Free Radical Biology & Medicine, Vol. 31, No. 10, pp. 1236–1244, 2001

0891-5849/01/$–see front matter

PII S0891-5849(01)00716-X

Original Contribution

DIETARY GLYCINE INHIBITS ACTIVATION OF NUCLEAR FACTOR KAPPA B AND PREVENTS LIVER INJURY IN HEMORRHAGIC SHOCK IN THE RAT

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(*Received* 28 *March* 2001; *Accepted* 28 *August* 2001)

Abstract—We investigated the effects of a glycine-containing diet (5%) on liver injury caused by hemorrhagic shock and resuscitation in rats. Anesthetized rats were bled to a mean arterial blood pressure of 35–40 mm Hg for 1 h and then resuscitated with 60% of shed blood and lactated Ringer's solution. Feeding the rats glycine significantly reduced mortality, the elevation of plasma transaminase levels and hepatic necrosis. The increase in plasma TNFα and nitric oxide (NO) was also blunted by glycine feeding. Hemorrhagic shock resulted in oxidative stress (significant elevations in TBARS and in the oxidized/reduced glutathione ratio) and was accompanied by a reduced activity of the antioxidant enzymes Mn- and Cu,Zn-superoxide dismutase, glutathione peroxidase and catalase, overexpression of inducible NO synthase (iNOS), and activation of nuclear factor kappa B (NF-κB). Glycine ameliorated oxidative stress and the impairment in antioxidant enzyme activities, inhibited NF-κB activation, and prevented expression of iNOS. Dietary glycine blocks activation of different mediators involved in the pathophysiology of liver injury after shock. © 2001 Elsevier Science Inc.

Keywords—Antioxidant enzymes, Glutathione, Glycine, Hemorrhagic shock, Nitric oxide, Nuclear factor κB, Oxidative stress, Free radicals

INTRODUCTION

Multiple organ failure, a systemic inflammatory process that leads to dysfunction of different vital organs, is a frequent complication after hemorrhagic shock and accounts for a high incidence of mortality [1]. Hepatic dysfunction plays a central role and may persist in those cases in which the reversal of the shock has been possible [2]. Although during the last two decades studies about the mechanisms and consequences of liver damage both in humans and animals models have been carried out, we still do not know enough, and the therapeutics must improve.

The pathogenesis of organ injury secondary to hypovolemic insults is still not completely understood, but both experimental studies and clinical observations indicate that macrophages are activated by translocated endotoxin-bacteria and ischemia/reperfusion [3]. Activated Kupffer cells release pathologically active substances such as inflammatory cytokines (including TNFα), reactive oxygen species (ROS), and nitric oxide (NO), all of which may participate in the mechanisms of hemorrhagic shock [3–6].

One pathway by which ROS, TNFα, and NO can contribute to liver injury is activation of inflammatory cascades through the transcription factor NF-κB, resulting in inflammation manifested by cytokine expression [7]. When activated, NF-κB translocates into the nucleus, where it binds to the promoter region of target genes [8]. NF-κB may therefore represent an important target for therapeutic blockade of inflammation and liver injury.

Glycine is a nonessential amino acid that has been reported to inhibit activation of macrophages and TNFα release [9,10]. This molecule reduces reperfusion injury [11], prevents liver damage after chronic exposure to alcohol [12], attenuates lipid peroxidation and glutathione depletion induced by different hepatotoxins [13], and very recently it has been reported that when iv injected prior to resuscitation, it reduces organ injury and mortality in rats with hemorrhagic shock [14]. Diets supple-

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Table 1. Components of Control and Glycine-containing Diets

	% Wt/Wt	
	Control diet	Glycine diet
Glycine	0.0	5.0
Casein	20.0	15.0
Sucrose	50.0	50.0
Cellulose	5.0	5.0
Corn oil	5.0	5.0
Mineral mixture	3.5	3.5
Vitamin mixture	1.0	1.0
DL-Methionine	0.3	0.3
Choline bitartrate	0.2	0.2
Corn starch	15.0	15.0

Rats were given free access to synthetic powdered diets containing 5% glycine or control diets and water for 4 d before experiments. The diet was also available to the appropriate group after shock was induced.

mented with glycine minimize injury by endotoxic shock [9], D-galactosamine [15], or cyclosporine A [16]. In any case, although the cytoprotective effect of glycine is well established, the mechanisms involved still must be fully clarified.

On the basis of the previous studies it could be proposed that, in hemorrhagic shock, activation of NF-κB, and upregulation of NO and cytokine production occur in association with oxidative stress, and that this activation contribute to pathological liver injury. Study of the beneficial effects of dietary glycine would provide further insight into the pathogenesis of hemorrhagic shock and would give opportunities for clinically useful therapeutical approaches.

MATERIALS AND METHODS

Experimental protocols

All experiments were performed in accordance with the *Guiding Principles for Research Involving Animals* (NAS) [17]. Male Wistar rats were obtained from Charles River, Barcelona, Spain. They were kept in a temperature- and humidity-controlled animal quarter under a 12 h light-dark cycle, with free access to tap water. Rats were fed a synthetic powdered diet containing 5% glycine and 15% casein (glycine diet) or 20% casein (control diet) for 4 d before experiments (Table 1). Average glycine consumed during the period of study was 0.97 $\pm$ 0.03 g.

Animals were anaesthetized with pentobarbital sodium (50 mg/kg body weight, ip). Body temperature was monitored by a rectal probe and maintained at 37°C with warming lamps. A caudal pressure transducer allowed continuous monitoring of mean arterial blood pressure. Hemorrhagic shock was induced over 5 min by withdrawing blood from a catheter inserted into the left carotid artery until arterial pressure was reduced to 35–40 mm Hg. Constant pressure was maintained with further withdrawal of small amounts of blood as necessary for 60 min. Rats were resuscitated by transfusion of 60% shed blood over 5 min. Simultaneously, lactated Ringer's solution (twice the shed blood volume) was given for about 1 h [6,14,18,19].

