Early resistance to cell death and to onset of the mitochondrial permeability transition during hepatocarcinogenesis with 2-acetylaminofluorene

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A hallmark of tumorigenesis is resistance to apoptosis. To explore whether resistance to cell death precedes tumor formation, we have studied the short-term effects of the hepatocarcinogen 2-acetylaminofluorene (AAF) on liver mitochondria, on hepatocytes, and on the response to bacterial endotoxin lipopolysaccharide (LPS) in albino Wistar rats. We show that after as early as two weeks of AAF feeding liver mitochondria developed an increased resistance to opening of the permeability transition pore (PTP), an inner membrane channel that is involved in various forms of cell death. Consistent with a mitochondrial adaptive response *in vivo*, (*i*) AAF feeding increased the expression of BCL-2 in mitochondria, and (*ii*) hepatocytes isolated from AAF-fed rats became resistant to PTP-dependent depolarization, cytochrome *c* release, and cell death, which were instead observed in hepatocytes from rats fed a control diet. AAF-fed rats were fully protected from the hepatotoxic effects of the injection of 20–30 μg of LPS plus 700 mg of D-galactosamine (D-GalN) × kg^{-1} of body weight, a treatment that in control rats readily caused a large increase of terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling-positive cells in liver cryosections and release of alanine and aspartate aminotransferase into the bloodstream. Treatment with LPS and D-GalN triggered cleavage of BID, a BCL-2 family member, in the livers of both control- and AAF-fed animals, whereas caspase 3 was cleaved only in control-fed animals, indicating that the mitochondrial proapoptotic pathway had been selectively suppressed during AAF feeding. Phenotypic reversion was observed after stopping the carcinogenic diet. These results underscore a key role of mitochondria in apoptosis and demonstrate that regulation of the mitochondrial PTP is altered early during AAF carcinogenesis, which matches, and possibly causes, the increased resistance of hepatocytes to death stimuli *in vivo*. Both events precede tumor formation, suggesting that suppression of apoptosis may contribute to the selection of a resistant phenotype, eventually increasing the probability of cell progression to the transformed state.

The role of mitochondria in cell death is being increasingly recognized (1). Mitochondrial dysfunction due to a permeability transition can precipitate a bioenergetic crisis with ATP depletion and Ca^{2+} dysregulation (2–4). On the other hand, mitochondria can release proteins that cause cell death through both caspase-dependent and caspase-independent mechanisms (5–9). Release of these apoptogenic factors is modulated by members of the BCL-2 family of proteins in a way that is consistent with their role in apoptosis. Antiapoptotic members inhibit (8, 10, 11) whereas proapoptotic members favor the release (12–16) in a process that is initiated by insertion of truncated BID in the outer mitochondrial membrane (17–19) and/or by the mitochondrial permeability transition (8, 16, 20–22). Defects of apoptosis are critical to both tumorigenesis and drug resistance (23). An involvement of mitochondria in the resistance of cancer cells to apoptosis is supported by many *in vitro* studies (24), but whether this holds true *in vivo* remains to be established.

The early formation of drug-resistant hepatocytes during feeding with the hepatocarcinogen 2-acetylaminofluorene (AAF) is well documented (25), but the basis for resistance and its potential role in tumorigenesis remain obscure. Metabolic activation of AAF is a prerequisite for the manifestation of both genotoxic and nongenotoxic effects (26). Previous work has shown that 2-nitrosofluorene, a metabolite of AAF, undergoes redox cycling after reduction by the mitochondrial respiratory chain (27) and is able to trigger opening of the permeability transition pore (PTP) (28). Yet, liver mitochondria isolated from rats fed with AAF were strikingly resistant to PTP opening well before the animals developed liver cancer, leading Neumann and Coworkers to propose that mitochondrial adaptation may play a role in the selection of resistant hepatocytes (28).

The present study was designed to investigate whether mitochondrial resistance is matched by an early inhibition of cell death regulation *ex vivo* and *in vivo*. We confirmed that within 2 weeks of feeding with a diet containing 0.01–0.04% (wt/wt) AAF, liver mitochondria developed an increased resistance to opening of the PTP (28). Consistent with a mitochondrial adaptive response *in vivo*, hepatocytes isolated from AAF-fed rats became resistant to PTP-dependent depolarization, cytochrome *c* release, and cell death. AAF-fed rats were fully protected from the hepatotoxic effects of the injection of *Escherichia coli* lipopolysaccharide (LPS) plus D-galactosamine (D-GalN) (29), a treatment that in control rats readily caused a large increase of terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL)-positive cells in liver cryosections and release of alanine and aspartate aminotransferase into the bloodstream. Treatment with LPS plus D-GalN caused BID cleavage irrespective of AAF feeding, whereas caspase 3 was cleaved only in animals fed a control diet. These results demonstrate that mitochondrial adaptation is an early effect of AAF feeding, which is likely to cause the increased resistance of hepatocytes to death stimuli *in vivo*.

