Superoxide generation from Kupffer cells contributes to hepatocarcinogenesis: studies on NADPH oxidase knockout mice

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We hypothesized that superoxide from Kupffer cells (KC) contributes to hepatocarcinogenesis. \(p47^{phox}^{-/-}\) mice, deficient in phagocyte NADPH oxidase and superoxide generation, received a single dose of the hepatocarcinogen diethylnitrosamine (DEN). The following hepatic effects were observed at time points between 30 min and 35 days. Liver damage after DEN was manifested by loss of body and liver mass and of liver DNA and by an increase in apoptosis, necrosis and signs of inflammation. These effects were massive in wild-type (wt) male mice, but only very mild in \(p47^{phox}^{-/-}\) mice. Regenerative DNA synthesis subsequent to liver damage was high in wt male mice, but weak in \(p47^{phox}^{-/-}\) mice. In females the apparent protection by \(p47^{phox}^{-/-}\) was less pronounced than in males. Therefore, further experiments were performed with males. In KC isolated from wt mice superoxide production was enhanced by DEN pretreatment in vivo. Also, in vitro addition of DEN to KC cultures induced superoxide release, similarly to lipopolysaccharide, a standard KC activator. Thus, DEN directly activates wt KC to produce superoxide. KC from \(p47^{phox}^{-/-}\) mice did not release superoxide. TNFα production by isolated KC was transiently depressed 0.5 h after DEN treatment in vivo, but recovered rapidly. In blood serum TNFα levels of wt mice were elevated for the initial 6 h. TNFα in KC cultures and in serum of \(p47^{phox}^{-/-}\) mice was reduced. DEN in vivo induced DNA damage (‘comets’) in hepatocytes. This damage was extensive in wt mice but much less in \(p47^{phox}^{-/-}\) mice. These studies suggest two conclusions: (i) superoxide generation by phagocytes during liver damage and inflammation aggravates genotoxic and cytotoxic effects in hepatocytes and may thus contribute to tumor initiation and promotion; (ii) DEN has a direct stimulatory effect on KC to release superoxide and TNFα.

Introduction

Hepatocellular carcinoma (HCC) is the third most frequent cause of cancer death worldwide. In Europe and the USA HCC incidence and mortality is less, but increasing (1–4). Etiological factors responsible for induction of HCC are well known: they include infection with hepatitis B or C virus, chronic ethanol abuse and dietary exposure to aflatoxin B1, as well as exposure to other genotoxic compounds, such as nitrosamines, from dietary sources or from tobacco smoke (5–8). Unfortunately, insufficient knowledge exists on the pathophysiological mechanisms triggered by these etiological factors. Such knowledge would be urgently needed in order to design strategies for secondary prevention and therapy. Animal and human data have shown that genotoxic and cytotoxic effects and the inflammatory responses they induce are key events to entrain hepatocytes on the pathway to hepatocarcinogenesis (9–13). In fact, HCC rarely develops in a liver in the absence of hepatitis, fibrosis and cirrhosis (3,4,6,8). Thus, interactions of hepatocytes with inflammatory cells resulting in an altered microenvironment appear of crucial importance in early and later stages of carcinogenesis.

Kupffer cells (KC), the macrophages of the liver, are key players in the regulation of inflammatory events in this organ. Their main function is defense from invading microorganisms and their toxins. Appropriate signals activate KC to release an ‘oxidative burst’ of superoxide and numerous other products with cytotoxic, pro-inflammatory and growth-promoting activity and to attract more immune cells to the liver (11,14–16). Superoxide is a prominent factor in host defense but may have detrimental effects if elevated secretion is excessive or persists for prolonged periods (12,13,17–19). It is metabolized to various reactive oxygen species (ROS), which damage cells and DNA and enhance release of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) (12,13,20–23). The purpose of the present study was to investigate whether KC and other phagocytes, by generation of superoxide, may contribute to early stages of hepatocarcinogenesis.