Immediately following resuscitation, catheters were removed, the vessels were occluded, and the wound closed. Animals were placed in a warming blanket until they regained consciousness and were observed for 20 h to assess survival.

Rats were assigned to the following experimental groups: (i) Sham: sham-operated rats not subjected to shock/resuscitation ($n = 8$). (ii) Shock: hemorrhage/resuscitation rats that had received control diet ($n = 8$). (iii) Shock + glycine: hemorrhage/resuscitation rats that were fed the glycine-supplemented diet ($n = 8$).

To eliminate the diurnal effects, all rats were killed at the same time of the day. After the animals were decapitated and exsanguinated, the livers were immediately removed and blood samples were centrifuged at 1800 $\times$ *g* for 15 min at 4°C to obtain plasma.

Plasma enzymes and protein

The plasma activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was estimated by commercially available kits (Boehringer Mannheim, Mannheim, Germany). Protein concentration was measured according to Lowry et al. [20].

Glutathione, antioxidant enzymes, and TBARS

Oxidized and reduced glutathione analysis was performed fluorimetrically by the method of Hissin and Hill [21]. For measuring liver enzyme activities samples were homogenized at 4°C and 750 $\times$ *g* for 15 min in an ice-cold medium containing 250 mM mannitol, 70 mM sucrose, and 2 mM EDTA (pH 7.4). Mitochondria were isolated by centrifugation of the supernatant at 12,000 $\times$ *g* for 15 min. The mitochondrial pellet was washed twice in isolation medium by centrifugation at 12,000 $\times$ *g* for 15 min and finally diluted to contain approximately 100 mg mitochondrial protein per milliliter. The supernatant fraction of the first 12,000 $\times$ *g* centrifugation was centrifuged again at 105,000 $\times$ *g* for 60 min, and the supernatant was retained as cytosol. Superoxide dismutase (SOD) (EC 1.15.1.1) was assayed according to Misra and Fridovich [22] at 30°C. Catalase (CAT) (EC 1.11.1.6) activity was determined by measuring the exponential disappearance of H_2O_2 at 240 nm [23]. The assay of glutathione peroxidase (GPx) (EC 1.11.1.19)

was carried out according to Flohe and Gunsler [24]. The amount of aldehydic products generated by lipid peroxidation was quantified by the thiobarbituric acid reaction [25].

Nitrite determination

Nitric oxide production was measured indirectly using a quantitative, colorimetric assay [26] based on the Griess reaction, a sensitive assay for nitrite ions.

TNFα

Plasma TNFα was determined by enzyme-linked immunosorbent assay (ELISA) kit (Diaclone Research, Besancon, France).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted with selective precipitation with guanidium thiocyanate. Later, cDNA was synthesized from 2.0 μg RNA using 5 μM random primers (Ambion, Austin, TX, USA) and 10U AMV Reverse Transcriptase (Promega, Madison, WI, USA). Polymerase chain reaction (PCR) on complementary DNA was performed by using primers specific for rat purchased from Biosource International (Camarillo, CA, USA). cDNA was subjected to quantitative real-time PCR with ABI Prism 7700 (PE Applied Biosystems, Foster City, CA, USA) using the primer 6FAM-TTT TTG TTG GTG GAA TCA ACT GCC TTC AC-TAMRA for quantification. The PCR-primer sequences for the Mn-SOD were (sense) 5′-GCG CAG ATC ATG CAG CTG-3′ and (antisense) 5′-GCC TGT GGT TCC TTG CAG-3′. For Cu,Zn-SOD, sense primer was 5′-ATG AAG GCC GTG TGC GTG-3′, corresponding antisense primer 5′-TCC ACC TTT GCC CAA GTC ATC-3′. For GPx, sense primer was 5′-TGG CAC AGT CCA CCG TGT AT-3′, corresponding antisense primer 5′-TCC ACC TTT GCC CAA GTC-3′. For CAT, sense primer was 5′-TGT CCT GGT CAG TCT TGT AAT G-3′, corresponding antisense primer 5′-AGC GAC CAG ATG AAG CAG TG-3′. For iNOS, sense primer was 5′-CTC TGA AGA AAT CTC TGT TC-3′, corresponding antisense primer 5′-TTG AGG TCT AGA GAC TCT GG-5′. The mRNA levels were normalized against β-actin mRNA. The amplified products for Mn-SOD, Cu,Zn-SOD, GPX, CAT, iNOS, and β-actin contained 368, 346, 575, 703, 352, and 457 base pairs (bp). After amplification PCR products were subjected to electrophoresis in 1% agarose gel and visualized by means of ethidium bromide staining. Fragments were then photographed using a Gelprinter plus photodocumentation system (TDI, Madrid, Spain).

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared from liver sections as described previously [27]. Activation of transcription factor NF-κB was examined using consensus oligonucleotides of NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′). Probes were labeled by T4 polynucleotide kinase as described [28]. Binding reactions included 10 μg of nuclear extracts in incubation buffer (10 mM Tris-HCl pH 7.5, 40 mM NaCl, 1 mM EDTA, and 4% glycerol and 1 μg poly(dI-dC). After 15 min on ice, the labeled oligonucleotide (30,000 cpm) was added and the mixture incubated 20 min at room temperature. For competition studies, 3.5 pmol of unlabeled NF-κB oligonucleotide were mixed 15 min before the incubation with the labeled oligonucleotide. The mixture was electrophoresed through a 6% polyacrimide gel for 90 min at 150 volts. The gel was then dried and autoradiographed at -70°C overnight. Signals were densitometrically analyzed.

Histology

For histological examination a piece of the liver was trimmed and fixed by immersion in 10% buffered formalin for 24 h. The blocks were dehydrated in a graded series of ethanol and embedded in paraffin wax. Serial 3 μm sections were stained with hematoxilin and eosin.

Statistical analysis

Means and SEMs were calculated. Significant differences between means were evaluated by analyses of variance and in the case of significance a Newman-Keul's test was also applied [29]. Survival data were evaluated with Fisher's exact test. A difference was considered significant when p was less than .05.

