First publ. in : Biochemical and Biophysical Research Communications ; 263 (1999),2. - S. 433-438

Prevention of Endotoxin-Induced Lethality, but Not of Liver Apoptosis in Poly(ADP-ribose) Polymerase-Deficient Mice

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Activation of poly-(ADP-ribose) polymerase (PARP) is often associated with cytotoxicity, but its precise role in shock-induced lethality and in different modes of tissue injury is still unknown. We took advantage of the existence of mice with a targeted deletion of the PARP gene (PARP−/−) to examine the differential sensitivity of wild-type (wt) and PARP−/− mice toward endotoxin (LPS)-induced lethality and different forms of liver damage. All PARP−/− animals survived high-dose (20 mg/kg) LPS-mediated shock, which killed 60% of wt animals. Moreover, LPS-induced necrotic liver damage was significantly reduced. In contrast, when apoptotic liver damage was induced via injection of low concentrations of LPS (30 μg/kg) into D-galactosamine-sensitized mice, or via activation of hepatic cell death receptors, PARP−/− animals were not protected. We conclude that PARP is involved in systemic LPS toxicity, while it plays a minor role in apoptotic liver damage mediated by TNF or CD95.

Poly-(ADP-ribose) polymerase (PARP) is involved in posttranslational modification of a large variety of proteins. PARP activity increases >100-fold upon DNA-damage and can lead to depletion of intracellular NAD and ATP stores (rev. in 1, 2). Pharmacological inhibition of the enzymatic activity of PARP can prevent such a suicidal (3) depletion (4) as well as necrotic cell death triggered e.g. by H_2O_2 (5). A great advance in the definition of PARP's causal role in pathology has been the development of mice with a deletion in the PARP gene (PARP−/−) (6, 7). Such animals are protected from neuronal necrosis following transient cerebral ischemia (8, 9), from intoxication with the Parkinson's disease-inducing neurotoxin MPTP (10), or from streptozotocin-induced diabetes (11, 12). In addition, PARP has been suggested to be involved in various models of ischemia-reperfusion damage, and in tissue injury and vascular hyporeactivity due to generalized inflammatory syndromes and shock (13). In apoptosis, PARP is frequently cleaved proteolytically and inactivated at early stages (14). This inactivation may be required for the proper execution of apoptosis, since the PARP-mediated ATP depletion may prevent caspase activation, and convert apoptosis to necrosis (15, 16). Accordingly, it has been suggested that inhibition of proteolytic PARP inactivation may increase the rate of necrosis and overall cell death triggered by apoptotic stimuli (17). All this suggests that inhibition of PARP may have a promising therapeutic potential for prevention of tissue injury (13, 18), and possibly lethality.

However, it has also been shown that PARP-deficient mice are strikingly more sensitive to DNA-damaging agents than corresponding wt animals (7), and that stabilization of PARP during apoptosis slows down cell death instead of accelerating it (19). In most models of apoptosis, neither the presence or the absence of PARP, nor its inhibition seem to be important (16, 19, 20, 21). Moreover, PARP−/− mice breed and grow normally (6, 7). This suggests that the essential apoptotic processes in development are not severely affected by the absence of PARP. It may therefore be speculated that PARP activation is causally involved only in specific types of cell loss, and that PARP inhibition would affect predominantly the non-apoptotic fraction of dying cells. Initial evidence for that has been provided in a cerebral stroke model (9), but confirmation of this hypothesis still requires further investigation.