As an established model we have used mice treated with a single high dose of the genotoxic carcinogen diethylnitrosamine (DEN). Current knowledge indicates that this agent is metabolically activated in hepatocytes by CYP2E1 and other enzymes (24–27). The electrophilic species formed bind covalently to multiple target sites in DNA, proteins and other cell constituents, resulting in DNA damage and mutations, as well as in cell death and subsequent regenerative proliferation (24–27). These events are considered early steps responsible for initiation and promotion of hepatocarcinogenesis as indicated by the eventual appearance of malignant tumors (28–31).

To test specifically for a role of phagocyte superoxide in carcinogenesis we studied the effect of DEN in mice deficient in phagocyte NADPH oxidase (PHOX for phagocyte oxidase). PHOX (also called NOX2 for NADPH oxidase) is a multi-protein enzyme generating superoxide in phagocytic cells.

Abbreviations: BrdU, 5-bromo-2’-deoxyuridine; DEN, diethylnitrosamine; HBSS, Hank’s balanced salt solution; HCC, hepatocellular carcinoma; HE, haematoxylin and eosin; KC, Kupffer cells; LPO, lipid peroxidation; LPS, lipopolysaccharide; MEME, Eagle’s minimal medium; NOX, NADPH oxidase; NPC, non-parenchymal cells; \(p47^{phox}^{-/-}\), \(p47^{phox}^{-/-}\) knockout; PHOX, phagocyte oxidase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SPF, specific pathogen-free; TNFα, tumor necrosis factor α; wt, wild-type.
The catalytic process involves electron transfer to oxygen along with proton (cation) translocation. Other NADPH oxidases (e.g. NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2) are present in non-phagocytic cells of many different tissues (32). PHOX is a complex of five major subunits, i.e. cytosolic proteins p40phox, p47phox and p67phox and membrane proteins p22phox and gp91phox (33-37). p47phox is essential for assembly and function of PHOX; elimination of the gene abolishes superoxide formation in phagocytes (33,35,38).

We found that male p47phox knockout mice are largely protected from DEN-induced hepatotoxicity and the ensuing inflammation and regenerative response; DNA damage as measured by the Comet assay was much less dramatic, as in wild-type (wt) mice. KC isolated from wt mice released high levels of superoxide after pretreatment with DEN in vivo and interestingly also after addition of DEN to the culture medium. These results strongly suggest that superoxide generation by phagocytic cells during liver damage and inflammation aggravates genotoxic and cytotoxic effects in hepatocytes and may thereby contribute to both initiation and promotion of hepatocarcinogenesis. The results are of interest also with respect to the mechanism of action of chemical carcinogens. They suggest that the model carcinogen DEN, in addition to its effect on hepatocytes, may directly activate KC to release superoxide.

Materials and methods

Mice

p47phox knockout (p47phox−/−) and wild-type (wt) B6,129 mice were kindly provided by Dr Steven M. Holland (Laboratory of Host Defences, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD); p47phox−/− mice were originally produced from embryonic stem cell 129/Sv-derived sperm and C57BL/6-derived eggs. All mice were bred in the breeding facilities of the University of Vienna (Institut für Versuchstierkunde und -genetik, Himberg, Austria). Mice were obtained by caesarean section and maintained under specific pathogen-free (SPF) barrier conditions until death. Mice were regularly checked for infections, because our initial experiments with p47phox−/− mice suffering with infections revealed much less distinct results.

Six-week-old (20 g) mice of both sexes were used for the experiments. They were adapted for at least 1 week in the animal laboratories at the Institute for Cancer Research (Vienna, Austria) under standardized SPF conditions (Macrolon cages, 2-3 mice/cage, 20 ± 2°C room temperature, 50-60% relative humidity and artificial lighting with lights on from 6 a.m. to 6 p.m.). Sterilized laboratory chow and water were given ad libitum.

Treatment and sampling

Animals were treated i.p. with a single dose of DEN (Sigma, Germany) at a dose of 90 mg/kg body wt, freshly dissolved in sterile 0.9% saline. Controls were given saline only. Body weights were taken at least four times per week.