Materials and Methods

Chemicals and Antibodies. LPS from *E. coli* serotype O111:B4, D-GalN hydrochloride, collagenase type IV, collagen type I, valinomycin, and AAF were purchased from Sigma. Calcium-green-5N and tetramethylrhodamine methyl ester (TMRM)

Abbreviations: AAF, 2-acetylaminofluorene; D-GalN, D-galactosamine; LPS, lipopolysaccharide of *Escherichia coli*; PTP, permeability transition pore; TMRM, tetramethylrhodamine methyl ester; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling.

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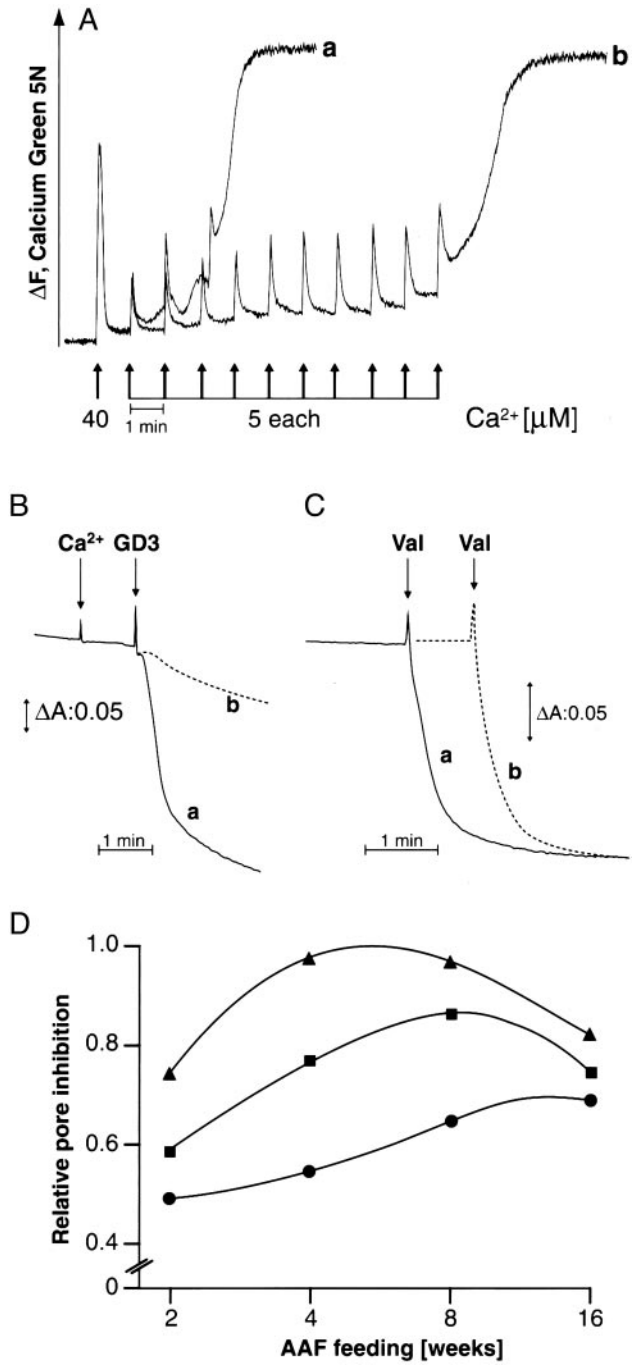

Fig. 1. Effect of AAF feeding on mitochondrial Ca^{2+} loading capacity and on PTP-dependent and valinomycin-dependent swelling. The incubation medium contained 200 mM sucrose, 10 mM Tris–Mops, 5 mM succinate/Tris, 1 mM P_i/Tris, 10 μM EGTA/Tris, and 2 μM rotenone. Final volume was 2 ml, pH 7.4, 25°C. All experiments were started by the addition (not shown) of 0.4 mg $\times$ ml^{-1} liver mitochondria prepared from rats fed either a control diet (traces a in all panels) or a diet containing 0.04% AAF (traces b in all panels) for 3–4 weeks. (*A*) The medium was supplemented with 1 μM calcium-green-5N, and the indicated concentrations of Ca^{2+} were added at the time points marked by arrows. (*B*) Where indicated 50 μM Ca^{2+} and 40 μM GD3 ganglioside were added. (*C*) The medium was supplemented with 1 mM KCl, and 1 μM valinomycin (Val) was added where indicated by the arrows. These experiments are representative of 10–45 replicates each. PTP adaptation (i.e., increased resistance to PTP opening relative to controls in protocols similar to those of *B*) was observed in mitochondria isolated from 45 of 50 AAF-fed animals. (*D*) Male Wistar rats were fed a diet containing 0.04% (▲), 0.02% (■), or 0.01% (●) AAF. At the indicated time points, rats were killed and liver mitochondria were isolated. Osmotic swelling of mitochondria (1 mg $\times$ ml^{-1}) was determined as shown in *B*, except that the concentration of P_i/Tris was 15 mM and the Ca^{2+} addition, which was sufficient to trigger mitochondrial swelling in the control mitochondria, was 80 μM. Data are expressed as inhibition of swelling rates, where 0 is the rate of control mitochondria (corresponding to $\Delta A/\Delta t$ values of 249 $\pm$ 9, 201 $\pm$ 34, 210 $\pm$ 82, and 232 $\pm$ 64 $\times$ 10^{-3} $\times$ min^{-1} for mitochondria from weeks 2, 4, 8, and 16, respectively) and 1 is the inhibition observed in the presence of 120 nM cyclosporin A (corresponding to $\Delta A/\Delta t$ values of 4 $\pm$ 2, 3 $\pm$ 2, 1 $\pm$ 1, and 10 $\pm$ 4 $\times$ 10^{-3} $\times$ min^{-1} for mitochondria from weeks 2, 4, 8, and 16, respectively). The rate of swelling was determined by linear regression of the A_{540} versus time diagram. Data represent mean values of four animals, and curves were fitted by interpolation. Standard deviations were omitted for clarity and did not exceed 0.1. Swelling rates for mitochondria from AAF-fed animals were significantly different from control values for all time points ($P <$ 0.001, Student's *t* test).

