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Effects of inhibitors of the activity of poly (ADP-ribose) synthetase on the organ injury and dysfunction caused by haemorrhagic shock

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1 Poly (ADP-ribose) synthetase (PARS) is a nuclear enzyme activated by strand breaks in DNA, which are caused by reactive oxygen species (ROS). Here we investigate the effects of the PARS inhibitors 3-aminobenzamide (3-AB), nicotinamide and 1,5-dihydroxyisoquinoline (ISO) on the circulatory failure and the organ injury/dysfunction caused by haemorrhage and resuscitation in the anaesthetized rat.

2 Haemorrhage (sufficient to lower mean arterial blood pressure to 50 mmHg for 90 min) and subsequent resuscitation with shed blood resulted (within 4 h after resuscitation) in a delayed fall in blood pressure to 66 ± 4 mmHg (control, $n=13$). This circulatory failure was not affected by administration (5 min prior to resuscitation) of 3-AB (10 mg kg^{-1} i.v., $n=7$), nicotinamide (10 mg kg^{-1} i.v., $n=6$) or ISO (3 mg kg^{-1} i.v., $n=6$).

3 Haemorrhage and resuscitation also resulted in rises in the serum levels of urea and creatinine. This renal dysfunction was attenuated by 3-AB and nicotinamide, but not by nicotinic acid ($n=7$), an inactive analogue of nicotinamide. Although ISO ($n=6$) also attenuated the renal dysfunction caused by haemorrhage and resuscitation, its vehicle (10% DMSO, $n=4$) had the same effect.

4 Haemorrhagic shock resulted in enhanced serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lipase, indicating the development of hepatocellular and pancreatic injury, respectively. Similarly, haemorrhagic shock also resulted in an increase in the serum levels of creatine kinase (CK) indicating the development of neuromuscular injury. This was attenuated by 3-AB and nicotinamide, but not by nicotinic acid. Although ISO also attenuated the liver, pancreatic and neuromuscular injury caused by haemorrhagic shock, its vehicle had the same effect.

5 Thus, activation of PARS contributes to the organ injury and dysfunction caused by haemorrhage and resuscitation in the rat.

Keywords: Haemorrhagic shock; oxygen radicals; poly (ADP-ribose) polymerase; multiple organ failure; trauma

Abbreviations: 3-AB, 3-aminobenzamide; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATP, adenosine triphosphate; HR, heart rate; ISO, 1,5-dihydroxyisoquinoline; MAP, mean arterial blood pressure; NAD, nicotinamide adenine dinucleotide; NO, nitric oxide; PARS, poly (ADP-ribose) synthetase; ROS, reactive oxygen species

Introduction

Poly (adenosine 5′-diphosphate ribose) synthetase (PARS, E.C. 2.4.2.30) is a chromatin-bound enzyme which is abundantly present in the nuclei of numerous cell types (Ikai & Ueda, 1983). Activation of PARS is triggered by single strand breaks in DNA and subsequently catalyzes the transfer of ADP-ribose moeities from NAD to various nuclear proteins including histones and PARS (automodification domain) itself (Ueda & Hayaishi, 1985; Lautier *et al*., 1993). This reaction leads to the generation of nicotinamide, which is an inhibitor (negative feedback) of PARS activity. Continuous or excessive activation of PARS produces extended chains of ADP-ribose on nuclear proteins and results in a substantial depletion of intracellular NAD. As NAD functions as an electron carrier in the mitochondrial respiratory chain, NAD depletion rapidly leads to a fall in intracellular ATP levels. Moreover, nicotinamide can be recycled to NAD in a reaction that consumes ATP. Thus, activation of PARS leads to a fall in ATP (by two different mechanisms) which can ultimately cause cell death (Berger, 1985; Schraufstatter *et al*., 1986a, b; Hyslop *et al*., 1988; Thies & Autor, 1991). Radicals including superoxide anions, hydrogen peroxide or hydroxyl radicals cause strand breaks in DNA, activation of PARS and depletion of NAD and ATP in cultured cells (Schraufstatter *et al*., 1986a, b; Hyslop *et al*., 1988; Thies & Autor, 1991). Although nitric oxide (NO) has been shown to generate strand breaks in DNA (Zhang *et al*., 1994; Heller *et al*., 1995), this effect appears to be mediated by peroxynitrite rather than NO itself (Szabo *et al*., 1996). Inhibitors of PARS activity attenuate the fall in NAD and ATP and improve survival of cultured cells (e.g. fibroblasts, endothelial cells, smooth muscle cells, human cardiomyoblasts; human proximal tubule cells) exposed to oxygen-derived free radicals (Schraufstatter *et al*., 1986a, b; Hyslop *et al*., 1988; Thies & Autor, 1991; Aalto & Raivo, 1993; Bowes *et al*., 1998; Chatterjee *et al*., 1999) or NO/peroxynitrite (Zhang *et al*., 1994; Heller *et al*., 1995; Szabo *et al*., 1996).