RESULTS

Body weight

Body weight gain of the control diet group and glycine-fed group during 4 d of feeding was 11.9 $\pm$ 1.2 g and 10.9 $\pm$ 1.6 g, respectively. There were no significant differences between the groups.

Effect of glycine on survival

Survival of rats is shown in Fig. 1. Animals with hemorrhagic shock that received the control diet had

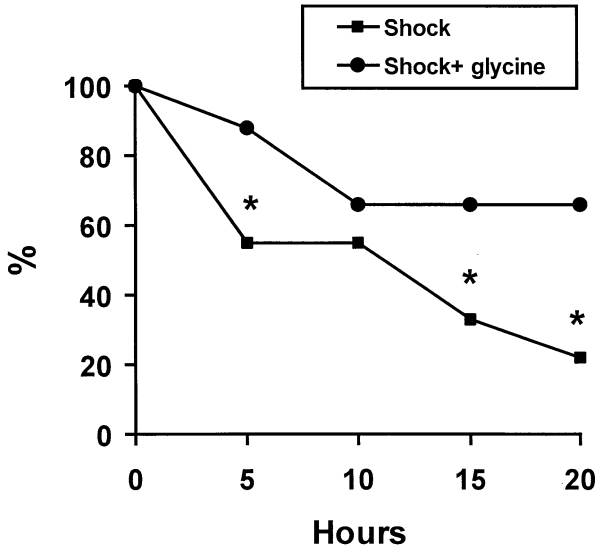

Fig. 1. Effects of glycine on survival rates after hemorrhagic shock. Survival rates were monitored for 20 h. Data represent percentage of surviving animals. Initial number $n = 9$. *$p < .05$ with Fisher's test.

survival rates of only 22% 20 h after shock. Mortality was partially prevented by glycine and the improvement was already significant from 5 h of resuscitation.

Plasma transaminases

AST increased 22-fold and ALT increased 8-fold 20 h after shock (Table 2). In the glycine-fed group data were significantly lower and AST and ALT were only elevated to values 68% and 64%, respectively, higher than levels observed in the controls.

Histological changes

As expected, hemorrhagic shock and resuscitation caused necrosis in pericentral areas and infiltration of macrophages and lymphocytes (Fig. 2). Vacuolar degeneration was also observed. These parameters were significantly reduced by glycine (Fig. 2).

Table 2. Effects of Glycine on Plasma Transaminase, TNFα, and Nitrite Levels

	Sham	Shock	Shock + glycine
ALT (U/l)	10.3 ± 1.1	77.7 ± 16.1*	16.9 ± 1.1*$^{\#}$
AST (U/l)	40 ± 6	899 ± 115*	67 ± 6*$^{\#}$
Nitrite (μmol/l)	12.4 ± 1.5	21.0 ± 3.9*	11.4 ± 1.0$^{\#}$
TNFα (pg/ml)	undetected	377 ± 68*	107 ± 30*$^{\#}$

Values are means ± SEM from 8 animals.
* $p < .05$ from sham group.
$^{\#}$ $p < .05$ from shock group.

Markers of oxidative stress

The cytosolic concentration of TBARS, a marker of lipid peroxidation, increased in the animals with hemorrhagic shock (+38%), while values did not significantly differ from the controls in rats treated with glycine (Table 3). A decrease in the hepatic concentration of GSH (−40%) and an increase in that of GSSG (+23%) were observed in hemorrhagic untreated rats. These effects were reverted by administration of glycine, with an almost complete normalization of the GSSG/GSH ratio in the animals receiving the supplemented diet (Table 3).

Antioxidant enzymes

Antioxidant enzyme activities were significantly reduced in hemorrhagic animals and recovered in those fed glycine. Thus, Mn-SOD was reduced by 49% and Cu,Zn-SOD by 23% and both normalized with glycine. Cytosolic GPx decreased by 26% and mitochondrial GPx by 54%; values returned to control by glycine. CAT decreased by 47% and almost completely recovered in glycine-treated rats (Table 3).

The mRNA levels of antioxidant enzymes were also determined (Fig. 3). In contrast to the marked decline in enzyme activity, Mn-SOD mRNA was 5.6-fold higher in hemorrhagic than in control rats, and GPx and CAT mRNA levels were elevated by 90% and 101%, respectively. Increases were partially prevented by glycine administration. The mRNA level of Cu,Zn-SOD was not significantly modified either in hemorrhagic or glycine-treated rats (Table 4).

*Plasma TNF*A

A marked elevation in plasma TNFα levels was found 1 h after shock. The increase in TNFα was clearly suppressed by glycine (Table 2).

NO and iNOS

Table 2 shows concentration of nitrites in plasma. This was significantly higher in hemorrhagic animals (+69%), coinciding with an increased expression of iNOS (+89%) (Fig. 4). Both these parameters tended to be normalized in rats receiving glycine.

Nuclear factor κB

Electrophoretic mobility shift assays revealed a clear activation of NF-κB in hemorrhagic rats, with values increased by 112% in comparison to control rats. Glycine inhibited the activation of NF-κB and no significant difference from controls could be appreciated (Fig. 5).