Different well-characterized models of cytokine-induced damage in the liver (22) offer an ideal opportunity to further explore the role of PARP in massive apoptotic organ damage. In addition, endotoxic shock is

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a well characterized model for testing the role of PARP in lethality due to a generalized systemic inflammatory response. High doses of endotoxin/lipopolysaccharide (LPS) cause necrotic liver damage (23) and systemic toxicity associated with a high rate of lethality (24) in mice. On the other hand, apoptotic hepatic damage is triggered by low doses of LPS or tumor necrosis factor (TNF) in D-galactosamine (GalN) sensitized mice or by CD95 stimulation in non-sensitized mice (25, 26, 27). By measuring biochemical endpoints representative for overall cell lysis (transaminase release), apoptosis (caspase-activation and DNA-fragmentation), and the inflammatory response (TNF-production, NO synthesis and survival) in these different models, we compared wt and PARP−/− mice. The results indicate that PARP is not important for cytokine-induced liver failure when the initiating mechanism is predominantly apoptotic.

MATERIALS AND METHODS

Materials. LPS from *Salmonella abortus equi* was purchased from Metalon (Aidenbach, Germany). Recombinant TNF-α (TNF) was generously provided by Dr. G. R. Adolf, Bender & Co. (Vienna, Austria). D-galactosamine was obtained from Roth Chemicals (Karlsruhe, Germany). Agonistic anti-CD95 antibody (Jo-2) was purchased from Pharmingen (Hamburg, Germany). The DNA fragmentation ELISA was obtained from Boehringer Mannheim (Mannheim, Germany). Ultrafree-MC microcentrifuge filter units were obtained from Millipore (Eschborn, Germany), Columbia blood agar plates from Haipha (Heidelberg, Germany). Unless further specified, all other reagents were purchased from Sigma (Deisenhofen, Germany).

Animals. PARP−/− mice were generated (6) and provided by Dr. Z.-Q. Wang (IARC, Lyon, France). These mice were crossed with C57BL/6 (wt) mice, and the resulting heterozygous litters were used for two cycles of further inbred breeding in the animal breeding station of the University of Konstanz. Offspring from heterozygous matings were genotyped by PCR and used for experiments. PARP expression in livers of wt mice was confirmed by Western blot using the antibody clone C2-10 (Pharmingen) as described earlier (28). Animals were housed at a constant day/night cycle of 12 h at 22°C and 55% humidity. For the GalN/TNFα and GalN/LPS experiments, mice (30 g) were starved overnight prior to the *in vivo* experiments. Experiments were generally started at 8 a.m. All animals received human care in adherence to the guidelines of the EU (European council directive 86/609/EEC) and the national German authorities, and followed the directives of the University of Konstanz ethical committee.

In vivo experiments. For shock experiments, LPS (20 mg/kg) was injected intraperitoneally (i.p.) in a volume of 300 μl. CD95 (4 μg/animal) was given intravenously (i.v.) in a volume of 300 μl saline (containing 0.1% human serum albumin). The sensitizing agent GalN (700 mg/kg) (25) was injected intraperitoneally in 300 μl saline 15 min prior to the injection of TNFα (10 μg/kg; i.v.) or of LPS (30 μg/kg; i.p.). Blood and tissue samples were obtained after lethal anesthesia of mice with 150 mg/kg pentobarbital plus 0.8 mg/kg heparin (i.v.). After midline laparatomy and opening of the chest, blood was withdrawn by cardiac puncture and immediately centrifuged for 2 min at 4°C at 13,000 $\times$ g to separate the plasma from the cellular fraction for determination of transaminase activities and nitrate/nitrite content.

After flushing with perfusion buffer (PB) (50 mM phosphate, 120 mM NaCl, 10 mM EDTA, pH 7.4), pieces of liver, caecum, colon and jejunum was frozen in liquid nitrogen, and stored at −80°C for the determination of caspase-3-like activity. Remaining parts of the liver were disintegrated in cold PB with three strokes of an Elvehjem type homogenizer. The 20% homogenate was centrifuged at 13,000 $\times$ g for 20 min, and the supernatant was used for quantitation of cytosolic oligonucleosomal DNA fragments (29).

