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# Prevention of Ischemia/Reperfusion Injury in the Rat Liver by Atrial Natriuretic Peptide

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**Background/Aims:** Atrial natriuretic peptide (ANP) protects against hypoxia/reoxygenation-induced damage of cultured hepatocytes, thus suggesting a therapeutic potential in the liver. Therefore, the effects of ANP on hepatic ischemia/reperfusion injury after warm ischemia were studied. **Methods:** Livers of male Sprague-Dawley rats subjected to 60 minutes of warm ischemia at 37°C were perfused in the presence or absence of 200 and 20 nmol/L ANP. **Results:** Sinusoidal lactate dehydrogenase efflux increased to  $2000 \pm 264$  and  $126 \pm 50$  mU·min<sup>-1</sup>·g liver<sup>-1</sup> after 1 minute and 60 minutes of reperfusion, but it only increased to  $1240 \pm 160$  and  $22 \pm 16$  mU·min<sup>-1</sup>·g liver<sup>-1</sup> in the presence of 200 nmol/L ANP during the preischemic and postischemic perfusion period. The postischemic bile flow ( $0.67 \pm 0.18$  μL·min<sup>-1</sup>·g liver<sup>-1</sup>) was significantly improved with 200 nmol/L ANP ( $0.92 \pm 0.05$ ) and showed a linear correlation to biliary glutathione excretion. In contrast, 20 nmol/L ANP had no protective effects. Administration of 200 nmol/L ANP during the preischemic perfusion period alone (but not after starting reperfusion) markedly preserved postischemic liver function. **Conclusions:** Continuous ANP administration or ANP pretreatment alone prevents hepatic ischemia/reperfusion injury, possibly because of influences on intracellular signal transduction processes. The correlation between bile flow and biliary glutathione excretion supports the hypothesis that biliary glutathione transport is one of the osmotic driving forces in postischemic bile formation.

Ischemia/reperfusion-induced injury of the liver is a major clinical problem after liver transplantation, partial hepatectomy, and shock. The mechanisms of ischemic liver injury are not yet fully understood. Depletion of adenosine triphosphate, disturbance of intracellular calcium homeostasis, and activation of phospholipase A<sub>2</sub> are proposed as major pathophysiological processes during ischemia leading to cell injury, but the sequence of these events remains controversial.<sup>1-4</sup> The reperfusion of ischemic organs may lead to the aggravation of ischemic injury, which is generally referred to as reperfusion injury.<sup>5</sup> Inflammatory products of activated Kupffer's cells and recruited granulocytes, such as reactive oxygen species, could contribute to hepatic reperfusion injury.<sup>6-11</sup>

The incomplete understanding of the ischemia/reperfusion injury may explain the lack of established pharmacological interventions preventing ischemic liver damage.

Recent studies suggest a therapeutic potential of the atrial natriuretic peptide (ANP), a circulating hormone released mainly by the atria of mammalian hearts in response to volume expansion or cardiac hypoxia.<sup>12,13</sup> ANP, infused upon reperfusion, preserves kidney function after renal ischemia.<sup>14,15</sup> As a possible mechanism, the antagonism of catecholamine-mediated renal vasoconstriction resulting in an increase of glomerular filtration rate and tubular flow and the prevention of intratubular obstruction by protein casts have been discussed. Furthermore, ANP protects cultured hepatocytes against damage induced by hypoxia/reoxygenation or oxidative stress.<sup>16,17</sup> It has been proposed that ANP exerts this cytoprotective effect by a cyclic guanosine monophosphate (cGMP)-mediated decrease of intracellular calcium.<sup>16</sup>

So far, the effect of ANP on the intact or ischemic liver has not been investigated. Therefore, we studied the effects of ANP on the ischemia/reperfusion damage after 60 minutes of warm ischemia using the isolated perfused rat liver. The sinusoidal release of lactate dehydrogenase (LDH), bile flow, and the carrier-mediated excretion of glutathione into bile<sup>18-21</sup> were determined as parameters of cell damage and postischemic liver function.

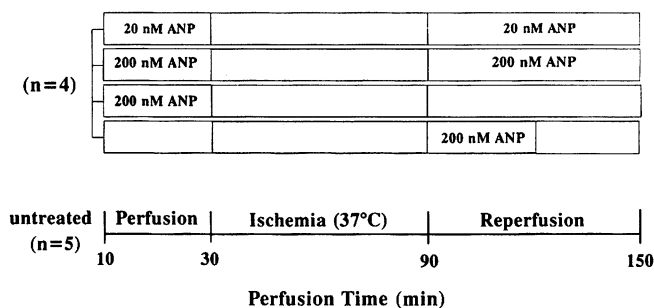
## Materials and Methods

### Perfusion of Rat Liver

Male Sprague-Dawley rats weighing 250–300 g were purchased from SAVO (Kisslegg, Germany) and housed in a climatized room with a 12-hour light-dark cycle. The animals had free access to chow (Standard-Diet, Altromin 1314 Lage, Germany) and water up to the time of the experiments. After anesthetizing the animals with pentobarbital (50 mg/kg body wt, intraperitoneally), the livers were perfused in situ with

**Abbreviations used in this paper:** ANP, atrial natriuretic peptide; DTNB, 5,5'-dithiobis(nitrobenzoic acid); GSH, reduced glutathione; GSSG, glutathione disulfide; LDH, lactate dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate.