There is good evidence that haemorrhagic shock is associated with the generation of reactive oxygen species (ROS) including superoxide anions and hydrogen peroxide (see McCord, 1985; Redl *et al*., 1993). An enhanced formation of peroxynitrite may contribute to the circulatory dysfunction in rats with haemorrhagic shock (Szabo *et al*., 1995). The overproduction of ROS in haemorrhagic shock leads to a

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considerable oxidant stress as indicated by lipid peroxidation (Fleckenstein *et al*., 1991; Hamano *et al*., 1993) as well as the consumption of the endogenous antioxidant, vitamin E (Fleckenstein *et al*., 1991). Interventions which reduce the generation or the effects of ROS exert beneficial effects in a variety of models of haemorrhagic shock (Allan *et al*., 1986; Sanan *et al*., 1989; Mannion *et al*., 1994; Daughters *et al*., 1996; Simon *et al*., 1996; Fan *et al*., 1998; Mota-Filipe *et al*., 1999).

It has been suggested that the activation of PARS plays a role in the cardiovascular failure associated with haemorrhagic shock (Szabo *et al*., 1998; Szabo, 1998), but there is little information regarding the role of PARS in the pathophysiology of the organ dysfunction/injury associated with haemorrhagic shock. Here we investigate the effects of the PARS inhibitors 3-aminobenzamide and nicotinamide on the circulatory failure and the organ injury and dysfunction (renal dysfunction, liver injury and dysfunction, pancreatic injury) caused by severe haemorrhage and resuscitation in the anaesthetized rat.

Methods

Surgical procedure

This study was carried out on 74 male Wistar rats (Tuck, Rayleigh, Essex, U.K.) weighing 250–320 g receiving a standard diet and water *ad libitum*. All animals were anaesthetized with thiopentone sodium (120 mg kg^{-1} i.p.) and anaesthesia was maintained by supplementary injections of thiopentone sodium as required. The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket. The right femoral artery was catheterized and connected to a pressure transducer (Senso-Nor 840, Senso-Nor, Horten, Norway) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate (HR) which were displayed on a data acquisition system (MacLab 8e, ADI Instruments, Hastings, U.K.) installed on an Apple Macintosh computer. The right carotid artery was cannulated to bleed the animals (see below). The jugular vein was cannulated for the administration of drugs. The bladder was also cannulated to facilitate urine flow and to prevent the possibility of the development of post-renal failure. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 15 min. Then, blood was withdrawn from the catheter placed in the carotid artery in order to achieve a fall in MAP to 50 mmHg within 10 min. Thereafter, MAP was maintained at 50 mmHg for a total period of 90 min by either withdrawal (during the compensation period) or re-injection of blood. At 90 min after initiation of haemorrhage, the shed blood was re-injected into the animal along with an equivalent volume of Ringers lactate solution.

Evaluation of the effects of various PARS inhibitors on the delayed circulatory failure and MODS: experimental design

In the first study aimed at elucidating the effects of inhibitors of PARS activity in haemorrhagic shock, all animals were randomized into nine groups (see Table 1). Different groups of animals were subjected to 90 min of haemorrhage followed by resuscitation with shed blood and an equivalent volume of Ringer's Lactate solution for 4 h and treated with either saline (vehicle for 3-AB and nicotinamide), 10% DMSO (vehicle for ISO), 3-AB, nicotinamide, ISO (chemically distinct inhibitors of PARS activity) or nicotinic acid (negative control for nicotinamide). In addition, we have evaluated the effects of saline (vehicle), 3-AB and nicotinamide in rats subjected to the same surgical procedure, but which were not subjected to haemorrhagic shock (sham-operated rats) (see Table 1).

Table 1 Experimental design

Group	*Protocol*	*Treatment*	*Dose*	n
1	Sham	Vehicle (saline)		6
2	Sham	3-Aminobenzamide	10 mg kg^{-1}	5
3	Sham	Nicotinamide	10 mg kg^{-1}	5
4	HS	Vehicle (saline)		13
5	HS	3-Aminobenzamide	3 mg kg^{-1}	8
6	HS	3-Aminobenzamide	10 mg kg^{-1}	7
7	HS	Nicotinamide	10 mg kg^{-1}	6
8	HS	Nicotinic Acid	10 mg kg^{-1}	7
9	HS	1,5-Dihydroxyisoquinoline	3 mg kg^{-1}	6
10	HS	Vehicle (DMSO)	10%	4

Animals were subjected to the surgical procedure alone (sham) or to haemorrhagic shock (HS).