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Histology and immunohistochemistry

Formalin-fixed liver specimens were transferred to 70% ethanol and embedded in paraffin. Tissue sections (5 μm) were stained with haematoxylin and eosin (HE). Liver pathology was scored according to the criteria of Chevallier and Scheuer et al. (39,40), with slight modifications as follows. Necrosis: no necrosis = 0; single necrotic cells per field = 1; small groups of necrotic cells per field = 2; confluent necrosis = 3. Inflammation: no or some (maximum 5) neutrophils per field = 0; 5-10 neutrophils per field = 1; 10-15 neutrophils per field = 2; >15 neutrophils per field = 3. A minimum of 10 central vein and 10 periportal fields each containing ~200 hepatocytes per field were analyzed. All slides were scored in a blind manner by one of the authors and by a recognized expert in rodent liver pathology. All slides were examined under 400x magnification.

Detection and quantification of apoptosis in liver, HE staining was used and morphological criteria were applied as previously described (41). Hepatocytes exhibiting chromatin condensation typical of the early stages of apoptosis as well as small membrane bounded bodies (apoptotic bodies) with or without chromatin located intra- or extracellularly were recorded. Apoptosis incidence was assessed by counting under 400x magnification (minimum of 2000 hepatocytes per liver). The incidence is presented as the total number of apoptoses expressed as a percentage of the total number of intact hepatocytes counted.

The number of hepatocytes in DNA replication was quantified after injection of BrdU. Sections were incubated for 1 h with rabbit anti-mouse BrdU antibody (1:200) (DAKO) (400x magnification, minimum of 1000 hepatocytes per slide, 3 slides per liver). Antibody binding was visualized using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The duodenum, a tissue with a high proliferation rate, was taken at necropsy and stained on the same slide as a positive control to confirm the systemic availability of BrdU.

Media and buffers

The pre-perfusion buffer consisted of Hank’s balanced salt solution (HBSS) buffered with 10 mM HEPES and equilibrated with carbon dioxide to pH 7.4 and supplemented with 2 U/ml heparin (REF Parfex). The collagenase perfusion buffer consisted of 100 U/ml collagenase dissolved in preperfusion buffer omitting calcium and with the addition of 10 mM calcium. Culture medium was William’s medium E supplemented with 100 nM dexamethasone, 0.4 nM glucagon, 7 nM insulin, 100 nM triiodothyronine, 150 mM ascorbic acid, 10 μg/ml gentamicin and 20 mM HEPES.

Isolation and culture of cells from the liver

KC and hepatocytes were obtained by collagenase digestion and differential centrifugation using Percoll (Pharmacia, Uppsala, Sweden) gradients as described elsewhere with slight modifications (42,43). Briefly, livers were perfused for 10 min with pre-perfusion buffer followed by collagenase perfusion until the liver was digested. The resulting suspension was centrifuged at 70 g for 5 min to separate hepatocytes (pellet) and nonparenchymal cells (NPC, supernatant).

For preparation of KC, the supernatant containing NPC was centrifuged at 650 g for 7 min at 4°C. Cell pellets were resuspended in HBSS and centrifuged on a density cushion of Percoll (25 and 50%) at 1800 g for 15 min at 4°C. The NPC fraction located between 25 and 50% Percoll was collected, centrifuged at 1000 hepatocytes per slide, 3 slides per liver). Antibody binding was visualized using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The duodenum, a tissue with a high proliferation rate, was taken at necropsy and stained on the same slide as a positive control to confirm the systemic availability of BrdU.

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To enrich the KC, the NPC fraction was seeded in 24- or 96-well plates and cultured for 30 min at 37°C in MEME ± 10% fetal calf serum. Within this period only KC adhere to the bottom of the plate. Non-adherent cells were removed by aspiration of the medium. Purity of KC cultures was evaluated by phagocytic uptake of FITC-labeled latex beads (1 μm diameter) by fluorometry as described elsewhere (45). Uptake of latex beads is a unique property of phagocytic cells; ≥ 5% of adherent cells phagocytosed latex beads, indicating that they were macrophages. Absence of hepatocytes was verified by morphological observation. In addition, analysis by RT-PCR for the hepatocyte-specific receptor PPARα did not give a signal in KC preparations (data not shown), although high levels of PPARα mRNA were demonstrated in hepatocyte preparations. KC were further cultured in phenol red-free RPMI-1640 medium (Gibco BRL Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum for 1 h before treatment in vitro.