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**Figure 1.** Experimental protocol. For detailed explanation, see Materials and Methods.

hemoglobin-free and albumin-free, bicarbonate-buffered Krebs-Henseleit solution (pH 7.4, 37°C) gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.<sup>22</sup> The perfusion medium was pumped through the livers with a membrane pump at a constant flow rate of 3.0–3.5 mL·min<sup>-1</sup>·g liver<sup>-1</sup> in a nonrecirculating fashion. The bile duct was cannulated with polyethylene PE 10 tubing, and bile was collected in preweighed tubes containing 4% sulfosalicylic acid to prevent autoxidation of reduced glutathione (GSH). After cannulation of the portal vein, the livers were perfused for 30 minutes. Thereafter, the perfusion flow was turned off for 60 minutes to produce warm ischemia (37°C). Then, the perfusion was continued for another 60 minutes. Portal perfusion pressure was monitored continuously during the total perfusion time (90 minutes). The study was registered with the local animal welfare committee.

### Experimental Design

Two groups of rats were studied with continuous liver perfusion without ischemia, and five groups were subjected to ischemia/reperfusion. Group 1 underwent continuous perfusion as follows: (1) controls, continuous perfusion for 90 minutes ( $n = 6$ ); and (2) 200 nmol/L ANP, continuous perfusion for 90 minutes with administration of dose between 40 and 70 minutes of perfusion ( $n = 4$ ). Group 2 was subjected to ischemia/reperfusion as follows (Figure 1): (1) *untreated*, 30 minutes of perfusion, 60 minutes of ischemia, and reperfusion for 60 minutes ( $n = 5$ ); (2) *20 nmol/L ANP*, 20 nmol/L ANP during 20 minutes until ischemia, 60 minutes of ischemia, and reperfusion for 60 minutes with 20 nmol/L ANP ( $n = 4$ ); (3) *200 nmol/L ANP*, 200 nmol/L ANP during 20 minutes until ischemia, 60 minutes of ischemia, and reperfusion for 60 minutes with 200 nmol/L ANP ( $n = 4$ ); (4) *200 nmol/L ANP before ischemia*, 200 nmol/L ANP during 20 minutes until ischemia, 60 minutes of ischemia, and reperfusion for 60 minutes without ANP ( $n = 4$ ); and (5) *200 nmol/L ANP after ischemia*, 30 minutes of perfusion, 60 minutes of ischemia, and reperfusion with 200 nmol/L ANP for 20 minutes after reperfusion ( $n = 4$ ).

Rat ANP (Novabiochem, Läufelfingen, Switzerland) was dissolved in perfusion buffer and infused into the portal inflow of the perfusion system.

### Analytical Methods

Glutathione disulfide (GSSG) in bile was measured by its reaction with nicotinamide adenine dinucleotide phosphate

(NADPH) catalyzed by GSSG reductase at 340–400 nm in a dual-wavelength spectrophotometer.<sup>23</sup> The concentration of GSH in bile and perfusate was measured together with GSSG in a kinetic assay using NADPH, GSSG reductase, and 5,5-dithiobis(nitrobenzoic acid) (DTNB).<sup>24</sup> GSH values were obtained by the difference between total glutathione (GSH + GSSG) and GSSG. The release of thiols into perfusate was determined by the reaction with DTNB at 412 nm.<sup>25</sup> Thiol concentrations were calculated assuming an extinction coefficient of  $E = 13.6 \text{ mmol} \cdot \text{L}^{-1} \cdot \text{cm}^{-1}$ .<sup>26</sup>

Glucose, lactate, pyruvate, and the activity of LDH released into the perfusate were analyzed according to standard tests.<sup>27</sup>

### Statistics

All data are expressed as the mean  $\pm$  SD. Statistical analysis of data was performed using analysis of variance.

### Results

#### ANP Administration and Continuous Nonischemic Liver Perfusion

During the perfusion period of 90 minutes, bile flow and the biliary excretion of GSH and GSSG declined

**Table 1.** Continuous Liver Perfusion in the Presence and Absence of 200 nmol/L ANP

	Minutes	Control	200 nmol/L ANP
Portal pressure (cm H <sub>2</sub> O)	30	4.6 $\pm$ 1.0	5.5 $\pm$ 1.3
	60	4.2 $\pm$ 0.7	5.7 $\pm$ 1.3
	90	3.8 $\pm$ 0.6	5.2 $\pm$ 1.8
LDH efflux (mU·min <sup>-1</sup> ·g <sup>-1</sup> )	30	4.6 $\pm$ 1.8	6.8 $\pm$ 3.7
	60	4.2 $\pm$ 1.2	6.4 $\pm$ 2.9
	90	10.4 $\pm$ 5.4	10.7 $\pm$ 4.0
Glucose efflux ( $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ )	30	0.90 $\pm$ 0.15	1.0 $\pm$ 0.24
	60	0.61 $\pm$ 0.23	0.60 $\pm$ 0.03
	90	0.58 $\pm$ 0.29	0.49 $\pm$ 0.16
Lactate efflux ( $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ )	30	0.77 $\pm$ 0.19	0.57 $\pm$ 0.24
	60	0.74 $\pm$ 0.29	0.41 $\pm$ 0.13
	90	0.63 $\pm$ 0.27	0.36 $\pm$ 0.12
Lactate-pyruvate ratio	30	5.2 $\pm$ 2.9	6.8 $\pm$ 3.5
	60	7.5 $\pm$ 3.2	6.4 $\pm$ 4.5
	90	6.4 $\pm$ 2.6	5.4 $\pm$ 1.1
Bile flow ( $\mu\text{L} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ )	30	1.31 $\pm$ 0.05	1.22 $\pm$ 0.21
	60	1.07 $\pm$ 0.1	1.09 $\pm$ 0.1
	90	0.94 $\pm$ 0.14	0.97 $\pm$ 0.11
Biliary GSSG release (nmol·min <sup>-1</sup> ·g <sup>-1</sup> )	30	0.70 $\pm$ 0.24	0.74 $\pm$ 0.19
	60	0.61 $\pm$ 0.13	0.60 $\pm$ 0.19
	90	0.52 $\pm$ 0.17	0.57 $\pm$ 0.21
Biliary GSH release (nmol·min <sup>-1</sup> ·g <sup>-1</sup> )	30	2.44 $\pm$ 0.80	2.19 $\pm$ 1.06
	60	1.73 $\pm$ 0.97	1.43 $\pm$ 0.74
	90	1.03 $\pm$ 0.60	0.97 $\pm$ 0.29