Quantification of organ function and injury

Four hours after resuscitation (end of the experiment), 1.5 ml of blood was collected into a serum gel S/1.3 tube (Sarstedt, Germany) from the catheter placed in the right carotid artery. The blood sample was centrifuged ($1610 \times g$ for 3 min at room temperature) to separate plasma. All plasma samples were analysed within 24 h by a contract laboratory for veterinary clinical chemistry (Vetlab Services, Sussex, U.K.). The following marker enzymes were measured in the plasma as biochemical indicators of multiple organ injury/dysfunction: (1) Liver injury was assessed by measuring the rise in plasma levels of alanine aminotransferase (ALT, a specific marker for hepatic parenchymal injury) and aspartate aminotransferase (AST, a non-specific marker for hepatic injury) (Baue, 1993). (2) Renal dysfunction was assessed by measuring the rises in plasma levels of creatinine (an indicator of reduced glomerular filtration rate, and hence, renal failure) and urea (an indicator of impaired excretory function of the kidney and/or increased catabolism) (see Thiemermann *et al*., 1995). (3) In addition, we have evaluated the rises in the serum levels of lipase, a specific indicator for the development of pancreatic injury. (4) Finally, we determined the increase in the serum levels of creatine kinase, an indicator for the development of muscle (skeletal or cardiac) or brain injury.

In order to ensure that the PARS inhibitor 3-AB does not interfere with the determination of any of the above parameters of organ injury, we have carried out the following experiment. Serum (250 μl) was obtained from seven rats (four subjected to haemorrhagic shock and three subjected to the surgical procedure) and then spiked with either 250 μl of saline or with 250 μl containing 0.24 mg ml^{-1} of 3-AB. Given an estimated blood volume of $\sim$25 ml of blood per rat, we have estimated the maximal concentration of 3-AB in the blood to be 0.12 mg ml^{-1}. The above assay has taken this estimation into account and, hence, the final concentration of 3-AB in the sample (comprising of 250 μl of serum plus 250 μl of saline containing 3-AB) was 0.12 mg ml^{-1}. We have then subjected this sample to analysis by VetLab and determined the following parameters: urea, creatinine, AST, ALT, CK and lipase.

Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, U.K.). Thiopentone sodium (Intraval Sodium®) was obtained from Rhône Mérieux Ltd. (Harlow, Essex, U.K.). All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, U.K.).

Statistical evaluation

All data are presented as mean ± s.e.mean of n observations, where n represents the number of animals or blood samples studied. For repeated measurements (haemodynamics) a 2-factorial analysis of variance (ANOVA) was performed. Data without repeated measurements (multiple organ injury/failure) was analysed by 1-factorial ANOVA, followed by a Dunnett's test for multiple comparisons. A P-value of less than 0.05 was considered to be statistically significant.

Results

Effects of PARS inhibitors on the delayed vascular decompensation (circulatory failure) caused haemorrhage

Baseline values of MAP in all groups of animals ranged from 123 ± 8 to 140 ± 6 mmHg, and were not significantly different between groups (Table 2). In sham-operated rats (no haemorrhage), neither administration of saline nor administration (at 90 min) of any of the PARS inhibitors had any effect on MAP (Table 2). In rats subjected to haemorrhage, resuscitation with shed blood led to an immediate increase in blood pressure from ~50 mmHg to 104 ± 3 mmHg. Thereafter, there was a progressive decline in MAP to approximately 65 mmHg at the end of the experiment (Table 2). None of the PARS inhibitors used attenuated the delayed fall in MAP associated with haemorrhage (Table 2).

Baseline values of heart rate (HR) in all groups of animals ranged from 360 ± 16 to 431 ± 12 beats per minute (b.p.m.), and were not significantly different between groups (Table 2). In sham-operated rats, neither administration of saline nor administration of any of the PARS inhibitors had any effect on MAP (Table 2). Haemorrhagic shock did also not cause a significant alteration in heart rate (Table 2, $P > 0.05$).

Effects of PARS inhibitors on the multiple organ dysfunction syndrome caused by haemorrhage in the rat

Effects on the renal injury/dysfunction In sham-operated rats, administration of saline, 3-AB (10 mg kg^{-1}), nicotinamide, nicotinic acid or ISO did not result in any significant alterations in the plasma levels of urea or creatinine (Table 4). When compared with sham-operated rats, haemorrhage/resuscitation resulted in significant rises in the plasma levels of urea (Figure 1a) and creatinine (Figure 1b), demonstrating the development of renal dysfunction. Treatment of rats subjected to haemorrhage and resuscitation with the PARS inhibitors 3-AB (10 mg kg^{-1}) and nicotinamide attenuated the renal dysfunction caused by haemorrhage and resuscitation. In contrast, the lower dose of 3-AB (3 mg kg^{-1}; Table 3) or nicotinic acid (negative control for nicotinamide, Figure 1) had no significant effect. Although ISO also attenuated the rise in the serum levels of urea and creatinine, its vehicle (10% DMSO) had a similar effect (Figure 1).