Hepatocytes obtained in the pellet after cell separation were washed twice with 30 ml of cold MEME and centrifuged at 15 g for 5 min. The pellet was resuspended in 14 ml of MEME and mixed with 15 ml of a 90% Percoll solution in HBSS and centrifuged at 50 g for 10 min. Viable cells were collected from the top of the gradient and washed once in cold MEME. Purity of hepatocytes was routinely verified by the presence of cytosolic β-glucosidase, which hydrolyzes resorufin-β-D-glucopyranoside to fluorescent.
Superoxide generation from Kupffer cells

Resorufin (45). More than 95% of cells contained the product. Hepatocyte viability was on average 83 ± 5%, as assessed by trypan blue exclusion (light microscopy).

Assay of superoxide release
KC (0.15 × 10^6/well) were seeded in 96-well microplates and superoxide was measured using the assay based on reduction of cytochrome c by superoxide. The specificity of the reaction was checked by addition of superoxide dismutase, which degrades superoxide. Assay conditions were modified in preceding studies (34) in order to obtain a time-saving, sensitive and highly reproducible method to measure superoxide production by KC.

The assay reaction mixture was prepared in HBSS with 2.2 mg glucose/ml immediately prior to use and 100 µl added directly to the cells after removal of the maintenance medium. The reaction mixtures contained 160 µM cytochrome c with or without 100 U/ml superoxide dismutase. Aliquots of 22 mM DEN or 10 ng/ml LPS were added as indicated. After 20 min exposure, reduction of cytochrome c was measured at 550 nm in a plate reader (Anthos HAT II). All cell preparations were assayed in quadruplicates. Background values from cells in situ without assay mixture were <5% of the sample readings. Blind values were obtained by measuring standard mixtures without cells. The ΔA550 values were converted to nmol cytochrome c reduced/10^6 cells, using a net extinction coefficient ΔE550 of 2.1 ± 10^6/M/cm (46). Superoxide production was calculated from the difference in the amount of cytochrome c reduced in the absence and presence of superoxide dismutase and expressed as release/m.

Measurement of TNFα levels in media of Kupffer cell cultures and in serum
KC (0.5 × 10^6/well) were seeded in 24-well dishes and cultured in phenol red-free RPMI-1640 medium for 4 h. Thereafter the medium was collected and stored at -80°C. After each experiment cell counts per well were determined. TNFα was assayed in KC medium and serum samples using an enzyme-linked immunosorbent assay kit according to the manufacturer's specifications (BD Biosciences, Franklin Lakes, NJ). All preparations were assayed in triplicate. Data are presented as pg TNFα/ml.

Real-time PCR of TNFα expression
Total RNA was extracted from frozen mouse liver samples (3–6 per time point) using the Trizol reagent (Invitrogen Life Technologies) according to the manufacturer's protocol and quantified spectrophotometrically. Quality of RNA preparations was checked on denaturing agarose gels and only samples with intact RNA were processed further. An aliquot of 2 µg total RNA was transcribed into c-DNA using a random hexamer primer and MMLV reverse transcriptase (Sigma) according to the manufacturer's protocol. Quantitative determination of gene expression levels was done on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the Taq Man Universal Master Mix (Applied Biosystems). The gene expression assay (Mm 00443258_m1) for TNFα amplification and assay Mm 99999915_g1 for GAPDH. Reactions were performed in duplicate. Results were normalized to GAPDH and relative gene expression levels were determined according to the ΔΔCt method (Applied Biosystems) using untreated animals as an internal control.

Single cell gel electrophoresis assay (SCGE, comet assay)
For SCGE experiments 200 µl portions of hepatocyte cell suspensions (30 × 10^6 cells) were transferred to Eppendorf cups and centrifuged (7 min, 80 g). The pellets were then mixed with 200 µl of low melting point agarose (0.5%, 37°C) (47,48). Subsequently, 100 µl of each mixture was spread on an agarose-coated slide, covered with a coverslip and the slide placed on a cooled metal plate for 3 min to solidify the agarose. Thereafter, coverslips were removed and the slides exposed for 24 h to lysis solution. Finally, the slides were rinsed with distilled water and electrophoresis buffer to remove salts, exposed to alkaline electrophoresis buffer (pH 13) for 40 min and subjected to electrophoresis for 20 min (300 mAh, 25 mV). Then the alkali was neutralized with Tris buffer, the slides rinsed with distilled water and methanol and the DNA stained with ethidium bromide (20 µM/ml).

