

Superoxide anions and hydrogen peroxide induce hepatocyte death by different mechanisms: Involvement of JNK and ERK MAP kinases

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Background/Aims: In liver diseases, reactive oxygen species (ROS) are involved in cell death and liver injury, but the mechanisms are not completely elucidated. To elucidate the mechanisms of hepatocyte cell death induced by the ROS superoxide anions and hydrogen peroxide, primary cultures of hepatocytes were exposed to the superoxide anion donor menadione (10–50 $\mu\text{mol/L}$) or H_2O_2 (1–5 mmol/L). Hepatocytes were also treated with caspases and MAPKs inhibitors, superoxide dismutase (PEG-SOD) and SNAP, a nitric oxide donor. Apoptosis was determined by measuring caspase-9, -6, -3 activation and cleaved PARP, and necrotic cell death by Sytox Green staining.

Results: (1) Menadione (50 $\mu\text{mol/L}$) induces JNK phosphorylation, caspase-9, -6, -3 activation, PARP cleavage and apoptosis. Superoxide anions-induced apoptosis is dependent on JNK activity. Menadione (50 $\mu\text{mol/L}$) induces the phosphorylation of ERK1/2 and this attenuates cell death. (2) H_2O_2 increases necrotic cell death at high concentration or when H_2O_2 detoxification is impaired. H_2O_2 does not activate MAPKs signalling. (3) PEG-SOD prevents ERK1/2-, JNK- phosphorylation, caspase activation and apoptosis induced by menadione. Glutathione depletion increases menadione-induced apoptosis. (4) SNAP abolishes menadione-induced apoptosis but increases necrotic cell death.

Conclusions: In normal hepatocytes, superoxide anions-induced caspase activation and apoptosis is dependent on JNK activity and totally abolished by superoxide scavengers.

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Keywords: Oxidative stress; Apoptosis; Necrosis; Signal transduction; Caspase; Glutathione.

1. Introduction

Reactive oxygen species (ROS) such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO^\cdot) are produced during normal intracellular metabolism, e.g. mitochondrial metabolism [1,2], and the action of P450 cytochromes [3] and superoxide dismutases [4] and by inflammatory cells. Oxidative stress is the inappropriate exposure to ROS and results from the imbalance between prooxidants and antioxidants leading to cell damage and tissue injury. ROS generation is increased in many pathological situations [5–7], including chronic viral hepatitis [8], alcoholic hepatitis, non-alcoholic fatty liver disease (NAFLD) [9] and chronic cholestasis. In liver diseases, excess of ROS can induce cell death by either

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Abbreviations: Ad5LacZ, adenoviral β -galactosidase; Ad5IkBAA, adenoviral dominant-negative IkB- α ; 3-AT, 3-amino-1,2,4-triazole; BSO, D,L-buthionine-(S,R)-sulfoximine; GPx, Glutathione Peroxidase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, Glutathione; GSH-MEE, Glutathione monoethylester; H_2O_2 , hydrogen peroxide; HO^\cdot , hydroxyl radicals; O_2^- , superoxide anions; MAPK, mitogen-activated protein kinase; MS, mercaptosuccinic acid; NO, Nitric oxide; PARP, Poly(ADP-ribose) polymerase; PEG-SOD, polyethylene glycol-superoxide dismutase; ROS, reactive oxygen species; SNAP, S-nitro-N-acetylpenicillamine; SOD, superoxide dismutase; TUDCA, Tauroursodeoxycholic acid.

apoptosis or necrosis. Apoptosis, or programmed cell death [10–12], is an active process characterized by cell shrinkage, chromatin condensation, formation of apoptotic bodies and activation of caspases [13–15]. In contrast, necrosis is passive and associated with ATP depletion, rupture of the plasma membrane and spilling of the cellular content eliciting inflammation [12].

Hepatocytes are equipped with several enzymatic antioxidant defences, including superoxide dismutases, that converts superoxide anions into H_2O_2 ; catalase and glutathione peroxidase (GPx) that decompose H_2O_2 into water [16]. In addition, a variety of non-enzymatic antioxidant defences exist, including glutathione [17,18] and vitamins A, C and E. Finally, ROS-induced activation of survival pathways such as MAPK [19] and NF- κ B, may attenuate their toxicity. The mode of oxidative stress-induced cell death is dependent on the variety of ROS and the cell type. Many studies have investigated the role of ROS on cell death in transformed hepatoma cells, although it is known that cell lines differ significantly from normal hepatocytes in many respects and in primary hepatocytes [20,21], however without making a distinction between necrotic and apoptotic cell death. Since in chronic liver diseases non-transformed hepatocytes are exposed to various ROS, it is of clinical importance to elucidate the mode and mechanism of cell death in normal hepatocytes. The aim of this study was to elucidate and compare the mechanisms of cell death induced by superoxide anions and H_2O_2 in normal, non-transformed hepatocytes. Furthermore, the role of MAPK signalling pathways, ROS detoxification mechanisms and different ROS scavengers were investigated in ROS-induced cell death.

2. Materials and methods

2.1. Animals

Specified pathogen-free male Wistar rats (220–250 g) were purchased from Harlan (Zeist, The Netherlands). They were housed under standard laboratory conditions with free access to standard laboratory chow and water. Experiments were performed following the guidelines of the local Committee for Care and Use of laboratory animals.

2.2. Rat hepatocyte isolation

Hepatocytes were isolated as described previously [22] and cultured in William's E medium (Life Technologies Ltd; Breda, The Netherlands) supplemented with 50 μ g/mL gentamicin (BioWhittaker, Verviers, Belgium) without the addition of hormones or growth factors. During the attachment period (4 h) 50 nmol/L dexamethasone (Sigma, St Louis, USA) and 5% fetal calf serum (Life Technologies Ltd) were added to the medium. Cells were cultured in a humidified incubator at 37 °C and 5% CO_2 . Hepatocyte viability was always more than 93% and purity more than 95% as determined by trypan blue staining.

2.3. Experimental design

Experiments were started 24 h after isolation of hepatocytes. Monolayer cultures were exposed to the commonly used intracellular superoxide

anions-donors menadione (2-methyl-1,4-naphthoquinone, Sigma) [23] and paraquat (50 mmol/L, Sigma); and the extracellular superoxide generator hypoxanthine (0.5 mmol/L) / xanthine oxidase (20 μ U/ml) (HX/XO, Roche Diagnostics), or H_2O_2 (MERCK, Haarlem, The Netherlands) for the indicated time. Hepatocytes were also treated with caspase-9 inhibitor (Z-LEHD-FMK, 0.2 μ mol/L), caspase-6 inhibitor (Z-VEID-FMK, 0.2 μ mol/L) and caspase-3 inhibitor (Z-DEVD-FMK, 0.05 μ mol/L), all obtained from R&D Systems, Abingdon, UK; and tauroursodeoxycholic acid (TUDCA, 50 μ mol/L). Depletion of intracellular reduced glutathione, was achieved using D,L-buthionine-(S,R)-sulfoximine (BSO, 200 μ mol/L, Sigma). Glutathione monomethylester (GSH-MEE; 5 mmol/L; Calbiochem) was used as a donor of glutathione. Glutathione assay was performed as described previously [24].