NOTE. Livers were continuously perfused with oxygenated Krebs-Henseleit buffer for 90 minutes (control,  $n = 6$ ) and compared with livers exposed to 200 nmol/L ANP from 40 to 70 minutes of perfusion time ( $n = 4$ ). Biliary and sinusoidal efflux rates are calculated from biliary and sinusoidal concentrations multiplied by the rate of bile flow and perfusate flow per minutes and grams liver weight. Data are given as mean  $\pm$  SD. There was no significant difference between ANP-treated livers and controls.



**Table 2.** Sinusoidal Efflux Rates of LDH, Thiols, GSH, Glucose, and Lactate During Reperfusion

	1 min of reperfusion		60 min of reperfusion	
	Untreated	200 nmol/L ANP	Untreated	200 nmol/L ANP
LDH ( $mU \cdot min^{-1} \cdot g^{-1}$ )	2000 $\pm$ 264	1240 $\pm$ 160 <sup>a</sup>	126 $\pm$ 50	22 $\pm$ 16 <sup>a</sup>
Thiol ( $nmol \cdot min^{-1} \cdot g^{-1}$ )	226 $\pm$ 44	137 $\pm$ 33 <sup>b</sup>	25.0 $\pm$ 3.5	19.1 $\pm$ 1.3 <sup>b</sup>
GSH ( $nmol \cdot min^{-1} \cdot g^{-1}$ )	91 $\pm$ 22	64 $\pm$ 12 <sup>b</sup>	14.7 $\pm$ 1.8	12.7 $\pm$ 2.3
Lactate ( $\mu mol \cdot min^{-1} \cdot g^{-1}$ )	12.3 $\pm$ 1.3	12.8 $\pm$ 1.4	0.20 $\pm$ 0.15	0.42 $\pm$ 0.19
Lactate-pyruvate ratio	ND	ND	9.1 $\pm$ 3.7	8.1 $\pm$ 5.2

NOTE. Livers were perfused for 30 minutes and then subjected to 60 minutes of warm ischemia. Sinusoidal efflux rates of untreated livers (n = 5) after 1 minute and 60 minutes of reperfusion are compared with livers exposed to 200 nmol/L ANP (n = 4) during the preischemic and postischemic perfusion period. Data are expressed as mean  $\pm$  SD.

ND, not determined.

<sup>a</sup>P < 0.01 (treated vs. untreated group).

<sup>b</sup>P < 0.05 (treated vs. untreated group).

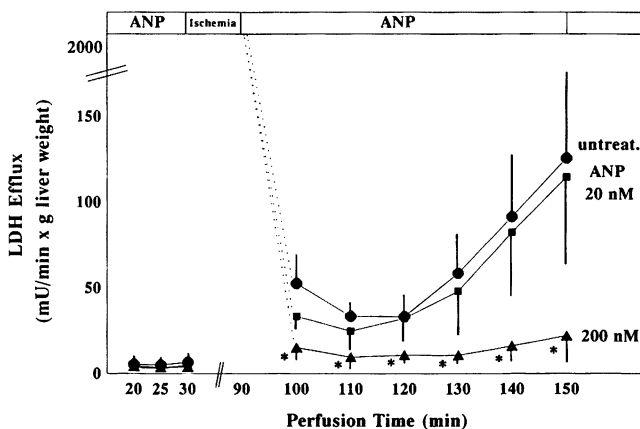
slowly (Table 1), which is comparable with previous results.<sup>28,29</sup> Thirty minutes after cannulating the portal vein, the biliary excretion of total glutathione (sum of GSH and GSSG) was  $3.92 \pm 1.26$  nmol  $\cdot$  min<sup>-1</sup>  $\cdot$  g liver<sup>-1</sup> (expressed in GSH equivalents) and decreased to  $2.07 \pm 0.92$  nmol  $\cdot$  min<sup>-1</sup>  $\cdot$  g liver<sup>-1</sup> at 90 minutes, corresponding to  $52\% \pm 7\%$  of the value at 30 minutes. Portal pressure remained nearly constant during the perfusion period. LDH release into the perfusate increased slightly, indicating negligible cell damage until the end of perfusion. Sinusoidal glucose and lactate efflux rates decreased continuously until the end of perfusion. When 200 nmol/L ANP was infused over 30 minutes, neither portal pressure nor bile flow nor biliary excretion of GSH and GSSG were affected. Thus, the excretion of total glutathione ( $1.95 \pm 0.74$  nmol  $\cdot$  min<sup>-1</sup>  $\cdot$  g liver<sup>-1</sup>;  $60\% \pm 11\%$ ) was similar to that of untreated controls at the end of perfusion. Equally, 200 nmol/L ANP did not influence the lactate-pyruvate ratio or the sinusoidal efflux rates of glucose, lactate, and LDH. Thus, bile acid-independent bile flow, biliary glutathione transport, and hepatic glucose production are not modulated by 200 nmol/L ANP.