were purchased from Molecular Probes. The mouse IgG1 antibody directed against BCL-2 was purchased from Transduction Laboratories (Lexington, KY). A rabbit polyclonal antiserum raised against the core subunit 2 (Core-2) of the mitochondrial bc_1 complex was kindly provided by Hermann Schägger. Antibodies against cytochrome *c* (clone 6H2.B4) and BID were from PharMingen, the antibody against cleaved caspase 3 was from Cell Signaling (CELBIO, Milan), and the antibody against actin was from Sigma. The rabbit polyclonal antibody raised against the rat bc_1 complex and the GD3 ganglioside were generous gifts of Roberto Bisson and Fidia Research Laboratories (Abano Terme, Italy), respectively.

Animals. Male albino Wistar rats (180–200 g) had free access to a standard diet (Altromin 1324, Altrogge, Lage/Lippe, Germany) or standard diet supplemented with 0.01–0.04% AAF. *In vivo* treatment was essential because concentrations up to 1 mM AAF had no effects on the PTP and on respiration when added directly to isolated mitochondria (results not shown). Animals were kept under controlled conditions of temperature and humidity on a 12-h light/12-h dark cycle. With the exception of Fig. 1*D*, experiments on mitochondria, hepatocytes, or living animals were carried out after 3–4 weeks of feeding with AAF or control diet. At this time point, mitochondrial PTP resistance was already significant (see Fig. 1*D*), whereas toxic effects of AAF-feeding were not apparent as assessed by monitoring the body weight, which did not show a significant difference relative to control animals (30). All preparations and treatments were always carried out in parallel for the two sets of feeding conditions.

Assays on Isolated Mitochondria. Rat liver mitochondria were isolated as described (27). Mitochondrial volume changes were determined from absorbance changes at 540 nm with an Aminco DW2000 spectrophotometer (SLM, Urbana, IL). The Ca^{2+} retention capacity of mitochondrial preparations was assessed fluorimetrically in the presence of the Ca^{2+} indicator calcium-green-5N with a Perkin–Elmer LS50B spectrofluorimeter exactly as described (31). All instruments were equipped with magnetic stirring and thermostatic control. The incubation conditions are specified in the figure legends.

Western Blot Analysis. For the experiments of Fig. 2, rat liver mitochondria were lysed in a buffer containing 50 mM NaF, 40 mM sodium pyrophosphate, 20 mM Tris·HCl (pH 8.0), 10 mM EGTA, 5 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 20 $\mu g \times ml^{-1}$ aprotinin, and 20 $\mu g \times ml^{-1}$ leupeptin for 1 h at 4°C, and the lysates were cleared by centrifugation at 14,000 $\times$ *g* for 10 min at 4°C. For the experiment of Fig. 4*E*, liver homogenates were extracted with 0.3 M mannitol/5 mM Tris–Mops (pH 7.4)/4 mM KH_2PO_4/1 mM EGTA/2 mM phenylmethylsulfonyl