Signal transduction pathways were blocked using MEK inhibitor U0126 at 10 μ mol/L to inhibit activation of ERK1/2 (Promega, Madison, USA), p38 inhibitor (SB203580, 10 μ mol/L) (Biomol, Plymouth Meeting, USA) and JNK inhibitor (SP600125, 10 μ mol/L, MERCK). H_2O_2 detoxification was inhibiting using the catalase inhibitor 3-amino-1,2,4-triazole (3-AT; 20 mmol/L) and the GPx inhibitor mercaptosuccinic acid (MS; 10 mmol/L, Sigma). All inhibitors were added 30 min before the addition of menadione or H_2O_2 .

Recombinant, replication-deficient adenovirus Ad5 κ BAA was used to inhibit NF- κ B activation as described previously and Ad5LacZ was used as control virus [25]. Hepatocytes were exposed to adenoviruses at a multiplicity of infection of 10, 15 h before exposure to superoxide anions or cytokine mixture [26]. Each experimental condition was performed in triplicate wells. Each experiment was performed, at least three times, using hepatocytes from different isolations.

2.4. Caspase enzyme activity assays

Caspase-3 enzyme activity was assayed as described previously [27]. Caspase-6 activity was assayed according to the manufacturer's instructions (BioVision).

2.5. Sytox Green and acridine orange nuclear stainings

To estimate necrotic cell death, hepatocytes were incubated 15 min with SYTOX green (Molecular Probes, Eugene, USA) nucleic acid stain, which penetrates cells with compromised plasma membranes but does not cross the membranes of viable cells or apoptotic bodies. Fluorescent nuclei were visualized using an Olympus CKX41 microscope at 450–490 nm.

Morphology of nuclei was examined with acridine orange as described previously [27].

2.6. Western Blot analysis

Western blot analysis of cell lysates was performed by SDS-PAGE followed by transfer to Hybond ECL nitrocellulose membrane (Amersham). An antibody against GAPDH (Calbiochem) and Ponceau S staining were used to ensure equal protein loading and electrophoretic transfer.

Caspase cleavage was detected using polyclonal rabbit antibodies against cleaved caspase-9, -6, and -3. Poly(ADP-ribose) polymerase (PARP) cleavage was detected with rabbit anti-PARP polyclonal antibody. PARP is a substrate of caspase-3 yielding a product of 89 kDa and it is considered a late marker for apoptosis. After Western blot analysis for p-p38 (rabbit polyclonal), p-ERK1/2 (monoclonal), and p-JNK (monoclonal, Santa Cruz Biotechnology), blots were stripped using 0.1% SDS in PBS/Tween at 65 °C for 30 min and incubated with antibodies against total-p38, total-ERK1/2 or total-JNK. Unless indicated otherwise, all antibodies were obtained from Cell Signalling Technology, and used at 1:1000 dilution.

2.7. Statistical analysis

All numerical results are reported as the mean of at least 3 independent experiments \pm SD. A Mann-Whitney test was used to determine the significance of differences between experimental groups. Statistical difference was accepted at $P < 0.05$.

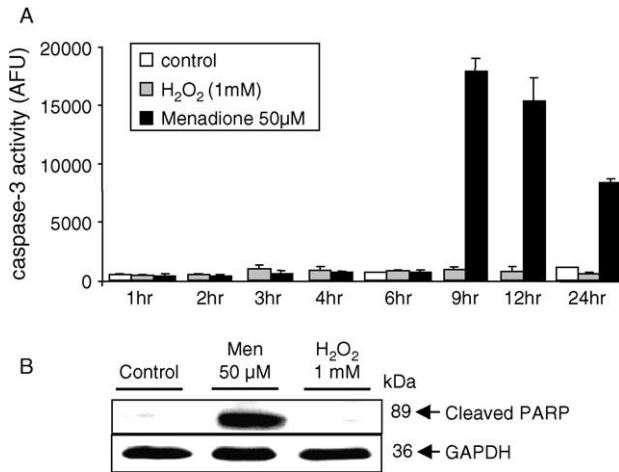


Fig. 1. The superoxide anion-donor menadione, but not hydrogen peroxide, induces caspase-3 activity and PARP-cleavage in primary rat hepatocytes. Cells were exposed to menadione (Men, 50 µmol/L) or hydrogen peroxide (H₂O₂, 1 mmol/L) for different time points. Controls: untreated cells. (A) Caspase-3 activity assay. (B) Western blot analysis against cleaved PARP (89 kDa) and GAPDH. Data represent mean of at least 3 independent experiments with $n=3$ per condition.

3. Results

3.1. Superoxide anions, but not hydrogen peroxide, induce apoptosis in primary rat hepatocytes

To investigate whether ROS induce apoptotic cell death in primary rat hepatocytes, cells were exposed to superoxide anion donors or H₂O₂.

Menadione increases caspase-3 activity, peaking between 9 and 12 h, and leads to PARP cleavage (Fig. 1A, B). Based on caspase-3 staining and nuclear morphology we estimate that at least 90% of cells were apoptotic. Lower concentrations of menadione did not induce caspase-3 activity, PARP cleavage or nuclear condensation (Fig. 2A, C). To verify these findings, we used paraquat and HX/XO as alternative superoxide-generators to reproduce the results obtained with menadione. Both paraquat and HX/XO induced caspase-3 activation, PARP cleavage and apoptosis in a time dependent manner, peaking at 4 and 9 h, respectively (Fig. 2B). In contrast, H₂O₂ is not apoptotic in primary hepatocytes at concentrations up to 5 mmol/L (Fig. 2C, D).

