 2004; 91(2):384–397.
[49] Yuyama K, Yamamoto H, Nishizaki I, Kato T, Sora I, Yamamoto T. Caspase-independent cell death by low concentrations of nitric oxide in PC12 cells: Involvement of cytochrome C oxidase inhibition and the production of reactive oxygen species in mitochondria. J Neurosci Res 2003;73(3):351–363.
[50] Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 1999; 397(6718):441–446.