Effects on the liver injury/dysfunction Neither saline nor any of the PARS inhibitors used caused any significant alterations in the plasma levels of AST or ALT in sham-operated rats (Table 4). In contrast, haemorrhage/resuscitation caused a significant increase in the plasma levels of AST (Figure 2a) and ALT (Figure 2b) and, hence, hepatocellular injury. Treatment of rats subjected to haemorrhage and resuscitation with the PARS inhibitors 3-AB and nicotinamide reduced the liver

Table 2 Alterations in mean arterial pressure (MAP) and heart rate (HR)

				Resuscitation (min)							
Group	n		*Baseline*	*30*	*60*	*90*	*120*	*150*	*180*	*210*	*240*
HS + saline	13	MAP	134 ± 4	104 ± 3	103 ± 2	95 ± 4	88 ± 4	80 ± 5	73 ± 5	71 ± 5	66 ± 4
		HR	399 ± 13	400 ± 9	409 ± 9	404 ± 7	411 ± 13	383 ± 16	396 ± 11	376 ± 21	386 ± 24
HS = 3-AB	8	MAP	131 ± 5	120 ± 5	110 ± 7	104 ± 8	102 ± 6	86 ± 7	83 ± 8	8 ± 7	82 ± 8
(3 mg kg^{-1})		HR	369 ± 16	402 ± 14	414 ± 16	395 ± 19	399 ± 16	412 ± 16	414 ± 13	416 ± 14	415 ± 26
HS + 3-AB	7	MAP	127 ± 6	112 ± 2	103 ± 3	102 ± 3	96 ± 4	89 ± 5	82 ± 5	76 ± 7	72 ± 9
(10 mg kg^{-1})		HR	360 ± 16	401 ± 27	424 ± 24	460 ± 33	413 ± 30	401 ± 34	382 ± 16	374 ± 13	371 ± 15
HS + Nic	6	MAP	123 ± 8	104 ± 6	99 ± 4	96 ± 5	96 ± 7	98 ± 3	83 ± 6	80 ± 6	82 ± 5
		HR	402 ± 15	414 ± 15	408 ± 14	387 ± 15	394 ± 20	396 ± 22	403 ± 20	400 ± 20	413 ± 18
HS + Nic A	7	MAP	131 ± 9	108 ± 9	104 ± 9	95 ± 8	91 ± 9	83 ± 9	77 ± 6	70 ± 8	71 ± 9
		HR	377 ± 8	394 ± 8	374 ± 14	374 ± 15	359 ± 13	372 ± 12	380 ± 10	378 ± 14	400 ± 18
HS + ISO	6	MAP	137 ± 3	117 ± 6	97 ± 7	99 ± 9	96 ± 9	96 ± 9	89 ± 9	82 ± 10	81 ± 11
		HR	414 ± 12	402 ± 9	388 ± 12	381 ± 15	373 ± 11	372 ± 12	372 ± 4	367 ± 9	376 ± 5
HS + DMSO	4	MAP	133 ± 6	112 ± 4	99 ± 4	98 ± 2	92 ± 7	91 ± 8	88 ± 7	80 ± 10	71 ± 9
		HR	393 ± 13	377 ± 15	381 ± 13	381 ± 14	376 ± 12	383 ± 10	387 ± 18	401 ± 31	386 ± 30
Sham + saline	6	MAP	129 ± 8	111 ± 5	105 ± 6	108 ± 8	105 ± 7	102 ± 6	99 ± 6	99 ± 9	100 ± 9
		HR	378 ± 24	379 ± 11	384 ± 15	392 ± 15	393 ± 12	401 ± 14	391 ± 10	394 ± 9	397 ± 11
Sham + 3-AB	5	MAP	140 ± 6	115 ± 5	113 ± 3	112 ± 4	110 ± 6	105 ± 7	96 ± 7	97 ± 7	95 ± 10
		HR	431 ± 25	396 ± 19	399 ± 13	402 ± 10	408 ± 24	388 ± 13	402 ± 18	414 ± 23	412 ± 21
Sham + Nic	5	MAP	129 ± 7	117 ± 8	116 ± 8	115 ± 10	112 ± 9	110 ± 7	107 ± 7	105 ± 8	106 ± 5
		HR	372 ± 33	386 ± 23	357 ± 21	391 ± 16	368 ± 12	358 ± 20	370 ± 8	398 ± 8	373 ± 8

Animals were subjected to the surgical procedure alone (sham) and treated with saline (vehicle, $n = 6$), 3–aminobenzamide (3-AB, 10 mg kg^{-1} i.v., $n = 5$) or nicotinamide (Nic, 10 mg kg^{-1} i.v., $n = 5$). In separate experiments, animals were subjected to haemorrhagic shock (HS) and treated with saline (vehicle, $n = 13$), 3-AB (10 mg kg^{-1} i.v., $n = 7$), Nic 10 mg kg^{-1} i.v., $n = 6$), nicotinic acid (NicA, 10 mg kg^{-1} i.v., $n = 7$), 1,5-dihydroxyisoquinoline (ISO 3 mg kg^{-1} i.v., $n = 6$) or dimethyl sulphoxide (10% DMSO, vehicle for ISO, $n = 4$).