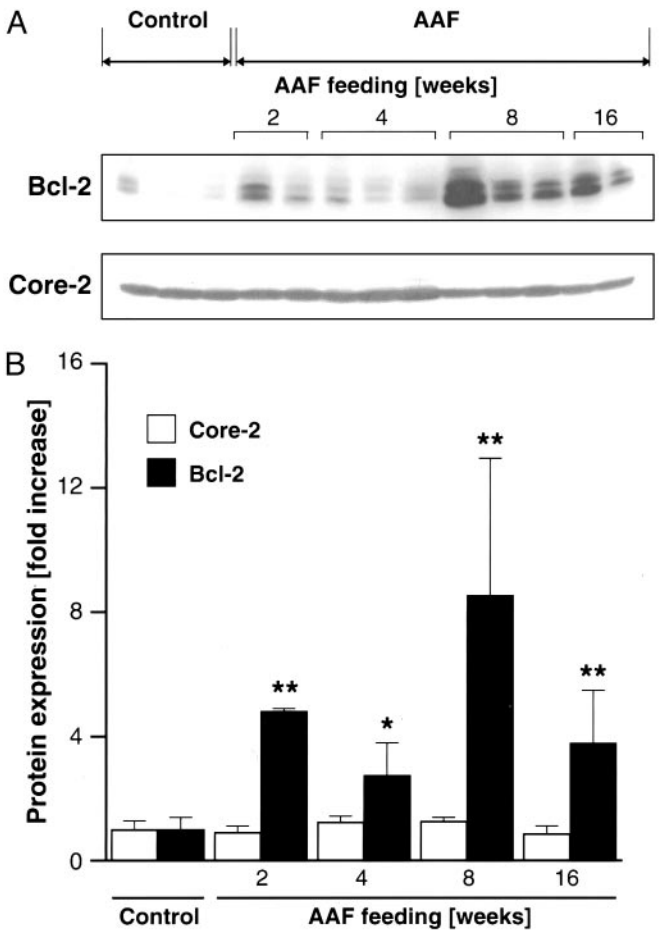

Fig. 2. Effect of AAF feeding on mitochondrial BCL-2 expression. (*A*) The levels of expression of BCL-2 and of complex III subunit Core-2 were determined by Western blotting of isolated mitochondrial proteins with antibodies against BCL-2 and Core-2. Animals were fed either a control diet or a diet containing 0.02% AAF for the indicated length of time, and each lane was loaded with mitochondrial proteins from one individual animal. (*B*) Protein expression levels were quantified as described in *Materials and Methods*, and the levels of expression were normalized to control values. Data represent mean values ± SD of three to five animals for each condition.

fluoride/10 $\mu g \times ml^{-1}$ aprotinin/1% Nonidet P-40 for 30 min at 4°C, and the lysates were centrifuged at 90,000 × *g* in a Beckman L7-55 ultracentrifuge. The extracts were diluted with 2× Laemmli gel sample buffer and boiled for 3 min. Identical protein amounts were separated by SDS/PAGE, electroblotted onto Immobilon-P poly(vinylidene difluoride) membranes (Millipore), and incubated with the antibodies as specified in the figure legends. For the experiments of Fig. 2, blots were scanned with the TLC Scanner 3 (Camag, Berlin) and quantified with the manufacturer's software, CATS 4.04.

Preparation of Hepatocytes. Hepatocytes were isolated by collagenase perfusion essentially as described by Berry and Friend (32). Hepatocytes were purified through a 40% (vol/vol) Percoll/Krebs–Henseleit Hepes buffer solution and rinsed free of Percoll with M199 medium supplemented with 24.7 mM Hepes and 25 mM bicarbonate (pH 7.2). Hepatocyte integrity was assessed by trypan blue exclusion and found to be always >85%. Cells were plated in six-well tissue culture plates coated with 0.01% (wt/vol) collagen type I (5×10^5 cells per well). The culture medium was M199 supplemented with 5% heat-inactivated FBS, 100 units × ml^{-1} penicillin, 100 $\mu g \times ml^{-1}$ streptomycin, 200 nM dexamethasone, 1 nM insulin, 12.5 mM Hepes, and 25 mM bicarbonate (final pH 7.2). After 16 h the culture medium was replaced by serum-free M199 medium supplemented with 100 units × ml^{-1} penicillin, 100 $\mu g \times ml^{-1}$ streptomycin, 12.5 mM Hepes, and 25 mM bicarbonate (pH 7.2), and the experiments were carried out as further described in the following paragraph.

Experiments with Isolated Hepatocytes. The mitochondrial membrane potential was assessed by epifluorescence microscopy based on the mitochondrial accumulation of TMRM. Briefly, hepatocytes were preincubated with 20 nM TMRM and 1.8 μM cyclosporin H in Hanks' balanced salt solution (HBSS) containing Ca^{2+} and Mg^{2+} (pH 7.4) for 20 min at 37°C, and changes of mitochondrial TMRM fluorescence were monitored with the Olympus IMT-2 inverted epifluorescence microscope equipped with a cooled charge-coupled device (CCD) camera exactly as described (33). The distribution of cytochrome *c* was determined exactly as described in ref. 34. Cell death was determined based on staining with propidium iodide. Cells were washed three times with HBSS, incubated with 2 μM propidium iodide in HBSS for 20 min, rinsed, and immediately analyzed with the Olympus IMT-2 epifluorescence microscope.

Treatment with LPS and D-GalN. Rats were treated with a single i.p. injection of 20–30 μg of LPS plus 700 mg of D-GalN × kg^{-1} of body weight. After 8 h blood samples were withdrawn from the tail vein, animals were killed by cervical dislocation, and livers were isolated for TUNEL. The TUNEL reaction was carried out on 10 μm-thick liver cryosections with an *in situ* cell death detection kit (Roche Molecular Biochemicals, Milan). Sections preincubated for 30 min with 5 $\mu g \times ml^{-1}$ DNase I (GIBCO/BRL) served as positive controls. An aliquot of serum was used to determine the concentration of tumor necrosis factor (TNF)-α, whereas the remainder was stored at −80°C until determination of serum enzymes was performed. TNF-α was determined by using a rat TNF-α immunoassay (Quantikine M, R & D Systems) according to the manufacturer's instructions. Serum levels of alanine aminotransferase and aspartate aminotransferase were determined according to standard procedures.