### Continuous Administration of ANP and Ischemia/Reperfusion Damage of the Liver

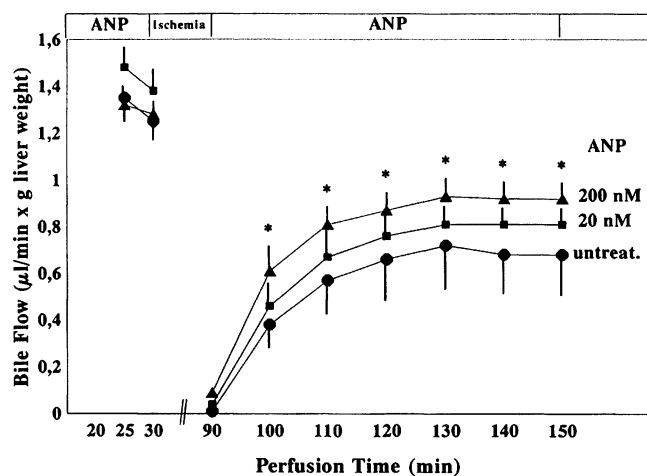
When livers were reperfused after 60 minutes of warm ischemia, LDH release markedly increased in the first minute of reflow to  $2000 \pm 264$  mU  $\cdot$  min<sup>-1</sup>  $\cdot$  g liver<sup>-1</sup> (Table 2) and decreased rapidly during the following 10 minutes of reperfusion, but did not return to basal values (Figure 2). LDH release again increased rapidly within 30 minutes of reperfusion, reflecting considerable cell damage. Administration of 200 nmol/L ANP during the preischemic and postischemic perfusion period resulted in a diminution of the peak LDH efflux ( $1240 \pm 116$  mU  $\cdot$  min<sup>-1</sup>  $\cdot$  g liver<sup>-1</sup>) 1 minute after starting reperfusion (Table 2). The LDH release then de-

creased to 7–14 mU  $\cdot$  min<sup>-1</sup>  $\cdot$  g liver<sup>-1</sup>, which was comparable with LDH efflux rates of livers continuously perfused without ischemia for the same duration (Table 1). In contrast, the time course of postischemic LDH release was uninfluenced in the presence of 20 nmol/L ANP.

In good agreement with previous results,<sup>28</sup> the sinusoidal efflux rates of thiols, GSH, and lactate were markedly increased during the initial washout phase (Table 2), reflecting accumulation of these metabolites in the extracellular space during ischemia. In ANP-treated livers, the efflux of thiols and GSH was significantly reduced (similar to LDH release), indicating that leakiness of cell membranes as a consequence of ischemic damage may be partially eliminated by ANP. During the following reperfusion period, the excretion of thiols and GSH by ANP-exposed livers was similar to that of untreated livers. The peak efflux rate of lactate was not affected by



**Figure 2.** Release of LDH into the perfusate during reperfusion of livers after 60 minutes of ischemia in the presence or absence of ANP. ANP, 20 nmol/L (■), or ANP, 200 nmol/L (▲), were infused for 20 minutes until perfusion was stopped and were infused again during the reperfusion period of 60 minutes (mean  $\pm$  SD; n = 4). \*P < 0.05 compared with untreated livers (●) (n = 5).



**Figure 3.** The effect of ANP on bile flow after 60 minutes of ischemia. ANP, 20 nmol/L (■), or ANP, 200 nmol/L (▲), were infused before and after ischemia as described in Figure 2 (mean  $\pm$  SD;  $n = 4$ ). \* $P < 0.05$  compared with untreated livers (●) ( $n = 5$ ).

ANP administration. After 60 minutes of reperfusion, lactate release was higher in ANP-treated livers, whereas the lactate-pyruvate ratio was unaffected (Table 1).

Postischemic bile flow reached  $0.68 \pm 0.18 \mu\text{L} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$  under control conditions and  $0.93 \pm 0.04$  in the presence of 200 nmol/L ANP (Figure 3). This significant improvement of postischemic bile flow was paralleled by an augmented biliary release of total glutathione (GSH plus GSSG) and GSSG, which recovered to  $50\% \pm 9\%$  vs.  $28\% \pm 15\%$  and  $78\% \pm 15\%$  vs.  $48\% \pm 23\%$  of preischemic efflux rates (Figure 4). Thus, the postischemic recovery of bile flow and biliary glutathione excretion were found to be similar to the values obtained in continuously perfused livers after 90 minutes. Again, 20 nmol/L ANP showed no significant effects on postischemic bile flow and biliary glutathione release (Figures 3 and 4).

During reperfusion, portal pressure increased markedly (Figure 5), reflecting an impaired hepatic microcirculation that may trigger postischemic cell damage and liver dysfunction. The time course of portal pressure during the reperfusion period was unaffected by administration of ANP (Figure 5), suggesting that the vasorelaxant nature of ANP may not be responsible for the observed protective effects.