Evaluation of SCGE
Slides were evaluated under a fluorescence microscope (Nikon 027012) with an automated image analysis system based on the public domain program NIH Image. Three slides per animal were analyzed; tail lengths of 50 cells/slide were evaluated.

Statistics
All data are given as means ± SD. At least 6 animals/group and time point were analyzed. Comparisons between groups were performed with one-way ANOVA followed by Dunnett’s test using statistical software SigmaStat. P < 0.05 was considered to indicate statistically significant differences.

Results
Effect of DEN on body and liver weights.
Wild-type (wt) and p47phox knockout (p47phox−/−) mice were treated with a single hepatotoxic dose of DEN (90 mg/kg body wt). Body and liver weights were recorded as indicators of extent and duration of intoxication. Untreated wt and p47phox−/− mice (Figure 1A and B, triangles) did not differ in body weight gain (30%) within 5 weeks. In contrast, after DEN treatment the body weights of wt males was decreased at 12 h, exhibited a maximal loss (−20%) on day 7 and then gradually recovered, regaining the initial body weight after 5 weeks (Figure 1A, squares). Interestingly, the body weight of p47phox−/− male mice after DEN (Figure 1B, squares) did not differ significantly from untreated p47phox−/− mice at any time during 5 weeks of observation.

When female wt mice were treated with DEN they displayed a similar decrease in body weight as males (−20%) (Figure 1C). Female p47phox−/− mice lost less body weight during the first week after treatment (−12%), subsequently recovered and showed similar body weights to untreated mice at 4 and 5 weeks.

Liver weight and liver/body weight ratios in untreated mice did not differ significantly between p47phox−/− and wt mice and remained almost unchanged within the observation period (data not shown). After DEN, liver weights decreased by up to 45% in wt males (Figure 2A). In contrast, liver weights of p47phox−/− mice remained almost unchanged (except for a moderate decrease 7 days after treatment). Likewise, liver/body weight ratios decreased in wt male mice on day 7 (−22%) and slowly recovered thereafter (Figure 2B). The reduction in both absolute and relative liver weights of wt mice was detectable at 12 h post-treatment. In contrast, liver/body weight ratios in treated p47phox−/− mice were not significantly altered at 12 h or any later time point.

In females a decrease in liver weight occurred in both wt and p47phox−/− mice, but was clearly less pronounced in the knockout mice (Figure 2C). Liver/body weight ratios decreased in wt females by 15% on day 5 but tended to be less reduced in p47phox−/− females at most time points (Figure 2D).

These data strongly suggest that deletion of the p47phox gene affords protection from DEN-induced toxicity, as indicated by losses in body and liver mass. Protection was almost complete in males and moderate in females.

Histology: inflammation, necrosis and apoptosis
To further characterize hepatic responses to DEN in wt and p47phox−/− mice we have analyzed liver pathology in HE stained liver sections. Representative slides taken 3 days after DEN are shown in Figure 3. Untreated liver from wt and p47phox−/− mice exhibited normal morphology (Figure 3A and B). DEN administration to wt male mice resulted in severe parenchymal damage as indicated by numerous necrotic hepatocytes with altered nuclei and eosinophilic cytoplasm. The necrotic cells were surrounded by clusters of inflammatory cells. Inflammatory, necrotic and apoptotic cells were predominantly seen around central veins (Figure 3C). Liver histology of male p47phox−/− mice after DEN treatment revealed much less damage (Figure 3D).

In an attempt to quantify the time course and extent of liver damage we scored liver sections for signs of inflammation and...
necrosis by using the arbitrary units described in Materials and methods. As shown in Figure 4A and B, the pathological scores were much higher and persisted longer in wt than in p47phox−/− male mice. Also, hepatocyte apoptoses occurred much more frequently in wt liver (1.7% at maximum) than in p47phox−/− liver (Figure 4C).