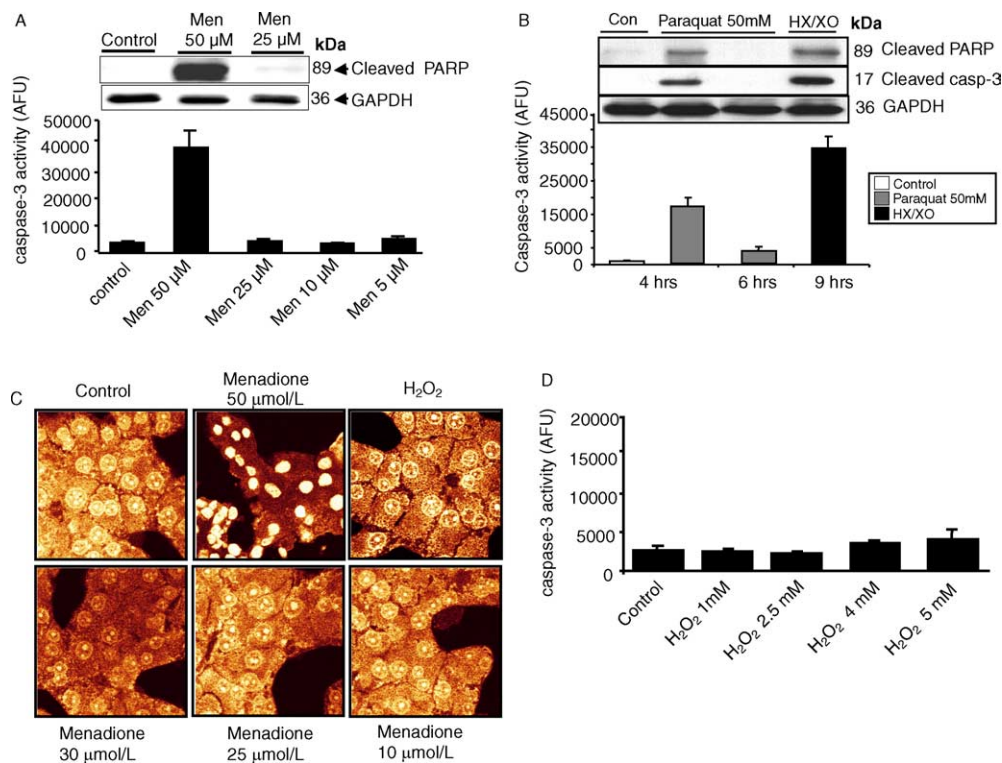


Fig. 2. (A) Menadione induces caspase-3 activity and PARP cleavage only at 50 µmol/L. Hepatocytes were incubated or not (controls) with different concentrations of menadione (Men) for 9 h. (B) Caspase-3 activity and Western blot. Paraquat (50 mmol/L) and hypoxanthine (0.5 mmol/L)/xanthine oxidase (20 µU/ml) (HX/XO) induce apoptosis, PARP and caspase-3 cleavage peaking at 4 and 9 h, respectively. (C) Menadione, but not hydrogen peroxide, induces nuclear condensation (apoptosis indicator), as determined by acridine orange staining, only at 50 µmol/L. Hepatocytes were treated with different concentrations of menadione (9 h) or H₂O₂ (1–4 mmol/L, 6 h). Original magnification: 1000×. (D) H₂O₂ does not induce caspase-3 activity in primary hepatocytes at concentrations up to 5 mmol/L.

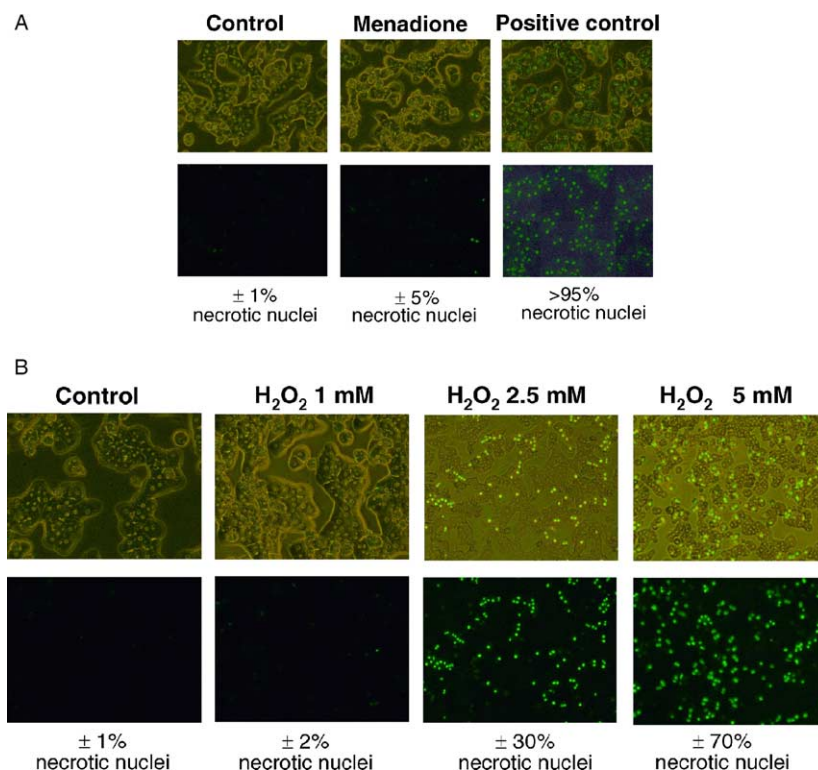


Fig. 3. Sytox Green nuclear staining of necrotic cells. (A) Menadione (Men, 50 $\mu\text{mol/L}$, 9 h) slightly increases necrotic cell death in primary hepatocytes (magnification: 40 \times). (B) H_2O_2 induces necrotic cell death only at high concentrations. Hepatocytes were exposed to increasing concentrations of H_2O_2 (1–5 mmol/L, 6 h). Upper panel phase contrast and fluorescence; lower panel fluorescence only. Magnification: 40 \times . [This figure appears in colour on the web.]

Of note, caspase-3 activity always correlated with the level of cleaved caspase-3 and cleaved PARP.

Menadione (50 $\mu\text{mol/L}$) induced only minimal necrotic cell death as determined by Sytox Green staining (Fig. 3A). At concentrations above 100 $\mu\text{mol/L}$ menadione, all hepatocytes became necrotic and detached. H_2O_2 at 1 mmol/L did not induce necrotic cell death but becomes necrotic above 5 mmol/L (Fig. 3B).

To further investigate whether H_2O_2 plays a role in cell death, experiments were performed in the presence of inhibitors of the H_2O_2 detoxification enzymes. In the presence of the catalase inhibitor 3-AT or the GPx inhibitor MS, caspase-3 activity level was only slightly increased (Fig. 4A), whereas necrotic cell death was highly increased when catalase was inhibited and only slightly increased when GPx was inhibited (Fig. 4B). In contrast, 3-AT treatment had no effect on menadione-induced apoptosis or necrotic cell death (Fig. 4C).

Taken together, these observations indicate that 50 $\mu\text{mol/L}$ menadione induces apoptosis in normal primary rat hepatocytes, whereas H_2O_2 causes necrotic cell death at high concentrations, or when H_2O_2 detoxification mechanisms are blocked.

3.2. Superoxide anions-induced apoptosis is dependent on caspase activation

Since apoptosis is usually associated with activation of caspases, we analyzed whether superoxide anions-induced apoptosis is mediated by caspase activation. Cleaved caspase-9, -6, and -3 were detected 9 hrs after treatment with 50 $\mu\text{mol/L}$ menadione, but not at lower concentrations. In contrast, H_2O_2 did not induce caspase-9, -6, or -3 activation in primary hepatocytes (Fig. 5A).

Superoxide anions-induced apoptosis is completely dependent on caspase activation, since treatment with caspase-9, -6, and -3 inhibitors totally abolished superoxide anions-induced apoptosis (caspase-3 activity and PARP-cleavage) (Fig. 5B).

Furthermore, caspase-9 inhibition blocked caspase-6 and -3 activation (Fig 5C), suggesting that caspase-9 is upstream of caspase-6 and -3, and that the mitochondria are involved in superoxide anions-induced apoptosis.