#### Effects of Preischemic or Postischemic ANP Administration on Ischemia/Reperfusion Damage

To investigate the importance of the temporal relationships of ANP administration and ischemia for the protective action of ANP in more detail, livers were perfused with 200 nmol/L ANP in the preischemic or postischemic perfusion period only. As shown in Figure

6, LDH release at 60 minutes of reperfusion was significantly reduced by preischemic ANP infusion for 20 minutes ( $23 \pm 5 \text{ mU} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$ ), comparable with the time course of LDH release of continuously ANP-treated livers. In contrast, LDH release was slightly, but not significantly decreased ( $70 \pm 42 \text{ mU} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$ ) when ANP was administered during the reflow period only. The postischemic bile flow was markedly improved by ANP pretreatment ( $0.86 \pm 0.16 \mu\text{L} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$ ) but not by postischemic infusion of ANP ( $0.64 \pm 0.23 \mu\text{L} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$ ) (Figure 7). Thus, ANP pretreatment only, but not postischemic ANP administration, protects the liver against ischemia/reperfusion-induced damage.

#### Postischemic Bile Flow and Biliary Excretion of Glutathione

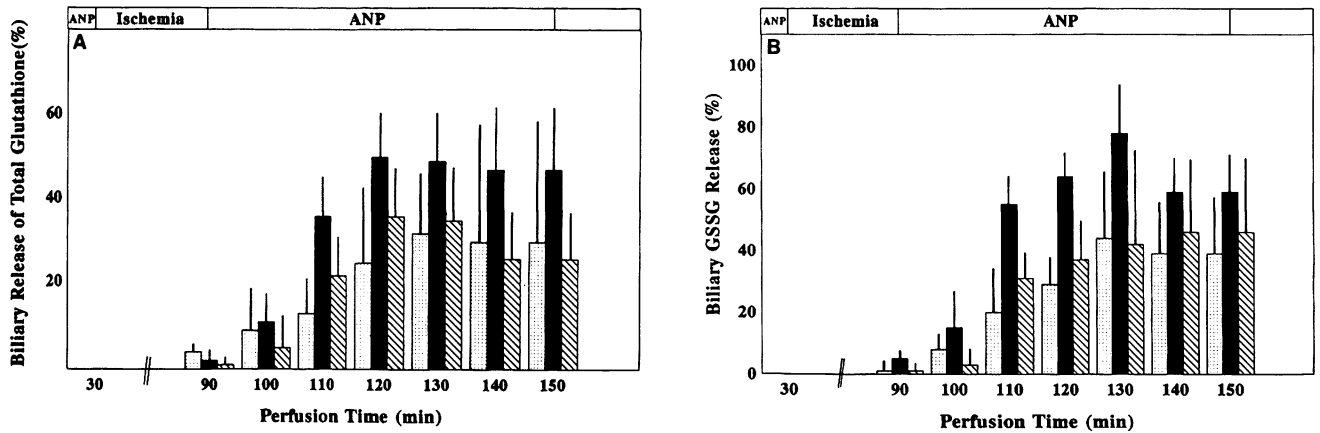
As shown in Figures 3 and 4, the improvement of bile flow by preischemic and postischemic ANP treatment was accompanied by a concomitant increase in biliary glutathione excretion. Similar results were obtained by preischemic ANP treatment, but not by postischemic administration. The observed changes in bile flow appeared to be independent of changes in bile acid excretion because a bile acid-free perfusion buffer was used. To examine whether postischemic bile flow depends on the biliary transport of glutathione, the data of all experimental groups were pooled to establish the correlation between bile flow and biliary excretion of total glutathione. Figure 8 illustrates the relation between bile flow and the rate of glutathione release after 60 minutes of continuous perfusion. A linear correlation ( $r = 0.96$ ;  $P < 0.001$ ) between bile flow and glutathione excretion was found, indicating that glutathione may be one of the osmotic driving forces in bile acid-independent bile formation also after hepatic ischemia.

Thus, preservation of postischemic bile flow by ANP can be partially explained as the consequence of improved biliary excretion of glutathione. The positive  $y$ -intercept in the plot ( $0.54 \mu\text{L} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$ ) indicates that in addition to glutathione, there are other compounds responsible for bile acid-independent bile secretion.

#### Discussion

##### Preservation of Postischemic Liver Function by ANP

The aim of the present study was to test the hypothesis that ANP protects against ischemia/reperfusion injury of the liver. This was based on observations that ANP prevented cell damage after hypoxia and reoxygenation in cultured hepatocytes.<sup>16</sup> Furthermore, ANP protects cultured hepatocytes against damage induced by

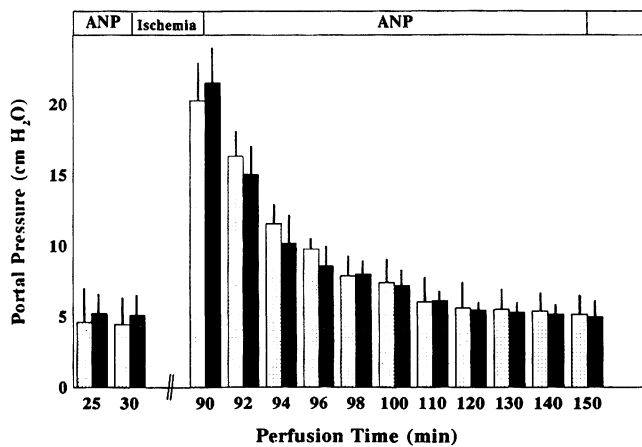


**Figure 4.** Relative increase of biliary GSSG and GSH release after 60 minutes of ischemia in the presence or absence of ANP. Rat livers were perfused with 200 nmol/L ANP (■), 20 nmol/L ANP (▨), or without ANP (□) (n = 5) as described in Figure 2. (A) Biliary efflux rates of total glutathione (GSH plus GSSG) and (B) GSSG (mean ± SD, n = 4) were compared with preischemic values in each group (100%). Biliary efflux rates of total glutathione and GSSG were improved by 200 nmol/L ANP but were not significantly different compared with the other groups.