Caspase-3-like protease activity. Activity of caspase-3-like proteases was assayed by following the cleavage of the fluorogenic substrate DEVD-afc (afc = 7-amino-4-trifluoromethylcoumarin). Cytosolic extracts derived from tissue samples were prepared by Dounce-homogenization of approximately 100 mg frozen sample in hypotonic extraction buffer (25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM PEFA-block and pepstatin, leupeptin and aprotinin 1 μg/ml each) and subsequently centrifuged for 15 minutes at 13,000 $\times$ g (4°C). Generation of free afc was followed in assay buffer (50 mM HEPES, pH 7.4, 1% sucrose, 0.1% CHAPS, 10 mM DTT, 50 μM fluorogenic substrate DEVDafc, Biomol, Hamburg, Germany) for 30 min at 37°C using a Victor-2 1420 Multilabel fluorimeter/counter (Wallac Instruments, Turku, Finland) set at an excitation wavelength of 385 nm and an emission wavelength of 505 nm. Protein concentrations of the corresponding samples were quantitated using the bicinchoininic acid method (Pierce, Rockford, IL). Specific caspase-3-like protease activity was calculated in pmol free afc per min and mg protein using afc standards. Control experiments confirmed that the activity was linear with time and with protein concentration under the conditions described above (20).

Cytotoxicity and DNA fragmentation Hepatocytolysis *in vivo* was determined by measuring the plasma activities of alanine aminotransferase (ALT) and aspartate-aminotransferase (AST) using an enzymatic analyzer (Eppendorf ACP 5040) (30). Data are expressed in U/l plasma. DNA fragmentation in murine livers was quantitated by measuring cytosolic oligonucleosome-bound DNA using an ELISA-kit (Boehringer, Mannheim, Germany) as described (29, 31).

Determination of TNF and nitrite/nitrate in plasma. Blood samples were withdrawn from the tail vein 90 min after LPS administration into heparinized microfuge tubes. For determination of TNF protein content, plasma was prepared and immediately frozen at −20°C until analysis by ELISA as described (32).

Nitrite and nitrate in plasma were measured essentially according to the method of Misko *et al.* (33). Plasma was filtered through an Ultrafree-MC microcentrifuge filter unit (Millipore, Eschborn, Germany) for 1 h at 21,000 $\times$ g in order to remove hemoglobin released by cell lysis, which might interfere with the colorimetric assay. Nitrate in serum was first reduced to nitrite by incubation with nitrate reductase from *Aspergillus* species. Nitrate reductase (14 mU in 20 mM Tris/HCl, pH 7.6) was added to 10 μl of filtrate and the reaction was started by addition of NADPH in Tris/HCl buffer to a final concentration of 100 μM and a final volume of 50 μl. After 5 min incubation at room temperature the reaction was terminated by dilution with distilled water. The whole reaction volume was transferred to 96-well microtiter plates, 10 μl sulfanilamide (1% in 1.2 M HCl) and 10 μl N-(1-naphthyl)ethylendiamine (0.1% in H_2O) were added and absorbance was read after 3 min incubation time at 560 vs. 690 nm on an ELISA reader.

Determination of aerobic colony-forming units (CFU) in blood. 100 μl blood were spread on Columbia blood agar plates (Haipha, Biotest, Heidelberg, Germany). Aerobic CFU were counted after overnight incubation at 37°C.

Statistical analysis. Statistical differences were determined by an unpaired t-test if applicable, or data were analyzed by two-way analysis of variance (ANOVA). In the case of inhomogenous variances, data were transformed before subjection to further analysis. $P < 0.05$ was considered significant.