3.3. Involvement of MAP-kinase signal transduction pathways in superoxide anions-induced apoptosis

Menadione (50 $\mu\text{mol/L}$) induced a marked increase in ERK1/2 phosphorylation within 2 h and JNK

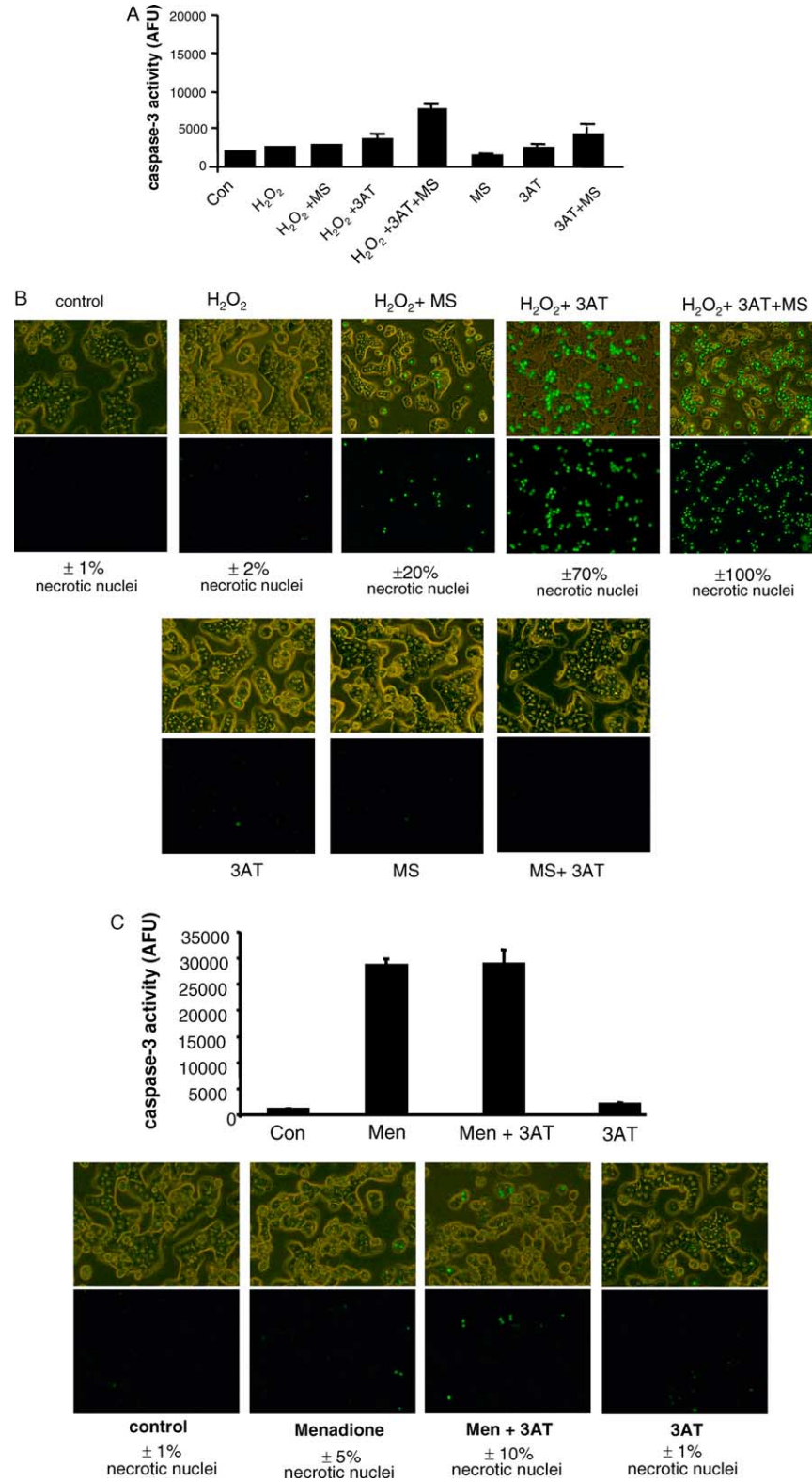


Fig. 4. (A) Hydrogen peroxide (H₂O₂) slightly increases caspase-3 activity in primary hepatocytes when H₂O₂ detoxification is impaired. Cells were exposed to H₂O₂ (1 mmol/L) for 6hrs in the presence of the catalase inhibitor 3-AT and/or GPx inhibitor MS. (B) H₂O₂ (1 mmol/L) induces massive necrotic cell death in hepatocytes with impaired H₂O₂ detoxification, as determined by Sytox Green nuclear staining. Magnification: 40×. (C) The catalase inhibitor 3-AT does not affect menadione-induced apoptosis (upper graph) or necrotic cell death (lower pictures). Upper panels phase contrast and fluorescence; lower panels fluorescence only. [This figure appears in colour on the web.]

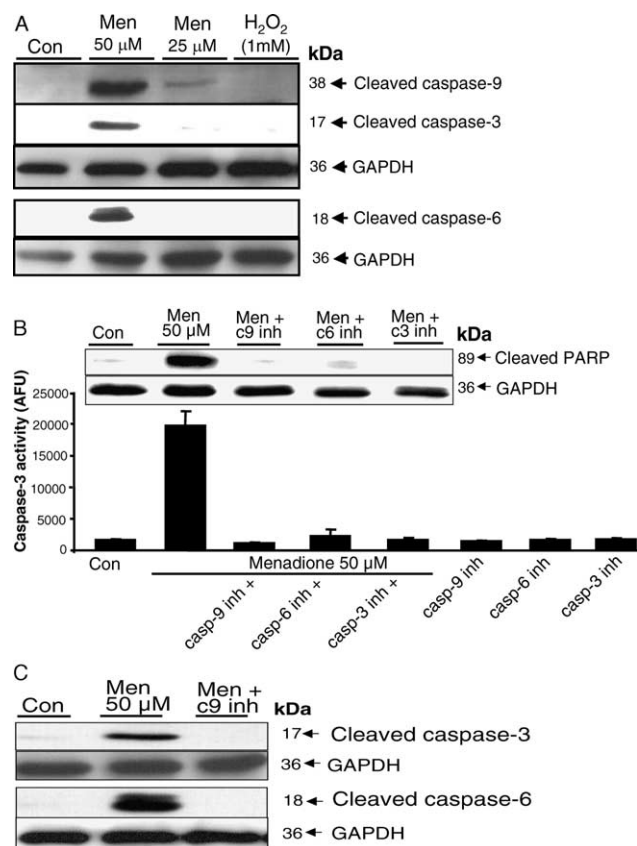


Fig. 5. Superoxide anions-induced apoptosis is dependent on caspase activation. Cells were incubated with menadione (Men, 50 and 25 $\mu\text{mol/L}$, 9 h) or hydrogen peroxide (H_2O_2 , 1 mmol/L , 6 h). (A) Western Blot analysis of caspase activation. Only 50 $\mu\text{mol/L}$ menadione, but not 25 $\mu\text{mol/L}$ menadione or H_2O_2 , induces caspase-9, -6, and -3 activation. (B) Addition of caspase-9, -6, and -3 inhibitors before and during menadione (50 $\mu\text{mol/L}$) treatment, abolishes caspase-3 activity and PARP-cleavage. (C) Caspase-9 inhibitor blocks caspase-6 and -3 cleavage.

phosphorylation within 1 h that persisted for 4 h. In contrast, 25 $\mu\text{mol/L}$ menadione did not induce p-ERK1/2 and only slightly induced p-JNK, whereas H_2O_2 did not induce p-ERK1/2 or p-JNK. These results indicate that superoxide anions induce JNK and ERK1/2 phosphorylation only at concentrations which induce apoptosis. Neither menadione nor H_2O_2 induced phosphorylation of p38 MAPK. No significant changes were detected in total-ERK, total-JNK or total-p38 after exposure to menadione or H_2O_2 (Fig. 6A).