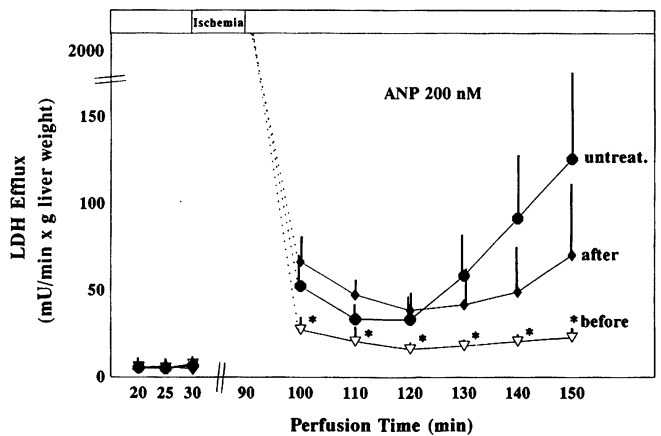
oxidants<sup>16,17</sup> that may contribute to hepatic ischemia/reperfusion injury.<sup>7</sup>

To investigate the influence of ANP on ischemia/reperfusion damage of the liver without additional effects of endogenous ANP,<sup>30</sup> we chose the model of the isolated perfused rat liver.<sup>31,32</sup> Reperfusion after 60 minutes of warm ischemia at 37°C resulted in a sustained increase of LDH efflux, indicating irreversible cell damage. Furthermore, bile flow (as an index of postischemic liver function)<sup>33</sup> recovered to only 50%–60% of values obtained in continuously perfused rat livers after the same perfusion period. These observations agree with previous reports on perfused rat livers subjected to 60 minutes of ischemia at 37°C<sup>34</sup> and prolonged warm ischemia (120 minutes) at room temperature.<sup>28</sup> Thus, a model of considerable hepatic ischemia/reperfusion damage was applied

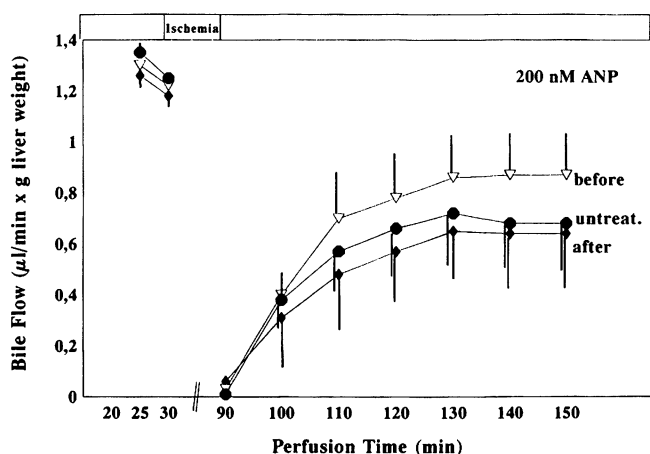
to study the pharmacological potential of ANP. Using this approach, we have shown that ANP protects the liver against ischemia/reflow-induced irreversible cell damage, yielding an improved postischemic liver function. The results showed that (1) 200 nmol/L ANP infused during the preischemic and postischemic perfusion period markedly reduced LDH release into the effluent perfusate on reperfusion, indicating diminution of cell damage; (2) postischemic recovery of biliary glutathione excretion and bile flow, which reflect postischemic liver function,<sup>33</sup> were improved and similar to values obtained in the continuously perfused rat liver after 90 minutes of perfusion; and (3) biliary parameters of continuously perfused livers were unaffected by ANP, rendering unlikely an ANP-induced stimulation of bile flow or transport processes that could mimic improved liver function.



**Figure 5.** Time course of portal pressure after ischemia in the presence and absence of ANP. ANP (200 nmol/L) was infused during the preischemic and postischemic perfusion period. Portal pressure was uninfluenced by ANP (■) compared with untreated livers (□) (n = 5; mean ± SD).



**Figure 6.** The effect of preischemic and postischemic administration of 200 nmol/L ANP on LDH release after 60 minutes of ischemia. ANP (200 nmol/L) was infused for 20 minutes until ischemia only (▽) and during the first 20 minutes after starting reperfusion (◆) (mean ± SD; n = 4). \*P < 0.05 compared with untreated livers (●) (n = 5).



**Figure 7.** The effect of preischemic and postischemic administration of 200 nmol/L ANP on bile flow after 60 minutes of ischemia. ANP (200 nmol/L) was infused as described in Figure 6. The recovery of bile flow was markedly improved by ANP pretreatment, but not significantly different compared with the other groups. ●, untreated livers; ∇, preischemic administration of ANP; ◆, postischemic administration of ANP (mean  $\pm$  SD; n = 4).