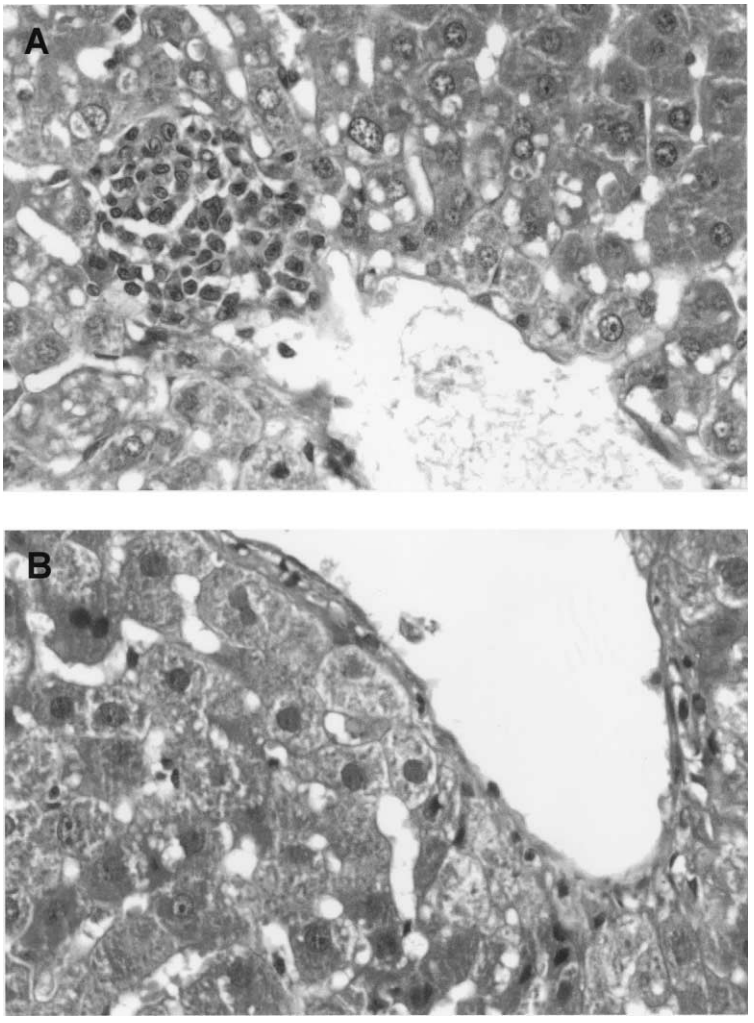

Fig. 2. Effects of glycine on liver histology. (A) Liver from a rat with hemorrhage/resuscitation that had received a control diet. (B) Liver from a glycine-fed rat with hemorrhage/resuscitation. Hematoxylin-eosin staining. 410×.

DISCUSSION

Our results indicate that dietary glycine improves survival and reduces liver injury caused by hemorrhagic shock, confirming previous data in hemorrhagic rats iv injected with glycine prior to resuscitation [14]. Although mechanisms for shock-related cell injury are still not completely understood and there are massive syner-

Table 3. Effects of Glycine on Liver TBARS, GSH, GSSG, and Antioxidant Enzyme Activities

	Sham	Shock	Shock + glycine
TBARS (nmol/mg prot)	7.59 ± 0.44	10.50 ± 0.80*	6.30 ± 0.64$^{\#}$
GSH (μmol/g liver)	3.58 ± 10.31	2.16 ± 0.10*	3.07 ± 0.09$^{\#}$
GSSG (nmol/g liver)	28.9 ± 3.0	35.3 ± 2.7*	31.1 ± 2.6
GSSG/GSHx100	0.81 ± 0.10	1.63 ± 0.11*	1.01 ± 0.07$^{\#}$
Cu,Zn -SOD (U/mg prot)	3.91 ± 0.26	3.03 ± 0.23*	4.11 ± 0.29$^{\#}$
Mn -SOD (U/mg prot)	1.97 ± 0.31	1.00 ± 0.12*	1.60 ± 0.25$^{\#}$
Cytosolic GPx (mU/mg prot)	365 ± 22	270 ± 19*	393 ± 22$^{\#}$
Mitochondrial GPx (mU/mg prot)	192 ± 14	89 ± 13*	180 ± 23$^{\#}$
CAT (K/mg prot)	0.28 ± 0.04	0.15 ± 0.01*	0.23 ± 0.03*$^{\#}$

Values are means ± SEM from 8 animals.
* $p < .05$ from sham group.
$^{\#}$ $p < .05$ from shock group.

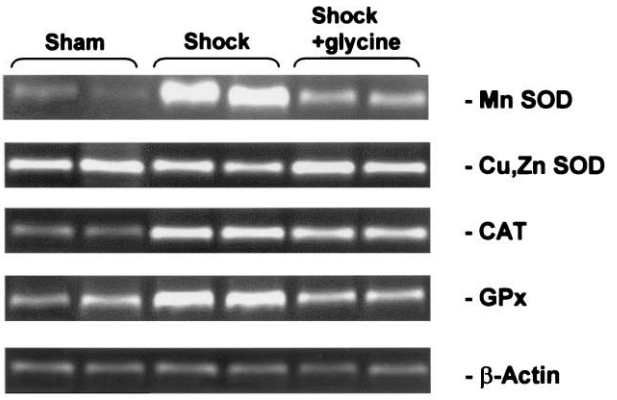

Fig. 3. mRNA expression of antioxidant enzymes in the different experimental groups. Total RNA was extracted and RT-PCR was carried out as described in Materials and Methods. β-Actin was used as an internal control. Representative RT-PCR reactions from $n = 5$ rats for group are shown.

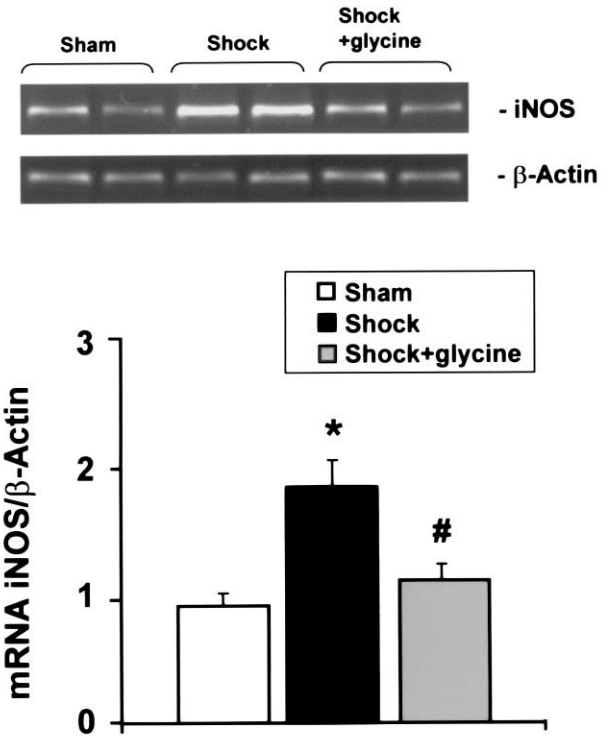

Fig. 4. iNOS mRNA expression in the different experimental groups. Total RNA was extracted and RT-PCR was carried out as described in Materials and Methods. β-Actin was used as an internal control. Top panel shows representative RT-PCR reactions from $n = 5$ rats for group. Bottom panel shows mean values ± SEM normalized to β-actin mRNA. *$p < .05$ from sham group; #$p < .05$ from shock group.

gism and redundancy in the pathways of inflammation associated with hemorrhagic shock, inflammatory cytokines, ROS, and NO appear to play a role in the pathogenesis of this condition [4–6].

