CMLS, Cell. Mol. Life Sci. 58 (2001) 1708–1714
1420-682X/01/111708-07 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Significance and molecular targets of protein kinase A during cAMP-mediated protection of cold stored liver grafts

S. Akbar and T. Minor*

Division of Surgical Research, University Clinic of Surgery, Sigmund Freud Str. 25, 53127 Bonn (Germany)
Fax: +49 228 287 5199, e-mail: umc20001@uni-bonn.de

Received 12 July 2001; received after revision 14 August 2001; accepted 14 August 2001

Abstract. The use of marginal donor livers is followed by a higher frequency of primary dys- or nonfunction after transplantation. The present study was designed to test the hypothesis that stimulation of the cAMP second-messenger signal pathway might protect the liver from ischemic injury, laying emphasis on the role of protein kinase A-mediated signal transduction.
Rat livers were harvested after 45 min of cardiac arrest and preserved in HTK solution for 24 h. Hepatic integrity was assessed thereafter using a blood-free reperfusion model.
Supplementation of the preservation solution with dibutyryl-cAMP (db-cAMP) promoted phosphorylation of BAD at Ser 112 and concomitantly mitigated mitochondrial release of cytochrome c into the cytosol. Apoptotic cell transformation was evident in reperfused livers by positive TUNEL-staining of sinusoidal lining cells and the detection of cleaved poly(ADP-ribose) polymerase (PARP) in tissue homogenates by western analysis. Treatment with db-cAMP was effective in minimizing both TUNEL staining and PARP cleavage and significantly reduced postischemic enzyme leakage of alanine aminotransferase to one half, while hepatic bile production was enhanced by approximately 60% when compared to untreated livers. This functional improvement was accompanied by a net amelioration of portal vascular conductivity. Inhibition of A kinase-anchoring protein with HT31 completely reversed any of the observed effects obtained by db-cAMP.
We conclude that enhancement of cellular cAMP signal maintains hepatic integrity during and after ischemic preservation which may be attributed to protein kinase A dependent phosphorylation of BAD in line with subsequent inhibition of mitochondria-initiated apoptosis of sinusoidal lining cells.

Key words. Apoptosis; BAD; iNOS; ischemia; nitric oxide; stellate cell.

The disparity between the increasing demand for liver grafts and the limited supply of donor organs has led to reconsidering of the use of marginal donor pools such as non-heart-beating donors (NHBDs) [1]. In contrast to acceptable long-term results of renal transplantation [2], liver transplantation from NHBDs still demonstrates poor outcomes, as indicated by a high incidence of primary graft non and dysfunction [3, 4]. Thus a better understanding of the pathophysiology during preservation of the grafts from NHBDs and to improving their tolerance to cold storage are of great clinical importance. Technical alternatives for the ischemic ex vivo storage of the graft were raised with the intention of achieving aerobiosis of the tissue. Continuous oxygenated perfusion at normothermia has been shown to improve the outcome of porcine liver grafts from NHBDs [5] as has gaseous aeration of the cold-stored liver by vascular persufflation with molecular oxygen [6].
Recent work from our group has found that tissue aerobiosis during ischemia enhances cellular concentrations

* Corresponding author.

of cyclic AMP (cAMP) [7]. Enhancement of cellular cAMP in turn has been proposed to be protective upon reperfusion after ischemic insult [8, 9]. Cyclic nucleotides are known to modulate cell death processes in many tissues, and cAMP has shown anti-apoptotic properties in various cell types [10, 11]. Nevertheless, proapoptotic effects of β-adrenergic agonism have also been reported in other models [12].

Apoptotic cell transformation has most recently been discussed as a culprit for postischemic organ failure in marginal donor livers [13, 14], but little information is currently available about the precise mechanistic role of cAMP-related signal cascades in postischemic liver failure. Harada et al. [15] have now demonstrated in vitro that cAMP-dependent protein kinase A (PKA) is able to phosphorylate the proapoptotic molecule BAD at Ser 112, inactivating BAD and reducing apoptotic cell death in isolated H-89 cells. Given that PKA activity decreases in liver grafts from NHBDs [16], the present study was designed to test the hypothesis that direct stimulation of the cAMP second-messenger signal pathway might protect the predamaged donor liver from cold preservation, laying emphasis on the role of PKA-mediated signal transduction.