In female wt mice liver damage as indicated by the scores was similar to that in males. p47phox−/− females tended to
have lower scores than wt mice (significant difference only at some of the time points studied). Thus, the apparent protection of the knockout mice was less marked in females than in males (Figure 4D – F). Consequently, further experimentation was done with male mice.

DNA content and DNA synthesis

Cell death by necrosis and apoptosis resulted in a 30% decrease in liver DNA in wt males at day 7 after DEN treatment, followed by partial recovery after ~3 weeks. In contrast, in p47phox/−/− males liver DNA content showed no decrease but tended to increase with body and liver growth (Figure 5A). We also determined the number of hepatocytes in DNA replication. In the liver of untreated p47phox/−/− and wt mice only low levels (maximum 0.4%) of DNA replication were observed. In DEN-treated wt mice DNA synthesis increased steeply from day 2 onwards, reached a maximum at day 3 and remained enhanced until day 35 (Figure 5B). As expected from necrotic and apoptotic counts (Figure 4), DNA synthesis increased only slightly in DEN-treated p47phox/−/− mice (Figure 5B). The increase (maximum 7.4% at day 3) in wt mice was significant when compared with both untreated wt and treated p47phox/−/− mice at all time points.

Superoxide release from Kupffer cells

To examine whether superoxide release from KC might be involved in DEN-induced hepatotoxicity in wt mice, we have isolated the KC fraction from the liver and measured superoxide anion release as described in Materials and methods. KC were obtained from untreated mice and at different times after DEN administration.

In KC from untreated wt and p47phox/−/− mice superoxide production was undetectable (Figure 6A, time 0). With increasing time after DEN treatment in vivo superoxide release from wt KC in vitro rose to a maximum at 6 h and declined thereafter. As expected, KC from DEN-treated p47phox/−/− mice did not generate measurable amounts of superoxide in vitro (Figure 6A).

DEN addition in vitro to KC from untreated wt mice dramatically increased superoxide production (Figure 6B, time 0). This effect of DEN in vitro was even more pronounced (up to 2-fold) when mice had been treated with DEN in vivo (Figure 6B). Obviously, DEN treatment in vivo primed KC for increased superoxide generation. KC from p47phox/−/− mice released a low level of superoxide in response to DEN in vitro, which may indicate a direct toxic effect of DEN on KC.

Lipopolysaccharide (LPS) is a well-known activator of KC. When added to KC in vitro superoxide release was enhanced to a similar extent as after DEN in vitro. LPS stimulation of superoxide production was also primed by DEN treatment in vivo (Figure 6C). LPS did not consistently enhance superoxide release in KC from p47phox/−/− mice.

TNFα after DEN treatment

We have measured TNFα in the medium of KC from wt and p47phox/−/− mice either untreated or treated with DEN. Basal TNFα production by KC from wt mice was 1.4-fold higher than in KC from p47phox/−/− mice (Figure 7A, time 0). When KC were isolated 0.5 and 1 h after DEN treatment in vivo TNFα release was dramatically suppressed in
cells of wt and p47phox<sup>−/−</sup> mice (Figure 7A). When isolated later KC from wt mice secreted TNFα in vitro at slightly increasing rates (at 6 h 1.4-fold of 0 h control), followed by a decline to 40% of control. TNFα production by KC from p47phox<sup>−/−</sup> mice also recovered from the initial drop and decreased by 12 h to 70% of the 0 time control level (Figure 7A).

Addition of LPS to the cultures increased TNFα production 3-fold in KC from untreated wt mice and 1.8-fold in KC from p47phox<sup>−/−</sup> mice (Figure 7B, time 0 compared with Figure 7A, time 0). When KC were isolated after DEN treatment in vivo TNFα release was first suppressed (at 1 h), recovered at 2 and 6 h and then decreased (Figure 7B). KC from p47phox<sup>−/−</sup> mice secreted less TNFα than wt counterparts at all time points investigated.