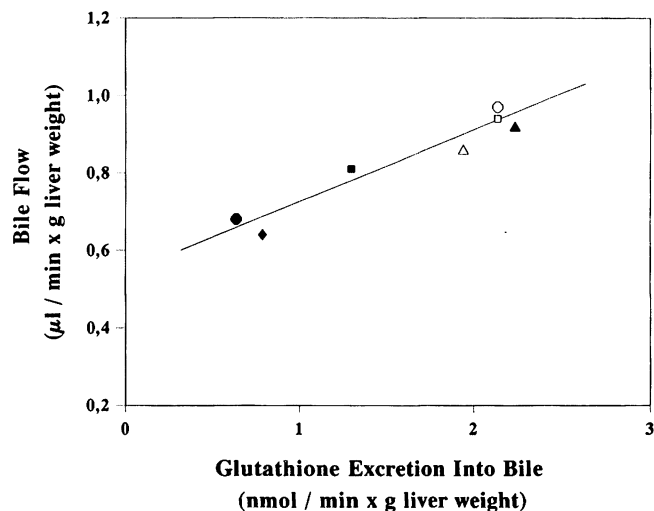
### Possible Mechanism of ANP Protection

After starting reperfusion, portal pressure transiently increased, then decreased and almost reached preischemic values after 10–20 minutes, reflecting impairment and consecutive redistribution of perfusate flow through the liver. The protective effects of vasodilating agents on liver function after long hypothermic preservation support the contention that an impairment of hepatic microcirculation may trigger postischemic cell damage.<sup>35</sup> We have recently shown that ANP antagonizes phenylephrine-induced vasoconstriction in the perfused rat liver.<sup>36</sup> Maximal vasodilating effects were observed at physiological ANP concentrations (40 pmol/L). In contrast, 200 nmol/L ANP had no effect on the time course of postischemic portal pressure. However, improvement of microcirculation need not necessarily result in changes of portal pressure, which may depend on the capacity of collateral shunt pathways. Moreover, 20 nmol/L ANP, three orders of magnitude higher than the physiological ANP concentration with maximal vasorelaxant effects, showed no protection. With respect to the vasodilating potency of ANP, the used concentrations are extremely high. Thus, different influences of both ANP concentrations on the hepatic microcirculation seem unlikely. These results suggest that the hepatic microcirculation is not improved by vasodilating effects of ANP or that improvement of microcirculation is not responsible for the observed protection. Consistent with this interpretation, infusion of 200 nmol/L ANP after starting reperfusion did not exhibit any significant effect on hepatic functional recovery, whereas ANP administration before ischemia improved postischemic liver func-

tion. Moreover, cultured hepatocytes subjected to hypoxia and reoxygenation were protected by preischemic ANP addition, but only to a minor extent by postischemic administration.<sup>16</sup> These findings lend additional support for a nonhemodynamic mechanism of ANP protection.

The beneficial effect of preischemic ANP treatment suggests that ANP-induced protective mechanisms must be initiated before ischemia to operate during ischemia. This view is supported by the marked reduction of LDH, thiol, and GSH efflux rates during the first minute of reperfusion in the presence of ANP. These molecules, which are not (or only to a minor extent) taken up by the liver, accumulate in the sinusoidal space as a consequence of increased cell leakiness during ischemia. The decreased efflux rates during the washout period thus reflect reduced damage of cell membranes during the ischemic period. Consequently, ANP protects against ischemic damage of the liver.

LDH efflux of untreated livers increased rapidly within 30 minutes of reperfusion, indicating aggravation of ischemic injury, which is generally referred to as reperfusion injury.<sup>5,37,38</sup> This later increase of LDH efflux was prevented by ANP. These findings suggest that ANP can



**Figure 8.** Relation between bile flow and glutathione excretion into bile after ischemia. For each experimental protocol, one data point is illustrated, representing bile flow and excretion of total glutathione (sum of GSH plus GSSG expressed as GSH equivalents) 90 minutes after starting reperfusion and 60 minutes after starting reperfusion, respectively. Treatment protocols were as follows: continuous perfusion (○, n = 6); continuous perfusion plus 200 nmol/L ANP from 40 to 70 minutes of perfusion time (□, n = 4); 60 minutes of ischemia (●, n = 5); 60 minutes of ischemia plus preischemic and postischemic administration of 200 nmol/L ANP (▲, n = 4); 60 minutes of ischemia plus preischemic administration of 200 nmol/L ANP (△, n = 4); 60 minutes of ischemia plus postischemic administration of 200 nmol/L ANP (◆, n = 4); and 60 minutes of ischemia plus preischemic and postischemic administration of 20 nmol/L ANP (■, n = 4). A linear correlation was found ( $r^2 = 0.96$ ;  $P < 0.001$ ) described by the equation  $y = 0.183x + 0.540$ .

also protect against hepatic reperfusion injury. Intracellular and vascular oxidant stress and the production of mediators of inflammation by activated Kupffer's cells may contribute to early reperfusion damage.<sup>9,10,11,34</sup> Besides these proposed pathomechanisms and their possible modulation by ANP, it is the extent of ischemic cell damage that may determine the vulnerability of hepatocytes to reperfusion injury. Then, prevention of hepatic reperfusion injury may be the consequence of the reduction of ischemic cell damage by ANP.