Materials and methods

Preparation of whole-cell lysates

Using an Ultra-Turrax (Janke and Kunkel, Staufen, Germany), frozen liver tissue was homogenized on ice in 30 vol of hypotonic lysis buffer, designed to prevent degradation of the phosphorylation sites during preparation (20 mM Tris HCl, pH 7.5, 50 mM NaF, 50 mM Na_3VO_4, 20 mM nitrophenyl phosphate, 1 mM ethyleneglycol tetraacetic acid, 1% Triton X 100, 5 mM freshly added benzamidine). The suspension was left on ice for 30 min and then centrifuged at 4 °C for 15 min at 15,000 g. Aliquots from the clear supernatant were diluted to 2 mg protein/ml after determination of protein content by Bradford's method [17].

Western Blot

Equal amounts of protein (20 mg) were suspended in Laemmli buffer and loaded on SDS-PAGE. After gel electrophoresis, proteins were blotted onto nitrocellulose membrane (0.2 µm) and homogeneous transfer confirmed by staining with Ponceau S. Membranes were then blocked for 1 h at room temperature with 5% non fat-dry milk in Tris-buffered saline + Tween 20 and subsequently incubated overnight with the respective primary antibodies: (BAD and cleaved poly(ADP-ribose) polymerase (PARP) (89 kDa) (Cell Signalling Technology) and inducible nitric oxide synthase (iNOS) (Transduction Laboratories). Proteins were then visualized on X-ray film via chemiluminescence after exposure to horseradish peroxidase-conjugated secondary antibody (Phototope; New England Biolabs, Schwalbach/Taunus, Germany).

Detection of free cytosolic cytochrome c

Fresh liver tissue was cut from the left median lobe at the end of reperfusion and gently dounce-homogenised on ice in 30 vol of lysis buffer including 280 mM sucrose, and intact mitochondria and nucleoli were then sedimented by centrifugation in the cold for 30 min at 15,000 g. The clear supernatants were collected frozen at –80 °C and later used for gel electrophoresis and Western blot using a polyclonal antibody against cytochrome c (Santa Cruz SC-7159).

Immunoprecipitation and detection of BAD phosphorylation

Total BAD was immunoprecipitated from the final tissue lysate after 18 h incubation and gentle shaking at 4 °C with a phosphorylation state independent rabbit anti-rat BAD antibody (1/200; New England Biolabs) and subsequent addition of 50 µl/500 ml of protein A sepharose beads for 2 h at 4 °C. After pulse centrifugation, the supernatant was discarded, the beads washed three times with ice-cold lysis buffer and resuspended in Laemmli buffer. Immune complexes were cleared from the beads by heating to 95 °C for 5 min and microcentrifugation. Equal amounts (10 µl) of the supernatant were used for SDS-PAGE and subsequent transfer on 0.2 µm nitrocellulose membrane. Blots were blocked for 1 h at room temperature and incubated overnight with monoclonal mouse antibody against phosphorylated BAD (Ser 112), 1/500, at 4 °C. Specific bands were then visualized using chemiluminescence techniques [18].

RT-PCR analysis of iNOS-mRNA

Total RNA was isolated from snap-frozen samples using the Rneasy kit (Quiagen, Valencia, Calif.). Complementary DNA was compounded by incubation with 5 µM $d(T)_{15}$ primer (Roche Diagnostics, Mannheim, Germany) and 5 U/µl SuperScript RT (Life Technologies, Grand Island, N.Y.). Real-time PCR of iNOS cDNA was performed using the Lightcycler technology with the commercial LightCycler Master Mix and a modified standard protocol (Eurogentec SA, Seraing, Belgium). The amount of specific mRNA in the tissue was quantified taking into account possible differences in loading by dividing the signal of each RNA sample hybridized to the iNOS probe by that hybridized to the reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) probe.