In serum from wt mice basal TNFα levels were 1.5-fold higher than in serum from p47phox<sup>−/−</sup> mice (Figure 7C). Following DEN administration to wt mice serum TNFα increased in a time-dependent manner, with a 1.8-fold increase between 2 and 6 h, and was back to control levels at 12 and 24 h. In p47phox<sup>−/−</sup> mice a similar increase occurred, but was less pronounced (maximal levels 1.6-fold above control). The difference between wt and p47phox<sup>−/−</sup> mice was significant (P < 0.05).

Expression of TNFα mRNA in liver (Figure 7D) showed a similar trend to TNFα levels in KC culture medium and in serum.

**Hepatocytes from p47phox<sup>−/−</sup> mice are less susceptible to DNA damage by DEN**

To study the question whether superoxide production by KC might contribute to DNA damage, single cell gel electrophoresis (comet assay) after alkaline treatment was performed. Hepatocyte suspensions were used immediately after preparation and the lengths of comet tails, reflecting the extent of DNA fragmentation, were measured. No evidence of damage was seen in hepatocytes isolated from untreated wt and p47phox<sup>−/−</sup> mice (Figure 8A and B). Two hours after DEN treatment in vivo wt hepatocyte nuclei displayed extensive DNA fragmentation (Figure 8C). This effect was much less pronounced in the p47phox<sup>−/−</sup> counterparts (Figure 8D).

We quantified the tail lengths of hepatocytes at various times after treatment of mice with DEN. The tail length of control hepatocytes ranged from 5.7 to 7.9 μm and was not significantly different in wt and p47phox<sup>−/−</sup> cells (Figure 9, time 0). Quantification of tail lengths after DEN exhibited a significant increase in DNA damage of wt hepatocytes already at 1 h (3-fold) and a further increase to a maximum 5-fold above...
controls at 2 h. An increase was also seen with \( p47phox^{-/-} \) cells, but was significantly less (Figure 9). The difference in hepatocyte DNA damage between \( \text{wt} \) and \( p47phox^{-/-} \) cells persisted at least up to 24 h after DEN.

These data suggest that superoxide from phagocytic NADPH oxidase is involved in the induction of hepatocyte DNA damage by DEN.

**Discussion**

The present study shows that absence of a functional PHOX in mice eliminates superoxide production by (isolated) KC and attenuates liver damage induced by DEN. This attenuation is indicated by quantitative differences in response to DEN of a number of end points including body and liver weights, liver histology, DNA synthesis and DNA damage. All these differences consistently suggest that phagocytic superoxide, directly or indirectly, contributes to toxic changes in the liver resulting from DEN treatment. This contribution seems more significant in male than in female mice, as indicated by the smaller protective effect seen in female than in male \( p47phox^{-/-} \) mice. The mechanism underlying this sex difference is currently not understood. Its elucidation is of great potential interest.

The product of PHOX, superoxide, is highly unstable and readily forms other ROS and reactive nitrogen species (RNS), including \( H_2O_2 \), \( OH^+ \) radicals and peroxynitrite (17,19,21,23). We have found that protein tyrosine nitration, an indicator of high levels of peroxynitrite in DEN-treated liver, is dramatically reduced in \( p47phox^{-/-} \) mice as compared with \( \text{wt} \) animals (manuscript in preparation). Obviously, less peroxynitrite is formed in \( p47phox^{-/-} \) mice. In addition, the release of phagocytic superoxide is regarded as enhancing the rate of secretion of TNF\( \alpha \) (20--23,49), as supported by the present data (Figure 7), and may likewise affect secretion of other cytokines by KC and related cells. Furthermore, lipid peroxidation (LPO), as observed after DEN (24,50), may be triggered by the superoxide burst and in turn give rise to the appearance of toxic products such as reactive aldehydes. Generation of superoxide involves electron transfer to oxygen along with cation (proton and potassium) translocation. This in turn results in activation of phagocyte proteases (51), which also might affect toxicity. In conclusion, the elimination of PHOX and of superoxide generation alters the formation of various toxic products.
intermediates, cytokines and proteases as well as the ionic milieu. All of these factors may contribute to liver injury by DEN (Figure 10).