Table 3 Dose Response to 3-AB

Biochemical parameter	*HS-Saline*	*HS-3AB* (3 mg kg^{-1})	*HS-3AB* (10 mg kg^{-1})
Urea (mM)	19±1	15±2	12±1*
Creatinine (μM)	108±9	81±9	50±4*
AST (iu L^{-1})	1216±163	1012±215	372±35*
ALT (iu L^{-1})	506±66	603±430	173±18
Lipase (iu L^{-1})	249±82	19±8*	5±2*
CK (iu L^{-1})	2494±564	2484±379	800±178*

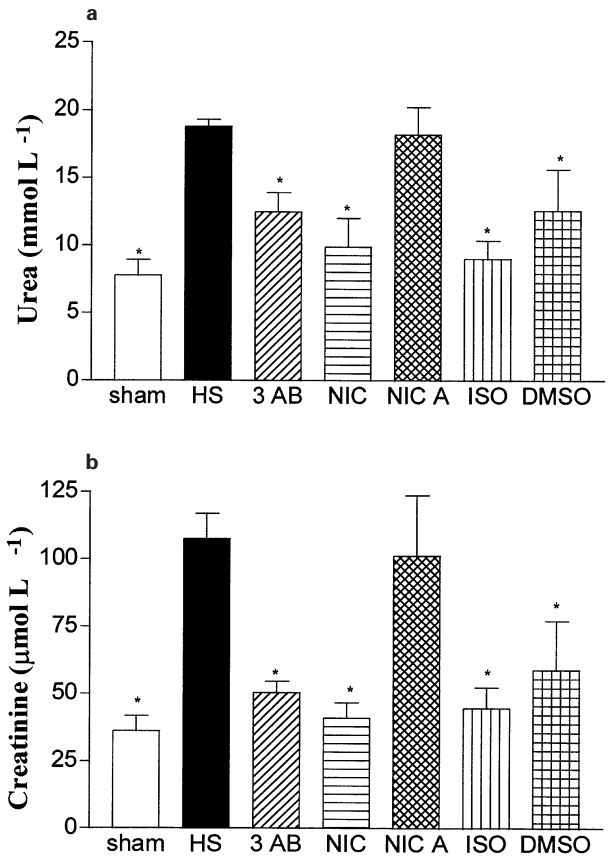

Figure 1 Alterations in the serum levels of urea (a) and creatinine (b). Animals were subjected to the surgical procedure alone and treated with saline (open column, $n=6$) or to haemorrhagic shock and treated with vehicle (HS, solid column, $n=13$), 3-AB (10 mg kg^{-1} i.v., $n=7$), Nic (10 mg kg^{-1} i.v., $n=6$), nicotinic acid (NicA, 10 mg kg^{-1} i.v., $n=7$), 1,5-dihydroxyisoquinoline (ISO, 3 mg kg^{-1} i.v., $n=6$) or dimethyl sulphoxide (10% DMSO, vehicle for ISO, $n=4$). *$P<0.05$ when compared to HS-control.

injury caused by haemorrhage and resuscitation. In contrast, the lower dose of 3-AB (3 mg kg^{-1}; Table 3) or nicotinic acid (negative control for nicotinamide, Figure 2) had no significant effect on the hepatocellular dysfunction caused by haemorrhagic shock. Although ISO also attenuated the rise in the serum levels of AST and ALT, its vehicle (10% DMSO) had a similar effect (Figure 2).

Effects on the pancreatic injury In sham-operated rats, neither administration of saline nor of any of the PARS inhibitors used had any significant effect on the plasma levels of lipase (Table 4). Haemorrhagic shock on the other hand resulted in significant rises in the plasma levels of lipase (Figure 3), demonstrating the development of pancreatic injury. Treatment of rats subjected to haemorrhage and resuscitation with