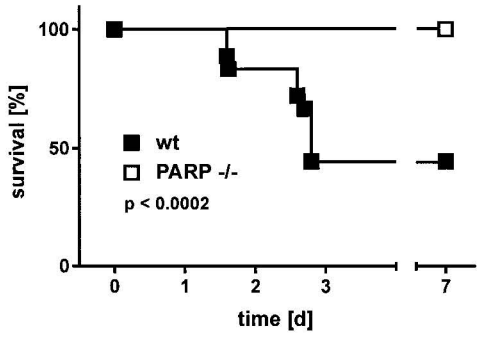

FIG. 1. Prevention of endotoxic shock lethality in PARP−/− mice. Twenty-four mice of each genotype were intraperitoneally injected with 20 mg/kg LPS, and survival was followed over 7 days. TNF concentrations in blood were measured in these animals 90 min after LPS injection. Statistical differences were evaluated by Log rank-test: $p < 0.0002$.

RESULTS

Protection of PARP−/− Mice from Endotoxic Shock

In an initial experiment, mice were injected with a high dose of LPS (20 mg/kg) that was lethal for >50% of wt animals within 72 h. Under these experimental conditions, PARP−/− mice were completely protected against endotoxin (Fig. 1). The systemic toxicity of LPS is not direct, but is rather conveyed by the production of endogenous mediators, the most relevant being TNF (34). To find out whether the absence of PARP may have an effect on TNF-production we measured serum concentrations of this mediator after LPS injection. High concentrations were detected both in wt (1930 ± 395 pg/ml) and in PARP−/− (1950 ± 330 pg/ml) mice, i.e. PARP deletion did not affect the production and release of this pivotal effector of the LPS response. Another pathogenic mediator of circulatory failure in shock is nitric oxide (NO). When we examined the blood nitrite/nitrate content as a measure of excessive NO-formation, we found a high release in both genotypes. Some modulation of NO production by PARP is suggested by the finding that nitrite/nitrate levels were significantly higher in wt mice than in PARP−/− mice. However, strong induction of NOS had occurred in the presence and absence of PARP (Table 1).

Differential Effects on Liver Injury in High-Dose and Low-Dose Endotoxin Models

In order to examine the endotoxin effects on the liver, we performed another high-dose LPS shock experiment. The initially observed protection from lethality by PARP deletion, and the lack of modulation of the TNF response were essentially confirmed (Table 1). In addition, examination of hepatic damage by quantitation of the release of liver transaminases also showed a partial but significant protection of PARP−/− mice. The liver damage in non-sensitized mice exposed to high doses of LPS has been shown to be strictly necrotic according to biochemical and morphological parameters (23). This was further confirmed by the lack of caspase activation detected in such livers (Table 1).

In contrast, liver damage triggered by low LPS doses in GalN-sensitized mice is predominantly apoptotic (23, 25). Since both the apoptotic and the necrotic damage are dependent on TNF we examined whether PARP deletion would also protect mice from apoptotic liver damage. When we injected mice with GalN *plus* low-dose LPS (30 μg/kg), we found an equally strong caspase induction in both groups (Fig. 2A). In addition, DNA-fragmentation (Fig. 2A), the release of transaminases (Fig. 2B) and the production of TNF (PARP−/−: 1490 ± 365 pg/ml; wt: 1195 ± 330 pg/ml) were similar in mice of both genotypes. Thus, PARP deletion seemed to reduce necrotic, but not apoptotic liver damage triggered by LPS.

Lack of Protection from TNF- or CD95-Mediated Hepatic Apoptosis

We further investigated the role of PARP in liver damage in models where toxicity was directly triggered via TNF or CD95 stimulation. These two different pathways (26) have been implicated in various forms of liver damage (22). First, we injected TNF into GalN-sensitized mice. Caspase activities were induced in the liver, and transaminases in the plasma rose drastically. However, no differences were observed in mice with or without functional PARP genes (Table 2). In order to exclude any possible obscuring effects of GalN

TABLE 1

Endotoxic Shock in wt and PARP−/− Mice

Endpoint [means ± SD; n = 10]	Genotype		wt *vs* PARP−/−
	wt	PARP−/−	
Survival [%]	40	100	$p < 0.01$
Nitrite/nitrate [μM]	230 ± 13	150 ± 10	$p < 0.01$
ALT [U/l]	570 ± 25	340 ± 15	$p < 0.01$
AST [U/l]	670 ± 45	495 ± 32	$p < 0.01$
TNF [pg/ml]	1760 ± 1290	1690 ± 1350	n.s.
Caspases (liver)	2 ± 1	2 ± 1	n.s.
Caspases (caecum)	45 ± 13	40 ± 7	n.s.
Caspases (colon)	175 ± 46	210 ± 65	n.s.
Caspases (jejunum)	410 ± 76	305 ± 45	n.s.