Previous studies have shown an early elevation in the plasma levels of TNFα after hemorrhagic shock, while treatment with anti-TNFα antibody decreases organ injury [30] and results in a significantly better survival rate [31]. In our study, elevation of TNFα levels was partially prevented by glycine feeding. A similar fact has been shown in rats with endotoxin shock that received a glycine-supplemented diet [9]. In isolated Kupffer cells from hemorrhagic rats it has also been demonstrated that glycine blunts the increased production of TNFα stimulated by endotoxin [14].

Hemorrhagic shock is also associated with overexpression of iNOS and increased NO production [4]. iNOS may be induced by TNFα and upregulated by hypoxia in vitro [32] and increased NO production has been confirmed by in vivo and in vitro studies to contribute to the pathogenesis of septic shock [4]. Moreover, selective inhibitors of iNOS, such as aminoguanidine, have been shown to improve survival rate in rodent models of hemorrhagic shock [33]. We found that up-regulation of iNOs was prevented by dietary glycine, which could explain the inhibition of NO production and contribute to the prevention of liver injury and improved survival after hemorrhagic shock.

Increased free radical production during hemorrhagic shock and resuscitation gives place to an increase in oxidative stress that would contribute to the hepatic damage [6,14,34]. Similar to previous reports by Rose et al. [19] or Bauer et al. [35], we found an increase in TBARS together with a decrease in the hepatic concentration of GSH and an increase in that of GSSG, which may be attributable to GSH consumption as a consequence of ROS formation. Feeding

Table 4. Effects of Glycine on Liver mRNA Antioxidant Genes

	Sham	Shock	Shock + glycine
mRNA Mn SOD (%)	0.53 ± 0.04	2.96 ± 0.16*	1.45 ± 0.17*#
mRNA Cu,Zn SOD (%)	1.98 ± 0.18	1.83 ± 0.10	1.99 ± 0.17
mRNA GPx (%)	1.33 ± 0.11	2.53 ± 0.12*	1.42 ± 0.07#
mRNA CAT (%)	1.00 ± 0.06	2.01 ± 0.06*	1.62 ± 0.08*#

Levels of mRNA were normalized to β-actin mRNA (means ± SEM) for 4–5 animals.
* $p < .05$ from sham group.
# $p < .05$ from shock group.

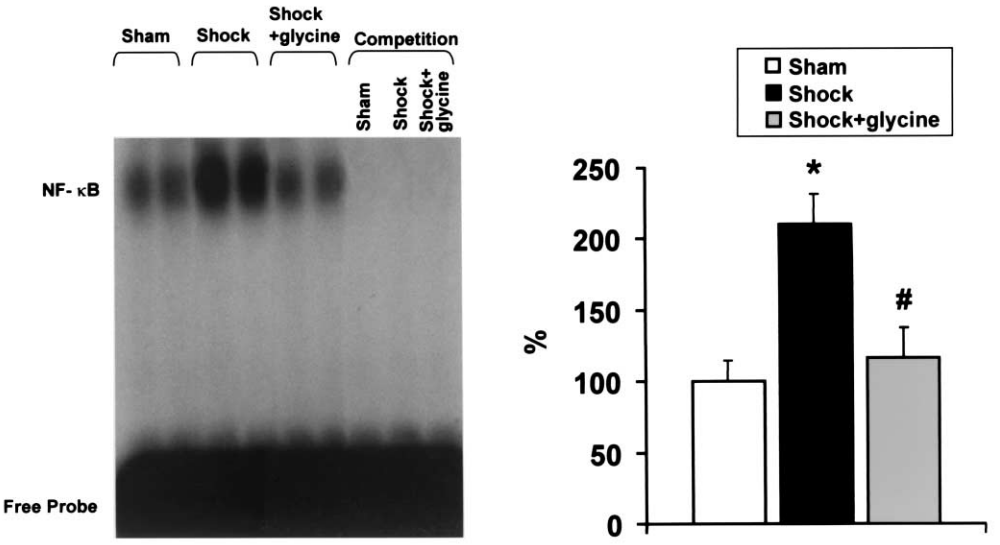

Fig. 5. NF-κB activation in nuclear extracts from rats in the different experimental groups. Left panel shows representative EMSA from $n = 5$ animals per group and competition experiments. Right panel shows mean values ± SEM expressed as percentage of sham animals. *$p < .05$ from sham group; #$p < .05$ from shock group.

the rats glycine significantly reduced those alterations. This finding suggests reduced lipid peroxidation and coincides with a previous report that acute administration of glycine reduces ROS activity as measured by spin resonance spectrometry [14].

ROS are kept at physiologically optimal levels under normal conditions by antioxidant defense systems, including nonenzymatic antioxidants such as glutathione and the enzymes SOD, GPx, and CAT [36]. The reduction in antioxidant defenses and decreased scavenging capacity reported in this study may contribute to oxidative stress in rats with hemorrhagic shock and could be explained by different factors. Decrease in catalase activity may be due to an inhibition by NO [37], while the drop of SOD activity could be also explained by NO inactivation [38] or the overproduction of the superoxide anion [39]. Inactivation of GPx after endogenous exposure to aldehydic by-products of lipid peroxidation [40] or NO [41] has also been reported. Impairment of the activity of antioxidant enzymes would increase the intracellular levels of the superoxide anion, which then rapidly reacts with NO to yield peroxynitrite [32], which has been demonstrated to inhibit a number of critical molecules in the mitochondrial respiratory chain and antioxidant enzymes such as Mn-SOD [4]. Depression of the protective capability against oxidative stress by antioxidant enzymes may lead to larger tissue damage and initiate a vicious cycle by increasing ROS production, thereby exceeding the antioxidant liver capacity and resulting in further oxidative damage. These self-amplifying cycles of damage were clearly blocked in the group of animals receiving glycine.