The lower dose of ANP (20 nmol/L) used in this protocol did not show any significant effects on hepatic functional recovery and cell damage after ischemia. This observation is quite consistent with experiments in the isolate-perfused kidney, which showed preservation of postischemic renal function by 300 nmol/L ANP, whereas 30 nmol/L ANP was without protective effects.<sup>15</sup> The physiological effects of ANP are mediated by ANP receptors<sup>39</sup> and may explain the concentration dependency of protection. Binding to the ANP-R1 receptor results in the activation of particulate guanylate cyclase, which converts guanosine triphosphate to cGMP, the second messenger that mediates most physiological effects of ANP.<sup>40,41</sup> Studies with cultured hepatocytes showed a concentration-dependent increase of cGMP that was maximal in the range of 0.1–1.0  $\mu\text{mol/L}$  ANP.<sup>16</sup> These data and the findings that ANP-mediated protection against hypoxic damage of cultured hepatocytes is eliminated in the presence of an inhibitor of guanylate cyclase<sup>16</sup> suggest a cGMP-mediated mechanism of cytoprotection. In contrast, the protective effect of ANP on hepatocytes can be blocked by pertussis toxin without lowering elevated cGMP levels, suggesting the involvement of G-proteins in the protective action of ANP.<sup>17</sup> Furthermore, ANP binds to ANP-R2 receptors, the physiological function of which is still poorly understood.<sup>39</sup> It has been suggested that the ANP-R2 receptor is associated with the activation of phospholipase C and increased formation of inositol phosphates.<sup>42</sup> Moreover, inhibition of sodium transport<sup>17,43</sup> and adenylate-cyclase<sup>44</sup> are discussed as ANP-R2 receptor-mediated processes; their role in prevention of ischemic organ damage has not been investigated.

Damage of cultured hepatocytes by oxidative stress and the accompanying increase in intracellular  $\text{Ca}^{2+}$  were simultaneously prevented by ANP, indicating that the maintenance of intracellular  $\text{Ca}^{2+}$ -homeostasis may contribute to the cytoprotective effects of ANP.<sup>16</sup> The increase in cytosolic  $\text{Ca}^{2+}$  is considered to be one of the principal factors responsible for the initiation of cell damage induced by ischemia, anoxia, or oxidative stress in the liver.<sup>2,45,46</sup> However, other studies showed no increase of intracellular  $\text{Ca}^{2+}$  until the onset of lethal cell injury,<sup>47</sup>

suggesting that  $\text{Ca}^{2+}$  increase is a consequence of cell damage. Thus, the modulation of other important pathophysiological processes by ANP, such as adenosine triphosphate depletion or cellular proteolysis during ischemia,<sup>48,49</sup> should be investigated to clarify the mechanism of ANP cytoprotection in further detail.

### Postischemic Bile Flow and Biliary Glutathione Transport

Because a bile acid-free perfusion buffer was used, the detected bile flow mainly represents the bile acid-independent bile formation,<sup>50</sup> which accounts for about half of the total canalicular bile flow in the rat. Ischemia/reperfusion injury impairs preferentially bile acid-independent bile flow.<sup>51</sup> To characterize postischemic bile formation in more detail, we quantified the biliary excretion of actively transported GSH<sup>20,21</sup> and GSSG,<sup>18,19</sup> which partially contribute to the bile acid-independent bile secretion in rat liver.<sup>52</sup> The biliary release of total glutathione and GSSG was markedly decreased during the reflow period. The carrier-mediated transport of molecules depends on their intracellular concentration. As shown previously, the intracellular concentration of GSH and GSSG is not substantially altered after 30 or 120 minutes of warm ischemia,<sup>28,53</sup> suggesting that biliary transport of GSH and GSSG is impaired after ischemia. ANP-treated livers showed an increased recovery of biliary glutathione and GSSG efflux during reperfusion. When postischemic bile flow was plotted against the biliary excretion of total glutathione, a linear correlation was obtained similar to studies under nonischemic conditions.<sup>52</sup> This shows that an increase in glutathione excretion is associated with an increase in postischemic bile formation. These findings provide strong support for the hypothesis that biliary transport of GSH and GSSG is one of the osmotic driving forces for hepatic bile formation after ischemia as well. However, these results cannot exclude the possibility that bile flow and glutathione transport are being affected in parallel but independently. The biliary GSSG-GSH ratio, a sensitive index of an intracellular oxidant stress,<sup>18,54</sup> and the sinusoidal efflux of GSH were not increased in the presence of ANP. This indicates that augmented biliary GSH and GSSG release is not the consequence of stimulated intracellular GSH synthesis or GSH oxidation to GSSG. It is more likely that improved transport of GSH into bile with subsequent autoxidation to GSSG or, in addition, preservation of both transport processes by ANP explain our results; this agrees with the finding that ANP did not increase the export of these molecules in continuously perfused livers. The enhanced transport of glutathione may explain the improvement of postischemic bile salt-independent bile flow. Other molecules additionally contribute to

posts ischemic bile flow, as shown by the positive  $\gamma$ -intercept in the plot of bile flow vs. glutathione export.

In conclusion, continuous preischemic and posts ischemic administration of 200 nmol/L ANP or preischemic ANP infusion alone reduced ischemia/reperfusion damage of the liver. This was accompanied by improved posts ischemic bile flow and biliary excretion of glutathione. Thus, evidence was found that ANP can act as a hepatoprotective hormone that preserves posts ischemic liver function after 60 minutes of warm ischemia. The strong correlation between posts ischemic bile flow and biliary glutathione excretion indicates that transport of glutathione is an important osmotic driving force for hepatic bile formation after liver ischemia. Finally, these results could suggest a therapeutic potential of ANP in liver resection and graft storage for liver transplantation, which requires further investigation using in vivo models.

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