Sense (5′-CACATCTGGCAGGATGAGAA-3′) and antisense (5′-GAAGGCGTAGCTGAACAAGG-3′) primers for iNOS (5 µM MgCl) and sense (5′-TGCCACTCAGAAGACTGTGG-3′) and antisense (5′-CAACGGATACATTGGGGGTA-3′) primers for GAPDH (2 µM MgCl)

were purchased from Metabion (Martinsried, Germany). Samples were denaturated at 95 °C for 30 s and subjected to 35 cycles at 95 °C for 1 s, 62 °C for 10 s, and 72 °C for 10 s. Measurement was done at 72 °C.

Animal model

In accordance with the German legislation on the protection of animals and the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 86-23, revised 1985), male Wistar rats with a body weight in the range of 250–300 g were used for the experiments.

Under general anesthesia (ketamine-hydrochloride/xylazine 90/10 mg/kg intramuscular) the abdomen was opened by midline incision, and cardiac arrest was induced by phrenotomy. Forty-five min after the heart had stopped beating, livers were excised and flushed with 60 ml of HTK preservation solution via a 16-gauge cannula inserted into the portal vein. The livers were randomly assigned to one of the following three groups: untreated (n = 6), harvest and preservation in HTK without additional treatment; cAMP (n = 6), dibutyryl-cAMP (db-cAMP, a membrane-permeable cAMP analogue; (Sigma Chemicals, Deisenhoven, Germany) was added to the HTK flushout and preservation solution at a final concentration of 2 µM; HT31 (n = 6), in addition to db-cAMP, stearated HT31, a novel, cell-permeable, and specific inhibitor of the protein kinase A (PKA)-anchoring protein (AKAP) (Promega, Mannheim, Germany) was also added to the HTK solution at a final concentration of 20 µM.

The livers were then stored in 125 ml of HTK solution at 4 °C for 24 h. During the cold storage period, a short 14-gauge polyethylene splint was telescoped into the suprahepatic vena cava and secured. The infrahepatic vena cava and phrenic veins were ligated.

Functional and structural integrity of the organs was tested thereafter upon reperfusion in vitro according to previously described techniques [19] in a recirculating system for 2 h at 37 °C with oxygenated (95% O_2-5%CO_2; $pO_2 > 500$ mmHg) Krebs-Henseleit buffer at a constant flow of approximately 3 ml/g × min.

Metabolic activity of the livers was approximated by the calculation of hepatic oxygen utilization. Perfusate samples were taken at the portal inflow and from the venous effluent and oxygen content was measured immediately in a pH-blood gas analyser (ABL 500 acid-base laboratory; Radiometer, Copenhagen, Denmark). Oxygen uptake of the livers was calculated from the differences between portal and venous sites and expressed as µl O_2/g liver per minute according to transhepatic flow and liver mass.

To measure bile production, the common bile duct of the livers was cannulated with 22 gauge polyethylene tubing. Bile was collected during the entire reperfusion period and hepatic bile production was calculated as µl/g per hour.

Hepatic leakage of cytosolic alanine aminotransferase (ALT) during reperfusion was determined in aliquots taken from the hepatic effluent using standard enzymatic methods.

To assess vascular conductivity, portal venous pressure was measured during isolated perfusion by means of a water column connected to the portal inflow line and precalibrated to the calculated flow of 3 ml/g per minute using PE-catheters of length and size identical to the one used for the perfusion of the livers. Total vascular resistance (TVR) was calculated from transhepatic flow and portal pressure and expressed in Pa s/ml^{-1} .

Hepatic release of NO was estimated in the effluent at the end of the experiments by measurement of the stable oxidation products, nitrate and nitrite. Nitrate was quantitatively reduced to nitrate using NADPH-dependent nitrate reductase [20] and total nitrite then determined colorimetrically by the Griess reaction, comparing absorption at 540 nm with external standards of sodium nitrite.