Note. Twenty mice of each genotype were intraperitoneally injected with 20 mg/kg LPS. In 10 animals per group, survival was followed over 72 h. TNF concentrations in blood were measured in these animals 90 min after LPS injection. The remaining animals were sacrificed after 24 h for determination of serum transaminase activities, blood nitrite/nitrate levels and tissue caspase activities (in pmol/mg protein/min afc-formation from DEVD-afc). In control animals nitrite/nitrate levels were 30 ± 2 μM, transaminase activities were <50 U/ml, TNF was <50 pg/ml and all caspase activities were <3 pmol/min/mg protein. Statistical differences were evaluated by Student's *t* test. n.s.: not significant.

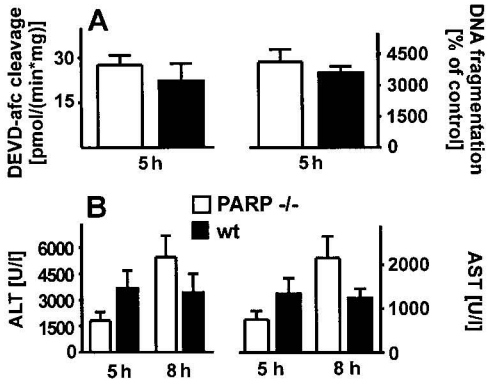

FIG. 2. Low-dose endotoxin-induced liver damage in GalN-sensitized wt and PARP−/− mice. Mice (n = 16 for each genotype) were intraperitoneally injected with 700 mg/kg GalN, and 15 min later with 30 μg/kg LPS. One group (n = 8) of animals was sacrificed after 5 h, and samples were prepared for measurement of hepatic DNA fragments, of serum transaminase activities (ALT and AST) and of liver caspase activities (measured by the cleavage of DEVD-afc). After 8 h transaminase activities were determined in another group (n = 8). (A) Data from the two genotypes were not significantly different according to statistical evaluation by Student's *t*-test. (B) Transaminase data were analyzed by 2-way ANOVA according to effects of time and genotype. Genotype differences were nonsignificant ($p > 0.05$).

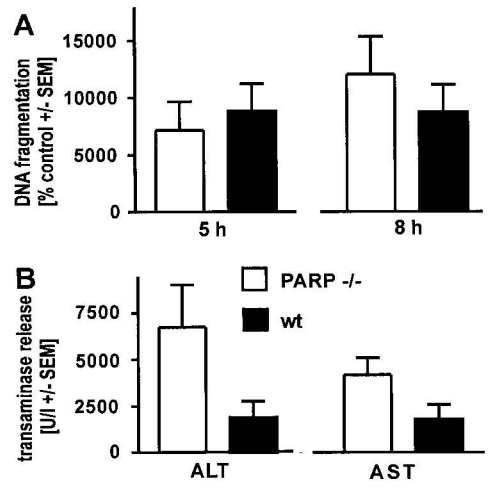

FIG. 3. CD95-induced hepatic apoptosis in PARP−/− and wt mice. Mice (n = 10 for each genotype) were intravenously injected with 130 μg/kg anti-CD95. (A) Animals were sacrificed after 5 h (n = 5) or 8 h (n = 5) for measurement of hepatic oligonucleosomal DNA-fragments, or (B) after 8 h (n = 5) for determination of transaminase activities (ALT and AST). DNA-fragmentation data from the two genotypes were not significantly different according to statistical evaluation by ANOVA. Transaminase data were analyzed by Student's *t*-test. Genotype differences were non-significant ($p > 0.05$).