Changes in mRNA levels of antioxidant genes in hemorrhagic shock were not entirely consistent with the changes in activities of corresponding enzymes. This coincides with previous data in different pathological situations. Thus, in experimental colitis SOD activities are suppressed while MnSOD mRNA level increases and that of Cu,Zn-SOD remains unchanged [41]. In liver of N1S1 transplanted hepatoma the mRNA levels of Cu,Zn-SOD and Mn-SOD show either no change or an increase, and those for glutathione peroxidase or catalase decrease while all enzyme activities are reduced [42]. Increased mRNA levels and decreased enzyme activities have been found with GPx in animals exposed to ethanol [43]. Whether the observed decrease in enzyme activities leads by autoregulatory loops to compensatory increase in mRNA levels or whether the decrease in activity is the result of decreased translation of mRNA or inhibition of enzymes cannot be deduced from this study. Several possibilities could, however, be pointed out. TNFα is known to induce Mn-SOD but not Cu,Zn-SOD [44], and previous reports have revealed a correlation between the activation of NF-κB and the induction of the Mn-SOD gene [45]. An inhibition of the translation of the Mn-SOD mRNA could be induced by NO [46] and, alternatively, NO could participate in posttranslational modifications of SODs, because nitration of tyrosine residues and inactivation of SOD by peroxynitrite has been reported [47]. Inactivation of GPx and catalase activities by exposure to oxidants and aldehydic by-products of lipid peroxidation has been previously reported [48].

In addition to promoting direct toxicity, oxidative stress

may also initiate or amplify inflammation through upregulation of genes involved in the inflammatory response. One such gene is NF-κB, whose activation results, in turn, in upregulation of TNFα and other inflammatory cytokines [49]. NF-κB becomes activated by a wide variety of stimuli, including growth factors, cytokines, NO, and oxidative stress [50]. ROS are potent activators of NF-κB, while antioxidants such as vitamin E or overexpression of antioxidants enzymes can block activation of NF-κB [51]. This transcription factor is required for the induction of iNOS gene in response to cytokines [32,52], and steady state of iNOS mRNA may also be affected at its gene transcription level by glutathione through an effect also closely related to NF-κB [53]. In a mouse model of hemorrhagic shock it has been reported that induction of iNOS is associated with activation of NF-κB and both are prevented by the infusion of an iNOS inhibitor [54]. In our experiments increased levels of iNOS mRNA, changes in the GSSG/GSH ratio, and oxidative stress were accompanied by NF-κB activation, and all these effects were blocked by dietary glycine. Regardless of the limitations of the study our findings suggest that NF-κB may be activated by ROS after hemorrhagic shock and play a role in the induction of iNOS gene transcription.

In conclusion, results obtained indicate that dietary glycine has potent protective effects against liver injury and death caused by hemorrhagic shock, preventing oxidative stress and blocking posttranslational inhibition of antioxidant enzymes, TNFα production, and iNOS overexpression. Although the causal relationship between the events here described and pathological changes is uncertain, the activation of NF-κB constitutes one mechanism by which hemorrhagic shock promotes liver injury and NF-κB activation was markedly attenuated by glycine. If shown to work, glycine might be included in the formulation of specific supplementary diets for situations, i.e., high-risk sports, in which hemorrhagic shock could be anticipated.

Acknowledgements — This work was partially supported by Novartis Nutrition AG. The authors acknowledge Dr. J. C. Fernández-Checa for technical assistance.