Statistical Procedures. Paired, two-tailed Student's *t* test was used to determine the statistical significance of differences between sample mean. $P < 0.05$ and $P < 0.01$ are denoted with one or two asterisks, respectively.

Results

In the experiments of Fig. 1*A*, we tested the mitochondrial Ca^{2+}-retention capacity, a sensitive measure of the propensity of mitochondria to open the PTP after Ca^{2+} uptake. Mitochondria from animals fed a control diet accumulated ≈125 nmol of Ca^{2+} per mg of protein before PTP opening, which can be identified by release of the accumulated Ca^{2+} (trace a). Mitochondria isolated from rats fed a diet containing 0.04% AAF for 3 weeks were more resistant, in that nearly twice as much Ca^{2+} was needed to trigger opening of the PTP (trace b). Mitochondria from AAF-fed rats became more resistant to other PTP-inducing stimuli. Fig. 1*B* shows that after the uptake of 100 nmol of Ca^{2+} per mg of protein (a load that was not sufficient to cause PTP opening *per se*), addition of GD3 ganglioside readily caused swelling of mitochondria from control rats (trace a). As expected of a PTP-dependent event, swelling was fully inhibited by cyclosporin A (results not shown, but see ref. 35). No swelling was observed after addition of GD3 ganglioside to mitochondria from AAF-fed rats (trace b). Similar results were obtained when mitochondria were treated with other PTP-inducing agents (acetoacetate, arachidonic acid, diamide, menadione, 2-nitrosofluorene, or uncoupler; results not shown). In contrast,