Next, the effect of MAPK inhibitors was determined after exposure to menadione or H_2O_2 . The MEK1/2 inhibitor U0126 markedly inhibited ERK1/2 phosphorylation and increased caspase-3 activity after treatment with menadione at 50 $\mu\text{mol/L}$. In contrast, inhibition of ERK-phosphorylation did not sensitize hepatocytes to apoptosis induced by a non-toxic concentration of menadione (25 $\mu\text{mol/L}$). Our data indicate that ERK1/2 MAPK, when phosphorylated, mediates protection against superoxide anions-induced apoptosis. Inhibition of the p38

MAPK pathway had no effect on menadione-induced apoptosis, indicating that p38 is not involved in superoxide anions-induced apoptosis (Fig. 6B).

On the other hand, the JNK inhibitor SP600125 [28] blocked caspase-9, -6 and -3 processing (Fig. 6C), caspase-6 activity and apoptosis (Fig. 6D) induced by 50 $\mu\text{mol/L}$ menadione. These results indicate that superoxide anions-induced apoptosis is dependent on JNK activity.

None of the MAPK inhibitors sensitized hepatocytes to death after exposure to H_2O_2 , suggesting that MAPK are not involved in the protection against H_2O_2 (not shown).

3.4. Role of NF- κB in superoxide anions-induced apoptosis

It has been proposed that ROS may activate NF- κB in various cell types. In our study, inhibition of NF- κB pathway by recombinant adenovirus expressing dominant negative I κB did not influence superoxide anions-induced apoptosis (Fig. 7A). Functionality of this virus was demonstrated by sensitizing hepatocytes to cytokine-induced apoptosis (Fig. 7B).

3.5. Role of glutathione in menadione-induced apoptosis

To provide a more mechanistic interpretation of superoxide action, we evaluated the role of glutathione in menadione-induced apoptosis. Menadione treatment reduced glutathione level approximately 10% compared to control level. Depletion of intracellular reduced glutathione (GSH) was achieved using BSO which inhibits γ -glutamyl-cysteinyl-synthetase, the rate limiting step in GSH biosynthesis. Addition of BSO, 12 h before and during menadione treatment, reduced GSH levels more than 80% and further increased superoxide anions-induced caspase-3 activity in primary hepatocytes. In contrast, addition of the glutathione donor GSH-MEE slightly inhibited menadione-induced apoptosis. Western blot against active caspase-3 confirmed the data obtained by caspase-3 activity assay (Fig. 8A, B).

3.6. Scavengers of superoxide anions prevent menadione-induced apoptosis

To investigate whether superoxide anions are directly involved in menadione-induced apoptosis in primary hepatocytes, the role of different superoxide anion scavengers was examined. Hepatocytes were treated 30 min before and during the addition of menadione with the nitric oxide donor SNAP or the cell-permeable superoxide dismutase mimic PEG-SOD [29]. Exposure to SNAP significantly reduced menadione-induced apoptosis (Fig. 9A) but dramatically increased necrotic cell death in hepatocytes (Fig. 9B).

In contrast, PEG-SOD reduced menadione-induced caspase-3 and -6 activities (Fig. 10A) but did not increase