REFERENCES

[1] Kobel, F.; Schreck, U.; Henrich, H. A. Involvement of liver in the decompensation of hemorrhagic shock. *Shock* **2:**281–288; 1994.
[2] Wang, P.; Ayala, A.; Dean, R. E.; Hauptman, J. G.; Ba, Z. F.; DeJong, G. K.; Chaudry, I. H. Adequate crystalloid resuscitation restores but fails to maintain the active hepatocellular function following hemorrhagic shock. *J. Trauma* **31:**601–606; 1991.
[3] Abello, P. A.; Buchman, T. G.; Bulkley, G. B. Shock and multiple organ failure. *Adv. Exp. Med. Biol.* **366:**253–268; 1994.
[4] Szabo, C.; Billiar, T. R. Novel roles of nitric oxide in hemorrhagic shock. *Shock* **12:**1–9; 1999.
[5] Jiang, J.; Diao, Y.; Chen, H.; Zhu, P.; Wang, Z. Effect of hemorrhagic shock on endotoxin-inducing TNF production and intra-tissue lypopolysaccharide-binding protein mRNA expression and their relationship. *Shock* **7:**206–210; 1997.
[6] Silomon, M.; Pizanis, A.; Rose, S. Oxyradical-mediated hepatocellular Ca^{2+} alterations during hemorrhagic shock and resuscitation. *Shock* **11:**193–198; 1999.
[7] García-Ruiz, C.; Colell, A.; Morales, A.; Kapolwitz, N.; Fernández-Checa, J. C. Role of oxidative stress generated from mitochondrial electron transport chain and mitochondrial glutathione status in loss of mitochondrial function and activation of transcription nuclear factor κB: studies with isolated mitochondria and rat hepatocytes. *Mol. Pharmacol.* **48:**825–834; 1995.
[8] Wulczyn, F. G.; Krappmann, D.; Scheidereit, C. The NF-κB/Rel and Iκ B gene families: mediators of immune response and inflammation. *J. Mol. Med.* **74:**749–769; 1996.
[9] Ikejima, K.; Iimuro, Y.; Forman, D. T.; Thurman, R. G. A diet containing glycine improves survival in endotoxin shock in the rat. *Am. J. Physiol.* **271:**G97–G103; 1996.
[10] Yang, S.; Koo, D. J.; Chaudry, I. H.; Wang, P. Glycine attenuates hepatocellular depression during early sepsis and reduces sepsis-induced mortality. *Crit. Care Med.* **29:**1201–1206; 2001.
[11] Zhong, Z.; Jones, S.; Thurman, R. G. Glycine minimizes reperfusion injury in a low-flow, reflow liver perfusion model in the rat. *Am. J. Physiol.* **270:**G332–G338; 1996.
[12] Iimura, Y.; Bradford, B.; Forman, D. T.; Thurmna, R. G. Glycine prevents alcohol-induced liver injury by decreasing alcohol in the stomach. *Gastroenterology* **110:**1536–1542; 1996.
[13] Deters, M.; Siegers, C. P.; Strubelt, O. Influence of glycine on the damage induced in isolated perfused rat liver by five hepatotoxic agents. *Toxicology* **128:**63–72; 1998.
[14] Zhong, Z.; Enomoto, N.; Connor, H. D.; Moss, N.; Mason, R. P.; Thurman, R. G. Glycine improves survival after hemorrhagic shock in the rat. *Shock* **12:**54–62; 1999.
[15] Stachlewitz, R. E.; Seabra, V.; Bradford, B.; Bradham, C. A.; Rusyn, I.; Germolec, D.; Thurman, R. G. Glycine and uridine prevent D-galactosamine hepatotoxicity in the rat: role of Kupffer cells. *Hepatology* **29:**737–745; 1999.
[16] Zhong, Z.; Connor, H. D.; Yin, M.; Moss, N.; Mason, R. P.; Bunzedah, H.; Forman, D. T.; Thurman, R. G. Dietary glycine and renal denervation prevents cyclosporine A-induced hydroxyl radical production in rat kidney. *Mol. Pharmacol.* **56:**455–463; 1999.
[17] National Academy of Sciences. *The guiding principles for research involving animals*. Bethesda, MD: National Institutes of Health; 1991.
[18] Marzi, I.; Maier, M.; Herzog, C.; Bauer, M. Influence of pentoxifylline and albifylline on liver microcirculation and leukocyte adhesion after hemorrhagic shock in the rat. *J. Trauma* **40:**90–96; 1996.
[19] Rose, S.; Pizanis, A.; Silomon, A. Altered hepatocellular Ca^{2+} regulation during hemorrhagic shock and resuscitation. *Hepatology* **25:** 379–384; 1997.
[20] Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, A. J. Protein measurement with the Pholin phenol reagent. *J. Biol. Chem.* **193:** 265–275; 1951.
[21] Hissin, P. J.; Hill, R. A. Fluorimetric method for determination of oxidised and reduced glutathione in tissues. *Anal. Biochem.* **74:**214–226; 1976.
[22] Misra, H. P.; Fridovich, I. The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* **247:**3170–3175; 1972.
[23] Aebi, H. Catalase in vitro. *Methods Enzymol.* **105:**121–126; 1984.
[24] Flohe, L.; Gunsler, W. A. Glutathione peroxidase. *Methods. Enzymol.* **105:**115–121; 1984.
[25] Ohkawa, H.; Ohnishi, N.; Yagi, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **95:** 351–358; 1979.
[26] Cattell, V.; Cook, T.; Moncada, S. Glomeruli synthesise nitrite in experimental nephrotoxic nephritis. *Kidney Int.* **38:**1056–1060; 1990.
[27] Essani, N. A.; McGuire, G. M.; Manning, A. M.; Jaeschke, H. Endotoxin-induced activation of the nuclear transcription factor NF-κB in hepatocytes, Kupffer cells and endothelial cells in vivo. *J. Immunol.* **156:**2956–2963; 1996.
[28] Morales, A.; García-Ruiz, C.; Miranda, M.; Mari, M.; Colell, A.; Ardite, E.; Fernández-Checa, J.C. Tumor necrosis factor increases