on potential protective effects of PARP-deletion, we then challenged non-sensitized mice with agonistic antibodies against CD95 (also known as fas or Apo-1), and compared the toxicity in both genotypes. A similarly strong increase in DNA-fragmentation was observed 5 h or 8 h after the injection (Fig. 3A) in the two groups of animals. Moreover, strongly increased caspase activities were detected after 5 h (wt: 20 ± 13; PARP−/−: 31 ± 20 [in pmol/min/mg]; $p > 0.05$) and 8 h (wt: 17 ± 7; PARP−/−: 32 ± 16; $p < 0.05$). The values were even higher in PARP−/− animals than in wt mice. This trend was confirmed by measurements of transaminase activities: the values were again consistently higher for PARP−/− than for wt mice. The enzyme release indicates severe liver damage in both genotypes (Fig. 3B). However, statistical significance was not reached. These data confirm conclusively that PARP does not play a role in mediating apoptotic liver damage, triggered by any of these three different stimuli.

TABLE 2

TNF-Induced Gastrointestinal Effects in GalN-Sensitized wt and PARP−/− Mice

Endpoint [means ± SD]	Genotype	
	wt	PARP−/−
ALT [U/l]	2970 ± 260	3250 ± 350
AST [U/l]	4780 ± 390	5540 ± 260
Caspases (liver)	23 ± 5	26 ± 9
Caspases (jejunum)	395 ± 330	345 ± 180

Note. Mice (n = 7 for PARP−/−; n = 11 for wt) were intraperitoneally injected with 700 mg/kg GalN, and 15 min later intravenously with 10 μg/kg TNF. Animals were sacrificed after 6 h for determination of serum transaminase and tissue caspase activities (in pmol/mg protein/min afc-formation from DEVD-afc). Data from the two genotypes were not significantly different according to statistical evaluation by Student's *t* test.

Inflammation-Induced Caspase Induction in the Intestine Occurs in the Absence of PARP

Another target organ of LPS is the intestine, where it causes the translocation of bacteria or endotoxins (35), and eventually bowel necrosis (36). We also observed that LPS shock induced marked DNA-fragmentation in the intestine (23). Therefore we examined here whether an altered apoptotic response in the intestine may contribute to the reduced LPS lethality in PARP−/− mice. We examined the induction of caspases as a convenient and quantifiable biochemical parameter for possible induction of apoptosis (37) due to inflammatory effects on the intestine. Endotoxic shock triggered a very pronounced increase of caspase activity in the gut (Table 1). This shows that this organ is affected by the systemic inflammatory response triggered by LPS, although its significance and the degree of additional necrotic damage or edema formation will require further investigation. Regarding induction of caspase activities, there was no difference between PARP−/− and wt mice. The same was true for bacte-

rial translocation into the blood, which resulted in the growth of an average of 5 colonies/100 μl in both genotypes. Interestingly, intestinal caspases were not only activated in the high-dose LPS model, but also by TNF (Table 2) or anti-CD95 injection (not shown). In all cases, caspases were activated to a similar degree in PARP-expressing and PARP-deficient mice.

DISCUSSION

We showed here that PARP is involved in endotoxin-induced and TNF-mediated lethality in mice, but not in the massive apoptotic liver damage triggered by TNF or CD95. In addition, we found that necrotic liver damage in endotoxic shock was reduced in PARP−/− mice. These data are well in agreement with other studies, showing a reduction of vascular hyporeactivity and of neutrophil-mediated injury by PARP inhibitors in various animal models of shock and systemic inflammation (rev. in 13). We extended these findings by showing that PARP ultimately affects the survival of the animals, and that inhibition of its enzymatic activity may in fact be life-saving. Moreover, the approach of using the genetically-targeted mice clearly defined the classical PARP enzyme as responsible for the effects. Recently, other enzymes with poly-(ADP-ribosylation) capacity (38, 39) have been described, but in LPS shock PARP itself appeared to play the pivotal pathological role.