KC activation, as indicated by altered release of superoxide and TNFα, was detected as early as 0.5 h after DEN administration. KC activation is known to attract other immune cells, including granulocytes. This is consistent with the present observation of increasing numbers of granulocytes in liver tissue with the progress of damage, a maximum being reached 2 days after DEN (Figure 3). Granulocytes also have PHOX and produce superoxide, along with other mediators of the immune response and toxicity (35,36). Therefore,

Fig. 7. Effect of DEN on TNFα in KC medium and in serum of p47phox−/− and wt mice. Isolation of KC and symbols are as in Figure 5. TNFα levels in medium of KC cultured for 4 h in the absence (A) or presence (B) of 10 ng/ml LPS. TNFα in serum collected from control and DEN-treated mice (C). TNFα liver mRNA expression (D). The expression level of untreated animals is set as 1 (dashed line). Dashed lines indicate control levels obtained from untreated mice. Data are means ± SD from three (A and B) independent cell preparations (see Figure 6) or 3–6 (D) or 6 (C) mice/group. Asterisks (*) indicate significant differences between wt and p47phox−/− mice. (A and B) Changes against zero time controls were not significantly different at 2 and 6 h but significantly different (*P < 0.05) at 12 h. (C) Serum TNFα levels were significantly increased (*P < 0.05) at 1, 2 and 6 h.

Fig. 8. Hepatocyte DNA damage of p47phox−/− and wt mice after DEN in vivo. Comet assays were performed with hepatocytes isolated from untreated wt (A) and p47phox−/− (B) mice as well as from wt (C) and p47phox−/− (D) mice 2 h after DEN in vivo.
the sequence of activation and immigration of phagocytic cells suggests that KC superoxide plays a primary role in the first stages of DEN intoxication while later superoxide from granulocytes and possibly other cells will aggravate the damage.

That KC activation and superoxide contribute to the hepatotoxic effects of DEN as indicated by the present work has, to our knowledge, not been reported before for DEN or other genotoxic carcinogens. However, similar conclusions were reported for some other hepatotoxicants. These include acetaminophen, endotoxin, carbon tetrachloride, galactosamine, 1,2-dichlorobenzene, allyl alcohol, cadmium and ethanol. With each of these compounds hepatotoxicity was attenuated or prevented by pretreatment of experimental animals with agents which block macrophage activity, such as gadolinium chloride (9,52–58).

The mechanism(s) by which DEN stimulates KC is not understood. DEN is known to be bioactivated in hepatocytes by CYP2E1 and other enzymes. The in vivo data would be compatible with the concept that DEN stimulates KC indirectly by diffusion of reactive metabolites or secondary toxic products from hepatocytes to KC. However, the present study shows that DEN has a stimulatory effect on isolated KC which do not contain any detectable hepatocyte contamination. This suggests that DEN, in addition to its known effect on hepatocytes, also has a direct action on KC (Figure 10). Whether this results from activation by CYP2E1, which is reported to occur (59,60), or by other mechanisms remains to be elucidated.

Cancer formation after DEN and other complete hepatocarcinogens is generally considered to result from two key effects: (i) genotoxicity leading to mutations and, in some hepatocytes, tumor initiation; (ii) cytotoxicity and cell death resulting in regenerative cell proliferation which preferentially occurs in initiated hepatocytes, i.e. tumor promotion. The present study shows that both key effects are more pronounced in the presence of functional PHOX. This implicates phagocyte activation and, most likely, formation of superoxide and its downstream ROS, RNS or LPO reaction products in hepatocarcinogenesis (Figure 10). Furthermore, promotion of tumor development by KC activation is supported by observations in a rat liver model. They indicate that the factors released from activated KC/NPC strongly enhance proliferation of initiated hepatocytes (Drucker et al., submitted for publication).

In conclusion, our data show that activation of KC and the resultant superoxide burst significantly contribute to the generation of toxic responses and, possibly, liver cancer. Since activation of macrophages and neutrophils occurs after very different pro-inflammatory challenges, including various toxic...
chemicals, some viral and other infections and during immune diseases, the mechanisms observed here with DEN in mouse liver may apply to other systems as well.

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