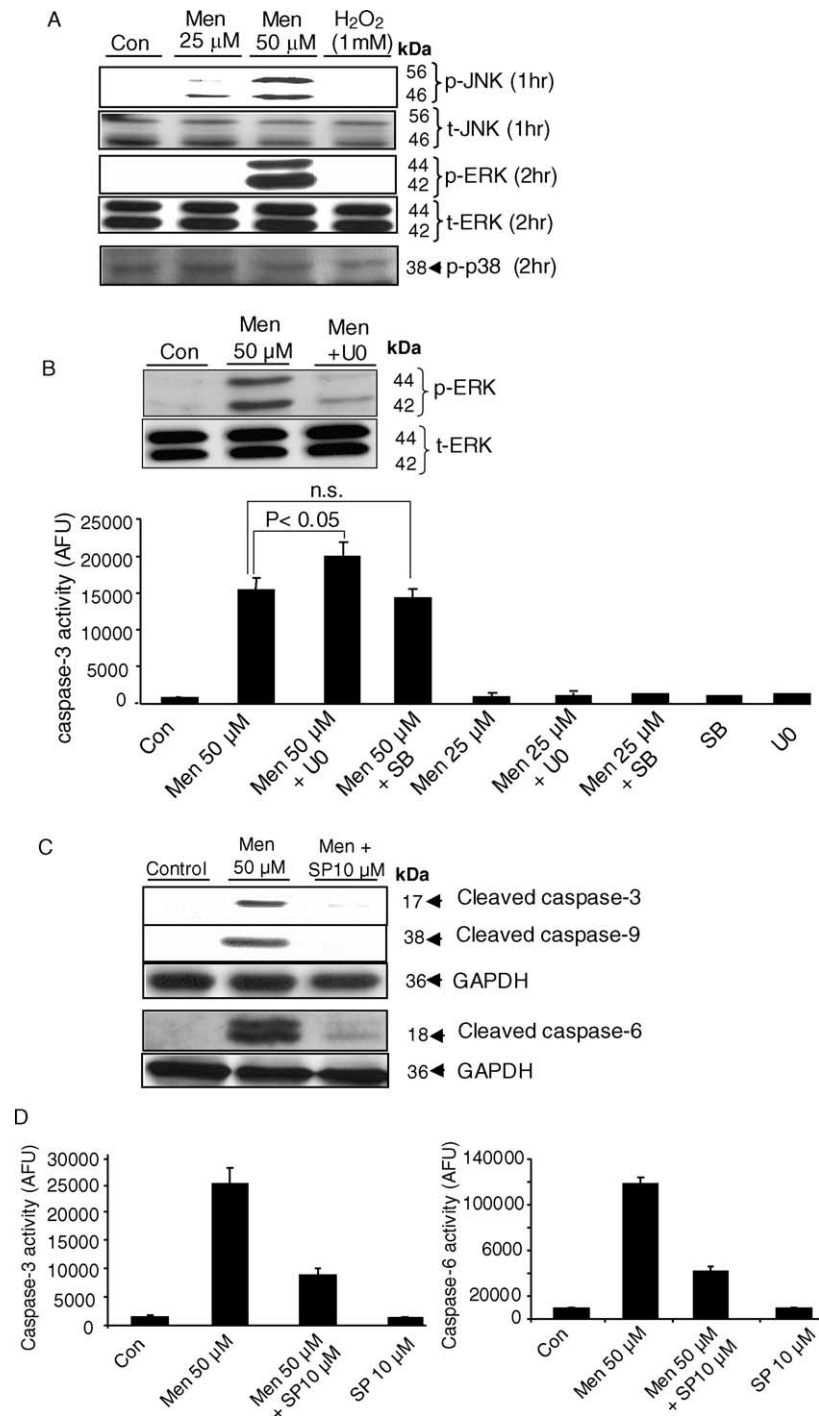


Fig. 6. (A) Phosphorylation of MAP kinases. Menadione 50 μ mol/L, but not menadione 25 μ mol/L or hydrogen peroxide (H₂O₂), induces p-JNK and p-ERK1/2 after 1 and 2 h, respectively; MAPK p38 was not phosphorylated by menadione or H₂O₂. (B) ERK activation attenuates menadione-induced apoptosis. Western blot demonstrates functionality of U0126, used as ERK inhibitor. Inhibition of ERK phosphorylation increases menadione-induced caspase-3 activity ($P < 0.05$; statistically significant according to Mann-Whitney *U*-test). (C) Caspase-9, -6 and -3 activation and (D) Menadione-induced caspase-6 activity and apoptosis are dependent on JNK activity. Cells were exposed to menadione with or without JNK inhibitor SP600125 (SP).

necrotic cell death (Fig. 10B). Moreover, PEG-SOD prevented superoxide anions-induced caspase-9, -6, and -3 activation and nuclear condensation (Fig. 10C, D). Additionally, PEG-SOD blocked JNK and ERK1/2 phosphorylation induced by 50 μ mol/L menadione (Fig. 10E).

These results indicate that superoxide anion scavengers protect against superoxide anions-induced apoptosis, suggesting that menadione-induced apoptosis is directly dependent on superoxide anions production. Finally, although highly effective against bile acid-induced apoptosis [27],

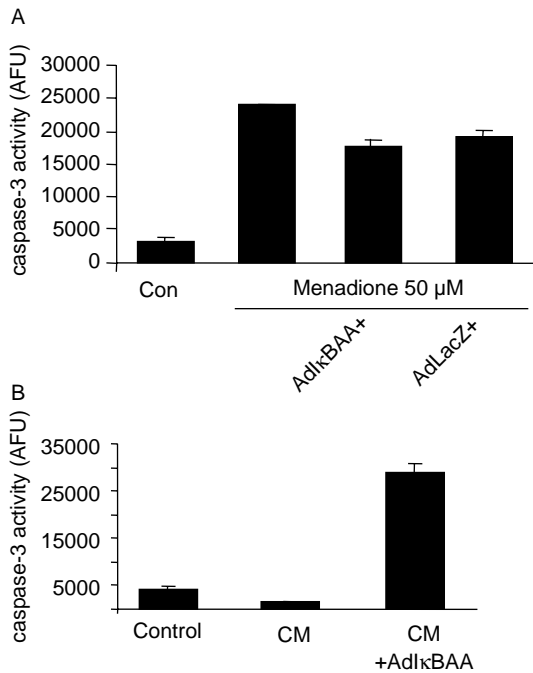


Fig. 7. NF- κ B-activation is not involved in menadione-induced caspase-3 activation. (A) Menadione-induced caspase-3 activity (50 μ mol/L, 9 h) is not changed by the inhibitor of NF- κ B pathway Ad5kBAA or Ad5LacZ (virus control). (B) Functionality of the NF- κ B inhibitor Ad5kBAA is demonstrated by increased caspase-3 activity after exposure to cytokines (CM: m-TNF- α (20 ng/ml), h-Interleukin-1 β (10 ng/ml) and r-Interferon- γ (10 ng/ml)).

the anti-apoptotic bile acid tauroursodeoxycholic acid, added before, at the same time or after menadione, did not protect against menadione-induced apoptosis (not shown).

4. Discussion

In this study we investigated the role of ROS in primary rat hepatocyte cell death. We demonstrate that the superoxide anion donor menadione mainly induces apoptosis and to a much lesser extent necrotic cell death in primary hepatocytes. In other studies, generation of superoxide in Sprague Dawley rats in vivo [30] and in isolated perfused rat liver [31] causes limited toxicity and only necrotic cell death. Several differences may explain these contrasting results: (1) Different rat strains differ with respect to the susceptibility to oxidant injury, (2) Site of ROS formation: diquat was added to the perfusion medium whereas in our study, superoxide-generators were added directly to the culture medium, which may result in different uptake and final intracellular concentration of the ROS-donor in vivo and in vitro. (3) Mode of cell death: in the previous in vivo studies, only LDH/AST/ALT leakage was used as a determinant of cell death, however apoptosis was not specifically analyzed. In our experiments, we observed that paraquat as well as HX/XO also induced apoptosis.

Furthermore, we show that H₂O₂ at concentrations below 5 mmol/L does not induce apoptosis or necrotic cell death in normal primary hepatocytes. Only at high concentration or when H₂O₂ detoxification (catalase and GPx) is impaired, H₂O₂ turns into a necrotic compound. Inhibition of catalase sensitizes hepatocytes to necrotic cell death much more than inhibition of GPx. This suggests that catalase is the principal H₂O₂ detoxification mechanism in hepatocytes. The high efficiency of detoxification, especially by catalase, explains why H₂O₂ is not or only moderately toxic at concentrations up to 4 mmol/L. A previous study [31] concluded that catalase contributes to only 20% of H₂O₂ detoxification. It is difficult to compare this in vivo study using diquat to generate H₂O₂, with our data using H₂O₂ added to the medium. In addition, the extent of catalase inhibition using a single intraperitoneal injection of 3-aminotriazole has not been verified. In our studies we use defined concentrations of H₂O₂ and 3-aminotriazole and, we observed a strong effect after inhibiting catalase. Other studies showed divergent effects of H₂O₂, reporting both necrotic and apoptotic cell death in hepatoma cell lines [32,33]. Apparently, the mode of cell death is dependent on the cell type, with hepatoma cell lines yielding different results than normal non-transformed hepatocytes. Indeed, it is well known that tumour cells exhibit increased intrinsic oxidative stress and have adapted to this situation by altered oxidative stress defence mechanisms [34,35]. Differences in the sensitivity to apoptotic stimuli between transformed hepatoma cells and normal non-transformed hepatocytes have been reported [36,37]. Therefore, results obtained using hepatoma cell lines cannot be extrapolated to normal, non-transformed hepatocytes.