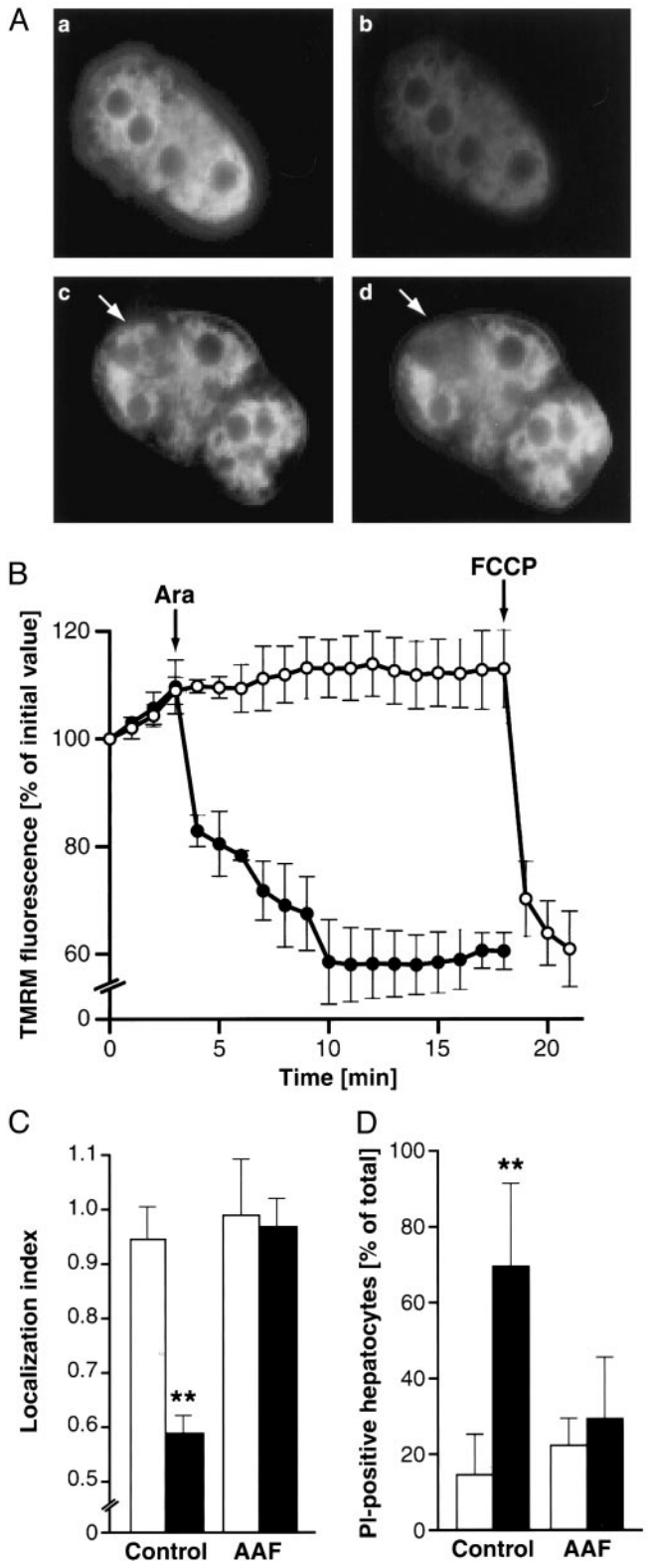

Fig. 3. Effect of AAF feeding on PTP-dependent mitochondrial depolarization, cytochrome *c* release, and cell survival after treatment of primary cultures of hepatocytes with arachidonic acid. Hepatocytes were isolated by collagenase perfusion as described in *Materials and Methods* from rats fed either a control diet or a diet containing 0.04% AAF for 4 weeks, and kept 24 h in the CO_2 incubator. (*A*) Hepatocytes were loaded for 20 min with 20 nM TMRM and transferred to the microscope stage. Shown are fluorescence images (magnification ×400) of hepatocytes from control (*a* and *b*) and AAF-fed animals (*c* and *d*) before (*a* and *c*) and 15 min after the addition of 200 μM arachidonic acid (*b* and *d*). (*B*) Time course of the mitochondrial fluorescence changes after the addition of 200 μM arachidonic acid (Ara) in hepatocytes from control (●) and AAF-fed (○) rats. Values (normalized to the initial fluorescence) report the mean fluorescence ± SD as determined in four independent determinations on the same preparations of hepatocytes and are representative of four separate experiments. Where indicated, 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added. (*C* and *D*) Two hundred micromolar arachidonic acid or vehicle was added to the hepatocytes, and the incubation was continued for a further 30 min [a time point at which the cytochrome *c* distribution was determined (*C*)] or 60 min [a time point at which nuclear staining with propidium iodide was determined (*D*)]. For both *C* and *D*, addition of vehicle or 200 μM arachidonic acid is denoted by open and filled bars, respectively. A lower localization index corresponds to a more diffuse distribution of cytochrome *c* relative to the bc_1 complex; see ref. 34 for details. Values refer to the mean ± SD of four independent determinations on the same preparations of hepatocytes and are representative of three (cytochrome *c*) or four (propidium iodide) separate experiments.

PTP-independent mitochondrial swelling by the addition of the K^+ ionophore valinomycin (Fig. 1*C*) caused an identical absorbance decrease in mitochondria from control and AAF-fed animals (traces a and b, respectively). Half-maximal PTP inhibition was already detectable after 2 weeks of treatment with the lowest AAF dose (0.01%) and reached a peak between 4 and 8 weeks (Fig. 1*D*). The inhibitory effect was transient, a decrease being detected with all AAF doses between 8 and 16 weeks of treatment, i.e., a time period that is characterized by a marked increase of focal lesions *in vivo* (30, 36). PTP resistance was matched by expression of mitochondrial BCL-2, which increased with the same time course as PTP adaptation (Fig. 2 *A* and *B*).

We next studied the responses of the PTP *in situ* in primary cultures of hepatocytes prepared from control and AAF-fed rats. In the experiments of Fig. 3*A*, hepatocytes were challenged with 200 μM arachidonic acid, a cytotoxic concentration (21, 37) that readily caused mitochondrial depolarization as measured by the release of intramitochondrial TMRM in hepatocytes from rats fed a control diet (*b*, compare with *a*). A striking resistance to mitochondrial depolarization was observed in hepatocytes prepared from AAF-fed rats (*d*, compare with *c*). The vast majority, but not all, hepatocytes from AAF-fed rats were resistant to arachidonic acid-induced mitochondrial depolarization. Indeed, the hepatocyte marked by the arrow in *c* and *d* underwent a mitochondrial depolarization that was indistinguishable from that of hepatocytes prepared from control animals. In Fig. 3*B*, the time course of the fluorescence changes induced by arachidonic acid in the two populations of hepatocytes is compared, confirming resistance to PTP opening *in situ* in hepatocytes prepared from AAF-fed rats. Control hepatocytes treated with arachidonic acid readily released mitochondrial cytochrome *c* (Fig. 3*C*) and became positive for nuclear staining with propidium iodide (Fig. 3*D*), whereas hepatocytes from AAF-fed rats retained cytochrome *c* in the mitochondrial compartment and remained impermeable to propidium iodide (Fig. 3 *C* and *D*, respectively).

To test whether AAF-feeding also conferred resistance to liver cytotoxic stimuli *in vivo*, we injected rats with a single bolus of LPS plus D-GalN, a well established protocol to induce acute hepatotoxicity through TNF-α release (29, 38). After 8 h of treatment with 20 μg of LPS plus 700 mg of D-GalN × kg^{-1} of body weight, liver cryosections revealed a sizeable number of TUNEL-positive hepatocytes in control but not in AAF-fed animals (Fig. 4*A b* and *e*, respectively). TUNEL positivity in control rats correlated with a significant number of apoptotic bodies in hematoxylin/eosin-stained slices, which were rarely detectable in slices from AAF-fed animals (data not shown). Inflammation, a frequent event in control rats that is indicated by lymphocyte infiltration of liver parenchyma, was absent in