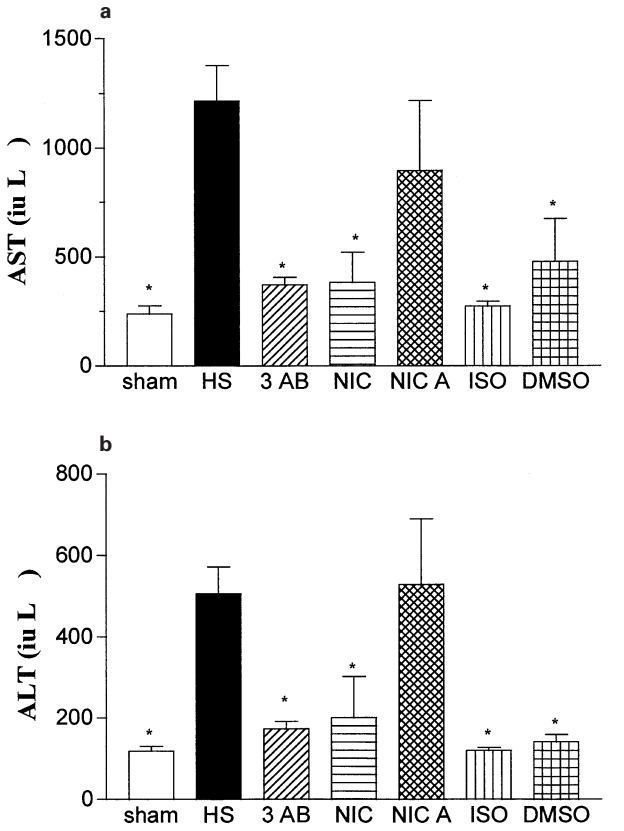

Figure 2 Alterations in the serum levels of aspartate aminotransferase (AST, a) and alanine aminotransferase (ALT, b). Animals were subjected to the surgical procedure alone and treated with saline (open column, $n=6$) or to haemorrhagic shock and treated with vehicle (HS, solid column, $n=13$), 3-AB (10 mg kg^{-1} i.v., $n=7$), Nic (10 mg kg^{-1} i.v., $n=6$), nicotinic acid (NicA, 10 mg kg^{-1} i.v., $n=7$), 1,5-dihydroxyisoquinoline (ISO, 3 mg kg^{-1} i.v., $n=6$) or dimethyl sulphoxide (10% DMSO, vehicle for ISO, $n=4$). *$P<0.05$ when compared to HS-control.

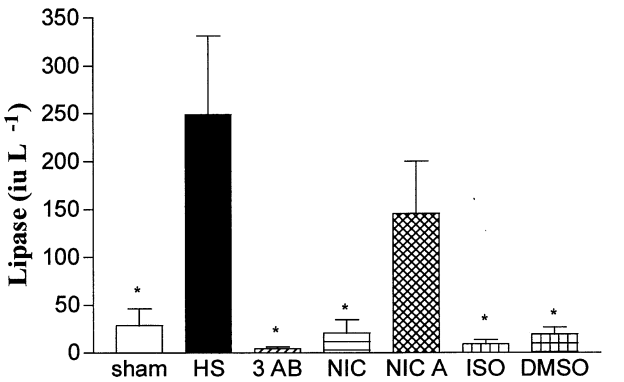

Figure 3 Alterations in the serum levels of lipase. Animals were subjected to the surgical procedure alone and treated with saline (open column, $n=6$) or to haemorrhagic shock and treated with vehicle (HS, solid column, $n=13$), 3-AB (10 mg kg^{-1} i.v., $n=7$), Nic (10 mg kg^{-1} i.v., $n=6$), nicotinic acid (NicA, 10 mg kg^{-1} i.v., $n=7$), 1,5-dihydroxyisoquinoline (ISO, 3 mg kg^{-1} i.v., $n=6$) or dimethyl sulphoxide (10% DMSO, vehicle for ISO, $n=4$). *$P<0.05$ when compared to HS-control.

the PARS inhibitors 3-AB (3 or 10 mg kg^{-1}; Table 3; Figure 3) or nicotinamide (Figure 3) reduced the pancreatic injury caused by haemorrhagic shock. In contrast, nicotinic acid

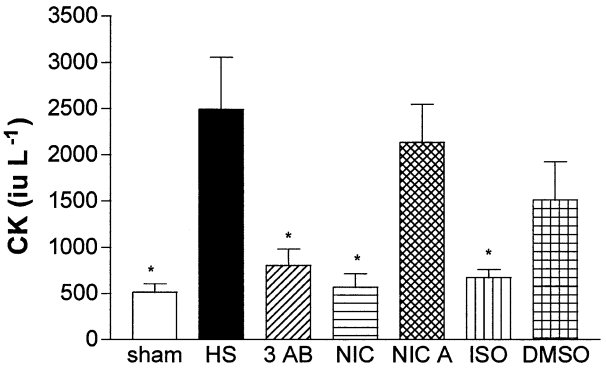

Figure 4 Alterations in the serum levels of creatine kinase (CK). Animals were subjected to the surgical procedure alone and treated with saline (open column, $n=6$) or to haemorrhagic shock and treated with vehicle (HS, solid column, $n=13$), 3-AB (10 mg kg^{-1} i.v., $n=7$), Nic (10 mg kg^{-1} i.v., $n=6$), nicotinic acid (NicA, 10 mg kg^{-1} i.v., $n=7$), 1,5-dihydroxyisoquinoline (ISO, 3 mg kg^{-1} i.v., $n=6$) or dimethyl sulphoxide (10% DMSO, vehicle for ISO, $n=4$). *$P<0.05$ when compared to HS-control.