hepatocellular glutathione by transcriptional regulation of the heavy subunit chain of γ-glutamylcysteine synthetase. *J. Biol. Chem.* **272:** 11369–11378; 1997.
[29] Snedecor, G. W.; Cochran, W. G. *Statistical methods.* Ames, IA: Iowa State University; 1980:185–191.
[30] Marzi, I.; Bauer, M.; Secchi, A.; Bahrami, S.; Redi, H.; Schlag, G. Effects of anti-tumour necrosis factor a on leukocyte adhesion in the liver after hemorrhagic shock: an intravital microscopic study in the rat. *Shock* **3:**27–33; 1995.
[31] Bahrami, S.; Yao, Y.; Leichtfried, G.; Redl, H.; Marzi, I.; Schlag, G. Significance of TNF in hemorrhage-related alterations, organ injury and mortality in rats. *Am. J. Physiol.* **272:**H2219–H2226; 1997.
[32] Liaudet, L.; García-Soriano, F.; Szabo, C. Biology of nitric oxide signalling. *Crit. Care Med.* **28:**N37–N52; 2000.
[33] Hua, T. C.; Moochhala, S. M. Influence of L-arginine, aminoguanidine and N°-nitro-L-arginine methyl ester on the survival rate in a rat model of hemorrhagic shock. *Shock* **11:**51–57; 1999.
[34] Guarini, S.; Bazzani, C.; Ricigliano, G. M.; Bini, A.; Tomasi, A.; Bertolini, A. Influence of ACTH-(1–24) on free radical levels in the blood of hemorrhage-shocked rats: direct ex vivo detection by electron spin resonance spectrometry. *Br. J. Pharmacol.* **119:**29–34; 1996.
[35] Bauer, C.; Walcher, F.; Holanda. M.; Mertzluff, F.; Larsen, R.; Marzi, I. Antioxidative resuscitation solution prevents leukocyte adhesion in the liver after hemorrhagic shock. *J. Trauma* **46:**886–893; 1999.
[36] Michiels, C.; Raes, M.; Toussaint, O.; Ramacle, J. Importance of Se-glutathione peroxidase, catalase and Cu,Zn-SOD for cell survival against oxidative stress. *Free Radic. Biol. Med.* **17:**235–248; 1994.
[37] Brown, G. C. Reversible binding and inhibition of catalase by nitric oxide. *Eur. J. Biochem.* **232:**188–191; 1995.
[38] Joe, B.; Lokesh, B. R. Studies on the inactivation of superoxide dismutase activity by nitric oxide from rat peritoneal macrophages. *Mol. Cell. Biochem.* **168:**87–93; 1997.
[39] Santiard, D.; Ribière, C.; Nordmann, R.; Houee-Levin, C. Inactivation of Cu,Zn-superoxide dismutase by free radicals derived from ethanol metabolism: a gamma radiolysis study. *Free Radic. Biol. Med.* **19:**121–127; 1995.
[40] Asahi, M.; Fujii, J.; Suzuki, K.; Seo, H. G.; Kuzuya, T.; Hori, M.; Tada, M.; Fuji, S.; Taniguchi, N. Inactivation of glutathione peroxidase by nitric oxide. Implication for cytotoxicity. *J. Biol. Chem.* **270:**21035–21039; 1995.
[41] Seo, H. G.; Takata, I.; Nakamura, M.; Tatsumi, H.; Suzuki, K.; Fujii, J.; Taniguchi, N. Induction of nitric oxide synthase and concomitant suppression of superoxide dismutases in experimental colitis in rats. *Arch. Biochem. Biophys.* **324:**41–47; 1995.
[42] Zhung, Y.; Juan, C.; Lee, H.; Yin, P.; Chi, C.; Ku, H.; Li, A.; Wei, Y.; Tsai, H. Oxidative stress is insignificant in N1S1-transplanted hepatoma despite markedly declined activities of the antioxidant enzymes. *Oncol. Rep.* **6:**1313–1319; 1999.
[43] Nanji, A. A.; Griniuviene, B.; Hossein Sadrzadeh, S. M.; Levitsky, S.; McCully J. D. Effect of type of dietary fat and ethanol on antioxidant enzyme RNA induction in rat liver. *J. Lipid Res.* **36:**736–744; 1995.
[44] Tsan, M. F.; White, J. E.; Treanor, C.; Shaffer, J. B. Molecular basis for tumour necrosis factor-induced increase in pulmonary superoxide dismutase activities. *Am. J. Physiol.* **259:**L506–L512; 1990.
[45] Xu, Y.; Kiningham, K. K.; Devalaraja, M. N.; Yeh, C.; Majima, H.; Kasarksis, E. J.; St. Clair, D. K. An intronic NF-kappaB element is essential for induction of the human manganese superoxide dismutase gene by tumor necrosis factor-alpha and interleukin-1beta. *DNA Cell. Biol.* **18:**709–722; 1999.
[46] Drapier, J. C.; Hirling, H.; Wietzerbin, J.; Kaldy, P.; Kuhn, L. C. Biosynthesis of nitric oxide activates iron regulatory factor in macrophages. *EMBO J.* **12:**3643–3649; 1993.
[47] Ischiropoulos, H.; Zhu, L.; Chen, J.; Tsai, M.; Martin, J. C.; Smith, C. D.; Beckman, J. S. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Arch Biochem. Biophys.* **298:**431–437; 1992.
[48] Povalarapu, R.; Spitz, D. R.; Sim, J. E.; Follansbee, M.; Oberley, L.W.; Rahemtulla, A.; Nanji, A. A. Increased lipid peroxidation and impaired antioxidant enzyme function is associated with pathological liver injury in experimental alcoholic liver disease in rats fed diets high in corn oil and fish oil. *Hepatology* **27:**1317–1323; 1998.
[49] Bowie, A.; O'Neill, L. A. Oxidative stress and nuclear factor-kappa B activation: a reassessment of the evidence on the light of recent discoveries. *Biochem. Pharmacol.* **59:**13–23; 2000.
[50] Nanji, A. A.; Jokelainen, K.; Rahemtulla, A.; Fogt, F.; Matsumoto, H.; Tahan, S. R.; Su, G. L. Activation of nuclear factor kappa B and cytokine imbalance in experimental alcoholic liver disease in the rat. *Hepatology* **30:**934–943; 1999.
[51] Nilakantan, V.; Spear, B. T.; Glauert, H. P. Liver-specific catalase expression in transgenic mice inhibits NF-κB activation and DNA synthesis induced by the peroxisome proliferator ciprofibrate. *Carcinogenesis* **19:**631–637; 1998.
[52] Hur. G. M.; Ryu, Y. S.; Yun, H. Y.; Jeon, B. H.; Kim, Y. M.; Seok, J. H.; Lee, J. H. Hepatic ischemia/reperfusion in rats induces iNOS gene transcription by activation of NF-κB. *Biochem. Biophys. Res. Commun.* **261:**917–922; 1999.
[53] Chen, G.; Wang, S. H.; Warner, T. D. Regulation of iNOS mRNA levels in endothelial cells by glutathione, a double-edged sword. *Free Radic. Res.* **32:**223–234; 2000.
[54] Hierholzer, C.; Harbrecht, B.; Menezes, J. M.; Kane, J.; MacMicking, J.; Nathan, C. F.; Peitzman, A. B.; Billiar, T. R.; Tweardy, D. J. Essential role of induced nitric oxide in the initiation of the inflammatory response after hemorrhagic shock. *J. Exp. Med.* **187:**917–928; 1998.

ABBREVIATIONS

ALT—alanine aminotransferase
AST—aspartate aminotransferase
bp—base pairs
CAT—catalase
cpm—counts per minute
EMSA—Electrophoretic mobility shift assay
GPx—glutathione peroxidase
GSH—reduced glutathione
GSSG—oxidized glutathione
iNOS—nitric oxide synthase
NF-κB—nuclear factor kappa B
NO—nitric oxide
Poly(dI-dC)—poly deoxyinosine-deoxycytidine
ROS—reactive oxygen species
RT-PCR—reverse transcription-polymerase chain reaction
SOD—superoxide dismutase
TBARS—thiobarbituric acid reactive substances
TNFα—tumor necrosis factor α