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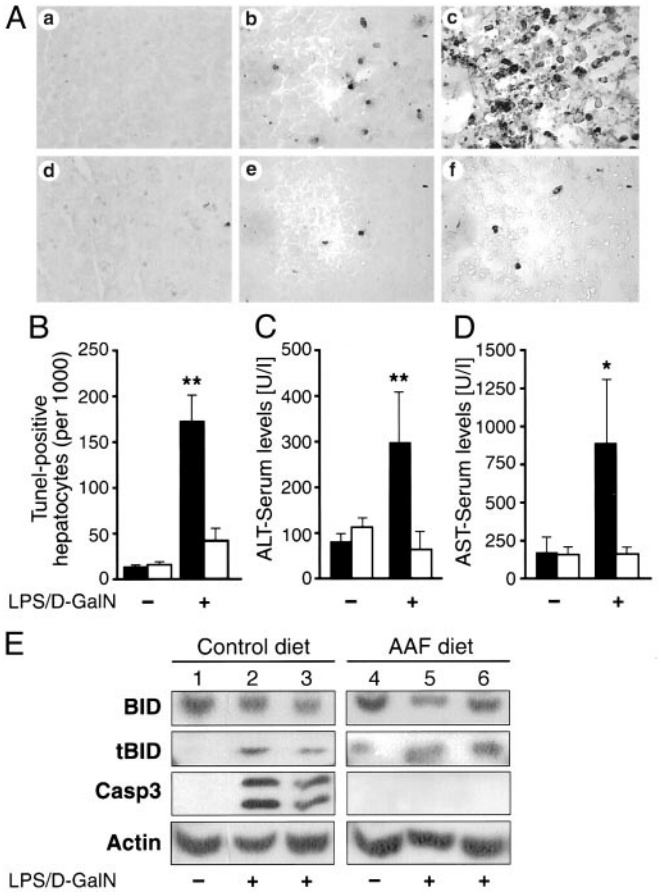

Fig. 4. Effect of AAF feeding on liver TUNEL staining, serum enzyme release, and BID and caspase 3 cleavage after treatment with LPS plus D-GalN *in vivo*. Rats fed a control diet or a diet containing 0.04% AAF for 3 weeks were treated with a single i.p. injection of vehicle (isotonic saline) or with LPS plus D-GalN. After 8 h blood was withdrawn from the tail vein to determine plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Extracts from liver homogenates and liver cryosections were prepared and analyzed by Western blotting and TUNEL staining, respectively (see *Materials and Methods* for details). (*A*) Magnifications of ×50 of TUNEL-stained cryosections from control animals (*a–c*) and AAF-fed animals (*d–f*) treated with saline (*a* and *d*), or with 20 μg of LPS (*b* and *e*) or 30 μg of LPS plus 700 mg of D-GalN × kg^{-1} of body weight (*c* and *f*). (*B*) Percentage of TUNEL-positive hepatocytes in control (filled bars) or AAF-fed (open bars) animals after treatment with saline (−) or 20 μg of LPS plus 700 mg of D-GalN × kg^{-1} of body weight (+). For each condition nuclear labeling was determined from 20 high-power fields, which corresponds to a total of ≈1,200–1,500 hepatocytes. Error bars denote the SD of six (treatment with LPS plus D-GalN) or four (treatment with saline) independent experiments. (*C* and *D*) Plasma levels of ALT and AST, respectively, in animals treated with a control diet (filled bars) or AAF diet (open bars) after injection of saline (−) or 20 μg of LPS plus 700 mg of D-GalN × kg^{-1} of body weight (+). Error bars denote the SD of five independent experiments. (*E*) Western blot analysis of BID, truncated BID (tBID), cleaved caspase (Casp) 3, and actin in liver extracts from control-fed (lanes 1–3) and AAF-fed (lanes 4–6) animals; samples in lanes 1 and 4 were from animals treated with saline, whereas samples in lanes 2, 3, 5, and 6 were prepared 8 h after treatment with 20 μg of LPS plus 700 mg of D-GalN × kg^{-1} of body weight. The same blot was sequentially probed with all antibodies after stripping, and exposure times were adjusted to the intensity of the signals.

AAF-fed rats (data not shown). We also treated one pair of rats with 30 μg of LPS plus 700 mg of D-GalN × kg^{-1} of body weight, a dose that caused diffuse hepatonecrosis in the control (Fig. 4*Ac*) but not in the AAF-fed animal (Fig. 4*Af*). The graph of Fig. 4*B* documents the protection obtained in AAF-fed rats challenged with 20 μg of LPS plus 700 mg of D-GalN × kg^{-1} of body weight. We measured the changes of TNF-α after the addition of the same amounts of LPS plus D-GalN in five AAF-fed and five control-fed rats. The peak TNF-α serum levels displayed the following changes (fold increase over the basal level): 9.1 ± 4.8 for the group of control-fed animals; and 9.1 ± 2.9 for the AAF-fed animals. The results obtained with TUNEL staining were consistent with serum alanine and aspartate aminotransferase levels measured 8 h after administration of LPS plus D-GalN (Fig. 4 *C* and *D*). Apical caspases were activated in both control and AAF-fed rats, as shown by cleavage of BID to truncated BID (tBID), whereas caspase 3 was cleaved only in control-fed animals (Fig. 4*E*). Note that a small amount of tBID could be detected in AAF-fed animals even in the absence of LPS plus D-GalN. Thus, resistance to LPS cytotoxicity is downstream of BID cleavage.