Table 4 Alterations in the serum levels or urea, creatinine, aspartate amino transferasae (AST), alanine aminotransferase (ALT), lipase and creatine kinase (CK)

Biochemical parameter	*Sham* (n=6)	*Sham 3-AB* (n=5)	*Sham Nic* (n=5)
Urea (mmol L^{-1})	7.8±1.2	8.5±0.6	7.4±0.4
Creatinine (μmol L^{-1})	36±6	42±3	40±7
AST (iu L^{-1})	238±38	223±40	206±15
ALT (iu L^{-1})	119±11	132±12	113±16
Lipase (iu L^{-1})	28.8±17.4	7.2±3.3	7.2±5.3
CK (iu L^{-1})	512±90	384±36	604±135

Animals were subjected to the surgical procedure alone (sham) and treated with saline (vehicle, $n=6$), 3-aminobenzamide (3-AB, 10 mg kg^{-1} i.v., $n=5$) or nicotinamide (Nic, 10 mg kg^{-1} i.v., $n=5$).

(negative control for nicotinamide) was without effect (Figure 3). Although ISO also reduced the rise in the serum levels of lipase, its vehicle had a similar effect (Figure 3).

Effects on the increase in the serum levels of creatine kinase Neither saline nor any of the PARS inhibitors used had any significant effect on the plasma levels of creatine kinase (Table 4). When compared with sham-operated rats, haemorrhage and resuscitation resulted in significant rises in the plasma levels of creatine kinase (Figure 4), indicating that an injury to either myocytes (skeletal or cardiac) or brain has occurred. Treatment of rats subjected to haemorrhage and resuscitation with the PARS inhibitors 3-AB and nicotinamide attenuated the rise in creatine kinase caused by haemorrhage and resuscitation. In contrast, the lower dose of 3-AB (3 mg kg^{-1}; Table 3) or nicotinic acid (negative control for nicotinamide, Figure 4) had no significant effect on the rise in the serum levels of CK. Although ISO also attenuated the rise in the serum levels of creatine kinase, its vehicle had a similar effect (Figure 4).

Effects of 3-AB on the determination of creatinine, urea, AST, ALT, CK and lipase

In a separate set of experiments, plasma samples obtained from rats subjected to haemorrhagic shock or sham-operation were spiked with 3-AB in order to evaluate whether 3-AB affects the determination of any of the above parameters of organ injury (see Methods for details). We found that 3-AB had no significant effect on the determination of creatinine (without 3-AB: 16±1 μM; with 3-AB 14±2 μM; $P>0.05$, $n=7$), urea (without 3-AB: 6±1 mM; with 3-AB 6±1 mM; $P>0.05$, $n=7$), AST (without 3-AB: 112±21 iu L^{-1}; with 3-AB 112±20 iu L^{-1}; $P>0.05$, $n=7$), ALT (without 3-AB: 64±8 iu L^{-1}; with 3-AB 64±8 iu L^{-1}; $P>0.05$, $n=7$), CK (without 3-AB: 190±38 iu L^{-1}; with 3-AB 229±57 iu L^{-1}; $P>0.05$, $n=7$) and lipase (without 3-AB: 19±3 iu L^{-1}; with 3-AB 14±4 iu L^{-1}; $P>0.05$, $n=7$).

Discussion

Haemorrhage for 90 min followed by resuscitation with shed blood (for 4 h) resulted in a substantial increase in the plasma levels of urea and creatinine indicating the development of acute renal dysfunction. Haemorrhage and resuscitation also caused an increase in the plasma levels of the transaminases AST and ALT, indicating the development of hepatocellular injury. Indeed, we have previously confirmed (by using light microscopy) that the model of haemorrhagic shock used here results in a substantial degree of tissue injury to the lung, kidney, intestine and liver (Mota-Filipe *et al*., 1999). In addition, haemorrhage and resuscitation resulted in a substantial increase in the serum levels of lipase indicating the development of pancreatic injury. Haemorrhage and resuscitation also caused a significant increase in the serum levels of creatine kinase, which is a cytosolic enzyme contained in large amounts in skeletal or cardiac muscle and in the brain. We report here that the administration (at 5 min prior to resuscitation) of three, chemically distinct PARS inhibitors (3-AB, nicotinamide and ISO) attenuated (i) the renal dysfunction, (ii) the liver injury, (iii) the pancreatic injury and (iv) the muscle and/or brain injury caused by haemorrhage and resuscitation.

We propose that the beneficial effects of 3-AB and nicotinamide are due to the ability of these agents to inhibit PARS activity. Although not very potent *in vitro*, 3-AB is a well-documented, water-soluble PARS inhibitor. The dose of 3-AB used in our study has been shown to inhibit PARS activity in the rat *in vivo*. As nicotinamide is a vitamin, it could be argued that the beneficial effects of nicotinamide observed in our study are due to non-specific effects of nicotinamide. This is, however, unlikely, as nicotinic acid, a structural analogue of nicotinamide, which does not inhibit PARS activity (Thiemermann *et al*., 1997; Bowes *et al*., 1998; 1999), failed to reduce the organ injury and dysfunction caused by haemorrhage and resuscitation. It could also be argued that the beneficial effects of nicotinamide could be secondary to the ability of nicotinamide to replenish intracellular NAD levels *via* the re-synthesis of NAD from nicotinamide (Carson *et al*., 1986). This is also very unlikely, as nicotinic acid, which can also be re-synthesized to NAD, did not reduce the organ injury and dysfunction caused by haemorrhage and resuscitation in the rat.