Induction of apoptosis by 50 μ mol/L menadione, as determined by PARP-cleavage and nuclear condensation,

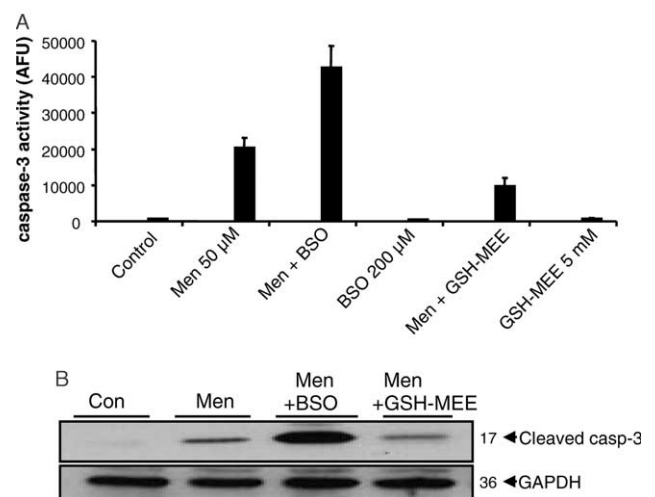


Fig. 8. (A) Caspase-3 activity assay. Addition of BSO (200 μ mol/L), 12 h before and during menadione treatment (9 h), further increased superoxide anions-induced caspase-3 activity in primary hepatocytes. In contrast, addition of GSH-MEE (5 mmol/L), a donor of glutathione, slightly inhibited menadione-induced apoptosis. (B) Western blot against active caspase-3.

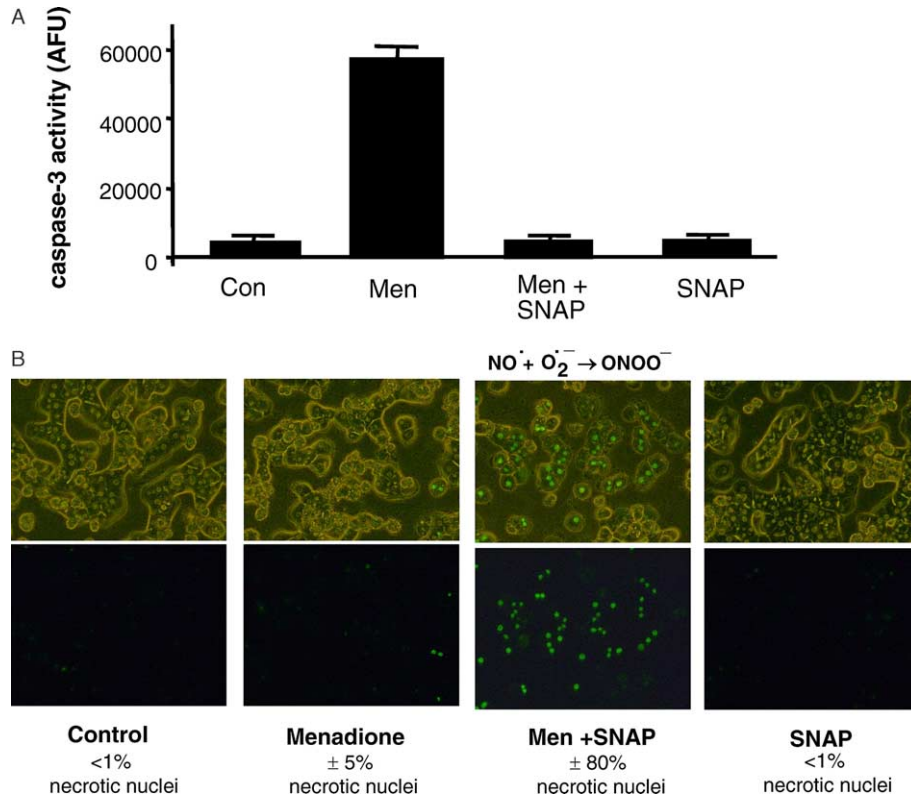


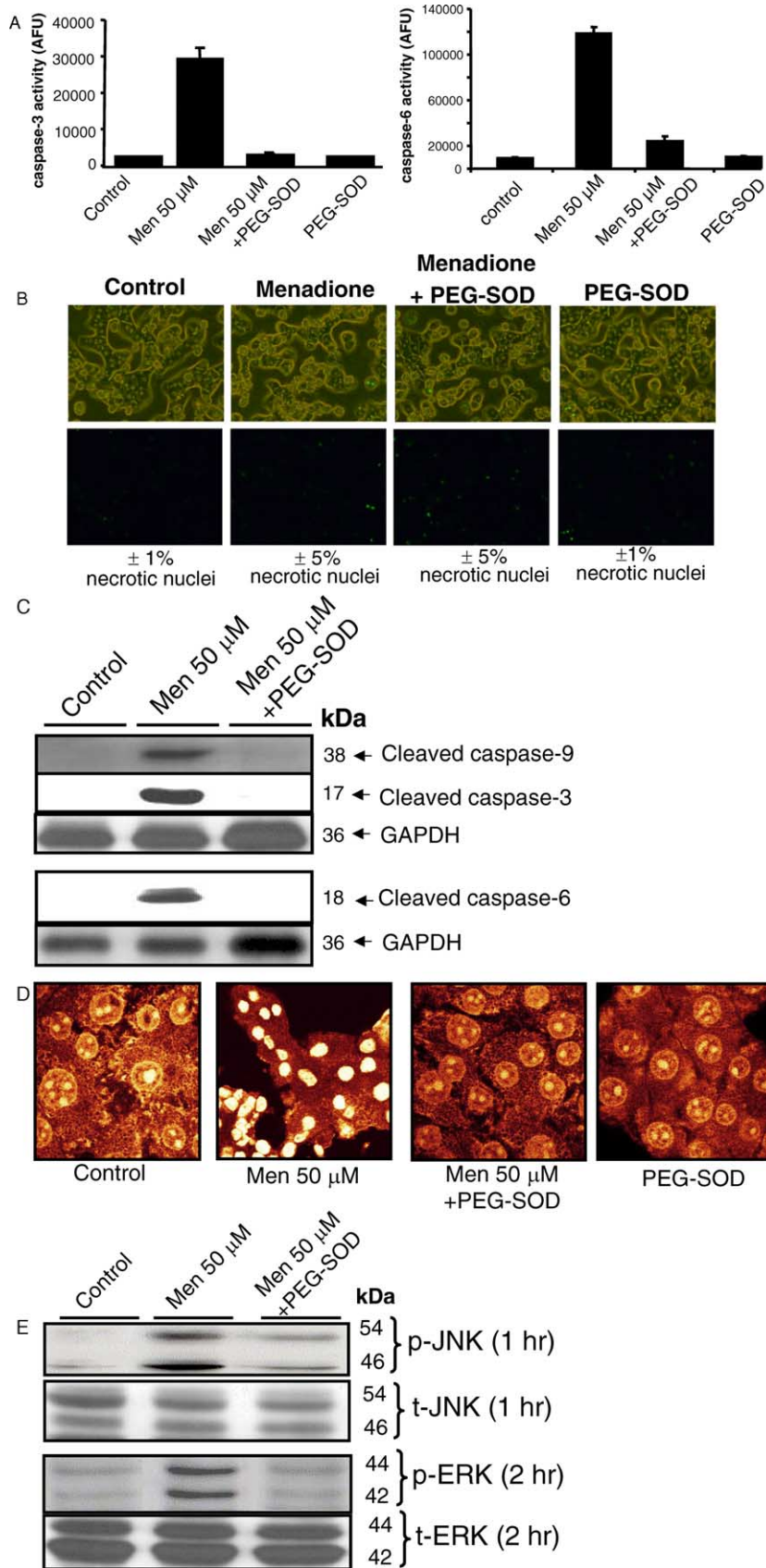
Fig. 9. Nitric oxide blocks caspase-3 activity induced by superoxide anions but increases necrotic cell death. Hepatocytes were incubated with menadione (Men, 50 $\mu\text{mol/L}$, 9 h) with or without the nitric oxide donor SNAP. (A) Caspase-3 activity (apoptosis indicator). (B) Sytox Green nucleic acid staining (necrotic cell death indicator). Original magnification: 40 \times . [This figure appears in colour on the web.]