PARP apparently did not affect liver injury triggered by apoptotic stimuli. Notably, CD95 and TNF-mediated hepatic failure cannot be classified as "silent mode of death," an epithet frequently attributed to apoptosis (40). On the contrary, here an initial phase of pure apoptosis with hardly any membrane lysis is followed by a phase of massive hemorrhage and cell lysis, associated with several typically necrotic phenomena (22, 25, 27). Unspecific tissue damage is particularly evident from the high AST/ALT activities and ratio produced by the very high TNF-dose used in the present study. Despite this extremely harsh damage at the borderline of apoptosis and necrosis, no involvement of PARP was detected. This is in line with previous findings that ischemia reperfusion injury, which in liver involves initial apoptotic processes and subsequent massive tissue damage (41), is also not dependent on PARP enzymatic activity (42). Thus, massive tissue damage can occur in the liver independent of PARP.

The dependence of cell death on PARP activation as a "suicide mechanism" (3), seems to have very specific requirements. Particularly compelling evidence for the role of PARP comes from cell death models with NO/$ONOO^-$ as essential mediator, such as cerebral ischemia, streptozotocin diabetes or MPTP intoxication (rev. in 18). In TNF-mediated liver injury, NO is rather a protective than a detrimental mediator (43). This may explain the lack of any PARP effect, even under conditions of wide-spread tissue necrosis. A second line of tissue protection due to PARP inhibition/deletion seems to involve the suppression of certain inflammatory responses. For instance, there is increasing evidence that PARP inhibition can reduce neutrophil infiltration. The underlying mechanisms still await elucidation, but it is possible that PARP regulates stress-induced transcription factors and the upregulation of adhesion molecules (13). Notably, it has been reported that PARP inhibition reduced $ONOO^-$ production in reperfused myocardium (44), and we have found here additional evidence for reduced NO production, since plasma nitrite/nitrate levels in endotoxic shock were reduced in PARP−/− mice. Although this reduction alone does not appear to be sufficient to explain the reduced lethality, a vicious circle of NO-mediated PARP induction and PARP mediated NO production may be envisaged.

In summary, we have shown here that PARP inhibition may prevent lethality and reduce necrotic hepatic damage in shock. The lack of inhibition of apoptotic tissue injury that we observed here opens interesting perspectives on the use of PARP inhibitors: on the one hand, PARP-inhibition may be synergistic in certain pathological settings with interventions that are more selective for apoptotic death. For instance, TNF or CD95 mediated liver apoptosis are efficiently prevented by the caspase-inhibitor Z-VAD-fmk (45), while caspase inhibition has no effect in endotoxic shock (46). A combination of caspase inhibitors and PARP inhibitors may be very beneficial in any type of mixed hepatic injury. On the other hand, it may frequently not be desirable to generally block apoptotic processes, even in disease settings. Apoptosis is often a physiologically required process, and damaged cells protected by caspase inhibitors may eventually die by necrosis and cause inflammation (15, 47). Where feasible, prevention of tissue injury by PARP inhibition appears to be more promising. Blockage of this enzyme appears to affect selectively toxic and pathologic degeneration events, while leaving apoptotic processes undisturbed.

ACKNOWLEDGMENTS

We thank H. Naumann for skilful technical assistance, Dr. Z.-Q. Wang for initially providing PARP−/− mice, and Dr. E. Ferrando-May for help with the genotyping of the mice. This study was supported by grants from the University of Konstanz AFF, the Deutsche Forschungsgemeinschaft (DFG) and the EEC Grants ENV4-CT96-0169 and 12029-97-06 F1ED ISP D.

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