We have recently documented that tempol, a small molecule which crosses biological membranes and functions as an intracellular radical scavenger, reduces the multiple organ failure caused by haemorrhage and resuscitation (Mota Filipe *et al*., 1999). The PARS inhibitors used here however, do not scavenge reactive oxygen-radicals (Bowes *et al*., 1998). Thus, we propose that the beneficial effects of PARS inhibitors are not due to their ability to scavenge radicals. This notion is

supported by the finding that these agents are unable to attenuate the development of single strand breaks in DNA caused by hydrogen peroxide in proximal tubule cells of the rat *in vitro* (Chatterjee *et al*., 1999). In contrast, desferrioxamine and catalase attenuate the DNA strand breaks caused by H_2O_2 in the same cells (positive control) (Chatterjee *et al*., 1999).

ISO is a more potent inhibitor of PARS activity than analogues of benzamide, such as 3-AB (Bowes *et al*., 1999). Although ISO abolished the renal dysfunction, the hepatocellular injury and the pancreatic injury associated with haemorrhage and resuscitation, a similar beneficial effect was also observed with its vehicle DMSO. As DMSO is a scavenger of hydroxyl radicals, it is possible that the observed beneficial effects of ISO are, at least in part, due to the ability of DMSO to scavenge hydroxyl radicals *in vivo*. Indeed, agents which either reduce the generation of hydroxyl radicals *in vivo* (e.g. deferroxamine) or agents which scavenge hydroxyl radicals (Mota Filipe *et al*., 1999) reduce the organ injury/dysfunction associated with haemorrhagic shock.

In principle, severe haemorrhage followed by resuscitation leads to ischaemia and reperfusion (injury) of target organs including the heart, liver, brain and kidney (Flaherty & Wesfeldt, 1988). There is good evidence that various, chemically distinct inhibitors of PARS activity (including 3-AB, nicotinamide and ISO) reduce the degree of tissue injury associated with regional myocardial ischaemia and reperfusion of the heart (Thiemermann *et al*., 1997; Zingarelli *et al*., 1997; 1998; Bowes *et al*., 1999), the brain (Eliasson *et al*., 1997), the gut (Cuzzocrea *et al*., 1997) and the kidney (Chatterjee *et al*., 1999). Most notably, the degree of tissue injury caused by ischaemia and reperfusion of the heart (Zingarelli *et al*., 1998; Walles *et al*., 1998a, b; Grupp *et al*., 1999) and brain (Eliasson *et al*., 1997) is attenuated in mice in which the gene for PARS has been disrupted by gene-targeting (PARS knock out or $-/-$ mice). We, therefore, propose that severe haemorrhage and resuscitation leads to organ ischaemia (McCord, 1985; Flaherty & Wesfeldt, 1988), the generation of oxygen- or nitrogen-derived free radicals upon reperfusion (Zweier *et al*., 1987; Nunes *et al*., 1995), strand breaks in DNA (Carson *et al*., 1986) and ultimately PARS activation. The resultant excessive activation of PARS contributes to the organ injury and dysfunction associated with severe haemorrhage and resuscitation.

The PARS inhibitor 3-AB (15 mg kg^{-1}) has been reported to attenuate the delayed circulatory failure (e.g. fall in blood pressure, cardiac output and stroke volume) associated with severe haemorrhage in the pig. Thus, it has been suggested (Szabo *et al*., 1998) that the beneficial effects of 3-AB in haemorrhagic shock are due to an improved cardiac performance. We have not measured the effects of any of the PARS inhibitors used in our study on cardiac performance. It should be noted that the mean arterial blood pressure of rats treated with 3-AB, nicotinamide or ISO were higher (at the end of the resuscitation period) than in the control group. Although consistent, the observed effect of the PARS inhibitors on blood pressure was small, but not significant. Thus, we provide no evidence that the PARS inhibitors used in this study attenuate the delayed fall in blood pressure caused by severe haemorrhage and resuscitation.

In conclusion, this study demonstrates that three chemically distinct inhibitors of PARS activity attenuate the renal dysfunction, the hepatocellular injury and the pancreatic injury associated with severe haemorrhage and resuscitation. As the beneficial effects of the potent and specific PARS inhibitor ISO were – in part – due to its vehicle, further studies with potent and specific inhibitors of PARS activity are warranted to ensure that the reduction by the agents of the multiple organ failure in haemorrhagic shock is indeed due to their ability to inhibit PARS activity.

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