consistently correlates with processing and activation of caspase-9, -6 and -3, and inhibitors of these caspases prevent PARP-cleavage. The induction of apoptosis and caspase activation by menadione is in accordance with previous studies [33]. In contrast with previous studies, we demonstrate that menadione-induced apoptosis is totally dependent on caspase activation, as shown by cleaved PARP. Our finding that caspase-6 and caspase-9 are activated by superoxide anions also represents a novel finding. We demonstrate that caspase-9 activation is upstream of caspase-6 and -3 activation. In type II cells, apoptosis is dependent on the release of pro-apoptotic factors from the mitochondria, that activate caspase-9 and subsequently caspase-3 [38–40]. Our demonstration of superoxide anions-induced caspase-9 activation indicates disruption of the mitochondria and release of pro-apoptotic factors [40].

Exposure to ROS has been shown to activate signal transduction pathways, which may modulate cell death [27, 41,42]. Our study shows that only apoptotic concentrations of menadione induces the phosphorylation of ERK1/2 MAPK and this activation attenuates cell death, since inhibition of ERK1/2 enhances superoxide anions-induced

apoptosis. However, activation of ERK1/2 is clearly not sufficient to prevent menadione-induced apoptosis, since superoxide anions also activate pro-apoptotic pathways, e.g. JNK, that overrule the protective ERK1/2 pathway. In contrast, non-toxic concentrations of superoxide anions or H_2O_2 do not phosphorylate ERK1/2 and, as expected, inhibition of ERK1/2 in these conditions does not sensitize primary hepatocytes to cell death. This is in accordance with previous reports, in which a protective effect of ERK1/2 activation against bile acid [27] and superoxide anions-induced apoptosis has been reported, although in the latter study a sensitizing effect of ERK1/2 inhibition to low concentrations of menadione was observed [43]. The explanation for this discrepancy could be that in the RALA cell line, ERK1/2 phosphorylation was also observed at low menadione concentrations, whereas in our study ERK1/2 phosphorylation was only observed at 50 $\mu\text{mol/L}$. In our study, we demonstrate that JNK phosphorylation and apoptosis are induced only with 50 $\mu\text{mol/L}$ menadione but not by H_2O_2 . Furthermore, we show that the inhibition of JNK blocks menadione-induced apoptosis, indicating that superoxide anions-induced apoptosis is dependent on JNK

Fig. 10. Superoxide dismutase prevents menadione-induced caspase-6 and -3 activity, processing of caspases and phosphorylation of JNK and ERK pathways but does not increase necrotic cell death. PEG-SOD (100 U/ml) was added 30 min before menadione (Men, 50 $\mu\text{mol/L}$) and cells were harvested 9 h after menadione addition. (A) Caspase-6 and -3 activity (apoptosis). (B) Sytox Green nucleic acid staining (necrotic cell death indicator). (C) Western blot analysis for cleaved caspase-9, -6, and -3. (D) Nuclear condensation determined by acridine orange staining. Magnification: 1000 \times . (E) Western blot analysis for phospho-JNK and phospho-ERK.



pro-apoptotic activity. The mechanisms by which JNK exert its pro-apoptotic properties are not completely understood. The fact that JNK inhibition blocks caspase-9 activation indicates that JNK triggers the mitochondrial pathway after menadione treatment. In many studies, an effect of JNK has been described at the level of the mitochondria triggering the mitochondria death pathway, including phosphorylation and activation of pro-apoptotic bcl-2 family members [43–49]. The p38 MAPK pathway is not involved in menadione-induced apoptosis.

Although the NF- κ B pathway is involved in protection against cytokine-induced apoptosis in primary rat hepatocytes [50,51], we demonstrate that the NF- κ B pathway is not involved in the protection against superoxide anions-induced apoptosis in hepatocytes.

We also evaluated the anti-apoptotic potential of several compounds that are considered to have therapeutic applications and demonstrate remarkable differences between different scavengers. Nitric oxide blocks apoptosis but increases necrotic cell death. This switch in the balance between apoptosis and necrotic cell death could be caused since NO \cdot is highly reactive with superoxide anions leading to the formation of the cytotoxic compound peroxynitrite (ONOO $^-$) [52]. Hence, the metabolism of ROS can switch the balance between apoptosis and necrotic cell death. Therefore, recent therapeutic strategies aimed at donating NO in liver diseases [53], should take into account the possibility of ROS generation in these diseases. In contrast, PEG-SOD totally inhibits caspase activation, abolishes apoptosis and does not increase necrotic cell death. In support, depleting the intracellular antioxidant glutathione increases and repleting glutathione decreases superoxide anions-induced apoptosis. Interestingly, and in contrast to previous studies using a hepatoma cell line [54], inhibition of catalase had no effect on superoxide anions-induced apoptosis, whereas PEG-SOD is completely protective. These data demonstrate that superoxide anions produced by menadione directly induce apoptosis in primary hepatocytes. Furthermore, PEG-SOD is able to block the phosphorylation of both JNK and ERK1/2, indicating that PEG-SOD completely scavenges superoxide anions. Indeed, PEG-SOD represents a potential strategy in therapy in liver diseases caused by oxidative stress, as shown in studies in which overexpression of superoxide dismutases inhibit apoptosis in alcohol-induced liver injury in rats [55,56]. Finally, the anti-apoptotic bile acid tauroursodeoxycholic acid, which inhibits apoptosis induced by toxic bile acids [27], did not attenuate superoxide anions-induced apoptosis, indicating that the mechanisms of bile acid and superoxide anions-induced apoptosis are different.

In summary, this study demonstrates major differences in the mechanisms and mode of cell death induced by superoxide anions and H $_2$ O $_2$ in primary, non-transformed hepatocytes and provides evidence for an anti-apoptotic role for ERK and a pro-apoptotic role for JNK MAP-kinases. Furthermore, we demonstrate that it is imperative to evaluate the consequences of any intervention in cell death on both

apoptotic and necrotic cell death. Chronic liver diseases, including alcoholic hepatitis, NAFLD, cholestasis and chronic viral hepatitis are almost invariably accompanied by exposure of hepatocytes to ROS and this exposure may contribute to liver damage. Therefore, knowledge about the mode of cell death and the mechanisms involved in ROS-induced damage to normal hepatocytes may contribute to the development of novel therapies.

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