To investigate whether discontinuation of the AAF diet would reverse the inhibition of the permeability transition, a group of four animals was fed a diet containing 0.04% AAF for 4 weeks, followed by control diet for a further 12 weeks. Measurements of PTP opening at this time indicated that inhibition of the permeability transition had been relieved by 80% in protocols identical to those described in Fig. 1*D*, and the animals regained sensitivity to hepatotoxicity after injection of LPS plus D-GalN (data not shown).

Discussion

An involvement of the mitochondrial permeability transition in the regulation of cell death has been demonstrated in several *in vitro* systems (39), but remains to be established *in vivo*, where evidence is limited to animal models of stroke (40, 41). In the present study we addressed the question of whether an increased resistance to PTP opening plays a role in carcinogenesis in a rat liver model.

We confirmed that mitochondria isolated from livers of AAF-fed rats are strikingly resistant to Ca^{2+}-dependent PTP opening well before the clonal expansion of focal lesions (28). Adaptation appears to be specific for the PTP, because the response of liver mitochondria from AAF-adapted rats is indistinguishable from that of control mitochondria when valinomycin-dependent K^+ uptake rather than PTP opening is used to induce swelling. These findings demonstrate that inhibition of swelling does not depend on mitochondrial structural constraints or on differences in formation of the proton electrochemical gradient, issues that had not been addressed in previous studies.

Increased resistance to PTP opening was matched, and possibly caused, by an increased mitochondrial expression of BCL-2. This is at apparent variance with a previous study on mitochondria from transgenic mice overexpressing BCL-2 in the liver, where no difference in PTP opening could be detected despite *in vivo* resistance to hepatotoxicity by anti-Fas antibodies (42). We note that the increased PTP resistance to Ca^{2+} is not an all-or-nothing event (Fig. 1) that can therefore be missed in protocols based on a single, large Ca^{2+} load (42), and that our data are in keeping with results obtained in neural cell lines where BCL-2 overexpression promoted an increased mitochondrial Ca^{2+} retention capacity (43).

PTP adaptation can also be detected in primary cultures of hepatocytes challenged with arachidonic acid, a potent inducer of cell death through the PTP (21). We assume that resistance to pore opening is an adaptation to reactive species generated by AAF metabolism in the liver. These species include 2-nitrosofluorene, which drains electrons from the respiratory chain at the level of the bc_1 complex, undergoes redox cycling with production of superoxide anion, and is a potent inducer of PTP opening (27, 28, 44). We think that up-regulation of BCL-2 may be a response to the increased oxidative stress (45), which would cause inhibition of the PTP and result in a decreased propensity to the release of cytochrome *c* and other apoptogenic factors (8).

It is remarkable that the opposite phenotype, i.e., an increased sensitivity to PTP opening *ex vivo* and increased liver apoptoses were observed in the $Sod2^{+/-}$ mice, which have a decreased ability to cope with oxidative stress (46). Our demonstration that AAF feeding induces an early resistance to the otherwise hepatotoxic treatment with LPS plus D-GalN clearly indicates that an anti-apoptotic response is initiated by AAF *in vivo*. Feeding with AAF did not affect the TNF-α response to LPS plus D-GalN and the formation of tBID, which depends on activation of apical caspases, but it did completely prevent the activation of caspase 3. These data indicate that in this model of hepatotoxicity cell death strictly depends on the mitochondrial pathway, and that the latter is selectively suppressed by AAF feeding.

The ability to evade apoptosis is an essential hallmark of both tumorigenesis and drug resistance (23), and the early appearance of hepatocytes resistant to apoptosis during AAF carcinogenesis may substantially promote the accumulation of mutations. The inhibition of cell demise would in turn interfere with the DNA damage response pathways, leading to the accumulation of unrepaired DNA lesions, genomic instability, and malignant progression (47, 48). In other words, the selection of resistant cells would represent a tumor-promoting property of AAF, and our data indicate that resistance is established through an epigenetic rather than a mutagenic process. First, resistance was observed before the clonal expansion of preneoplastic cells (30), suggesting that cells acquire resistance to cell death stimuli before the selection of critical mutations in genes of growth-regulating proteins, and before their transformation into autonomously growing cancer cells (49). Second, increased resistance was fully reversible after returning to the control diet, a feature that is characteristic of epigenetic processes, whereas a phenotype generated by mutations is presumed to be irreversible (50, 51).

In summary, our results support a sequence of events in which the chronic challenge to the liver by AAF metabolites triggers epigenetic responses that include mitochondrial PTP adaptation. The benefit of resistance to AAF toxicity would be outweighed by the persistence of mutated cells, eventually resulting in the formation of tumors. It will be interesting to identify the relevant changes by comparing the protein expression profiles of liver mitochondria from control and AAF-fed rats.

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