Statistics

Data are expressed as the mean ± SEM. Stochastic significance of differences was assessed using one-way analysis of variance and multiple comparison of the means with the SNK-test.

Results and Discussion

Supplementation of the preservation solution with db-cAMP resulted in a significant increase in BAD phosphorylation at Ser 112 as shown by Western analysis after immunoprecipitation of BAD (fig. 1). In addition, phosphorylation and thus inactivation of BAD were almost entirely counteracted in the presence of HT31, a peptide which inhibits the anchoring of PKA to mitochondrial membranes.

Since BAD phosphorylation is expected to release anti-apoptotic proteins of the Bcl family (e. g., Bcl-xl or Bcl 2) from dimerization with BAD [15], and these proteins are known to prevent translocation of intramitochondrial cytochrome c into the cytosol [21], we looked for free cytosolic cytochrome c in the reperfused liver homogenates (fig. 1). While only traces were found in non-ischemic control preparations, cytosolic translocation of cytochrome c was present in the untreated group. According to its effect on BAD phosphorylation, preservation with db-cAMP was accompanied by a relevant reduction in free cytosolic cytochrome c, which, however, was found to be counteracted by the addition of HT31.

In the cytosol, cytochrome c associates with Apaf-1 to eventually cleave procaspase-9, thus initiating a caspase cascade which leads to programmed cell death [15]. In cells undergoing apoptosis, PARP is cleaved by caspases resulting in the appearance of a PARP fragment of ap-

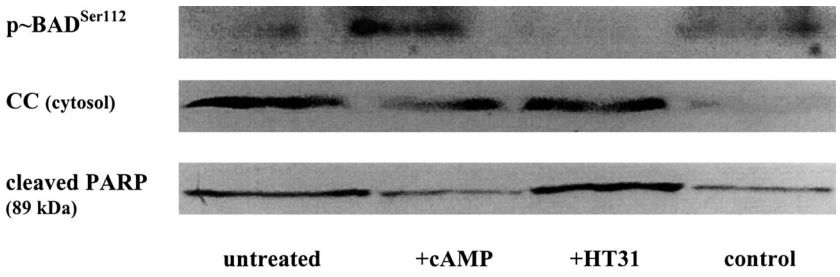

Figure 1. Effect of cAMP on BAD phosphorylation and induction of apoptosis after liver preservation. Upper panel: Western blot of immunoprecipitated BAD, phosphorylated at Ser 112. Middle panel: Western blot analysis of free cytochrome c in the cytosolic fraction of liver homogenates. Lower panel: apoptotic cleavage of PARP, visualized by the accumulation of the 89 kDa fragment. Blots are representative of three individuals per group with similar results. From left to right: (i) untreated livers; (ii) +cAMP, addition of 2 µM db-cAMP to the preservation fluid; (iii) +HT31, addition of db-cAMP (2 µM) and HT31 (20 µM), an inhibitor of protein kinase A- anchoring protein to the preservation fluid; (iv) control lysates from non-ischemic livers processed as a reference. Mean relative densitometric intensities in percent of control values were (untreted vs cAMP vs HT31 groups) 35 ± 3 vs 207 ± 34* vs 37 ± 20 for BAD phosphorylation, 763 ± 171 vs 273 ± 14* vs 631 ± 134 for cytosolic cytochrome c, and 292 ± 19 vs 134 ± 28* vs 348 ± 87 for PARP cleavage, * $p < 0.05$ vs untreated or HT31).

proximately 89 kDa, which can be used as a readout of cellular apoptosis.

Notable accumulation of this cleaved PARP fragment was found in untreated livers (fig. 1). In contrast, PARP cleavage was markedly attenuated by approximatively 50% after enhancement of the cAMP second-messenger signal pathway but remained, nonetheless, prominent when PKA anchoring was simultaneously inhibited with HT31.

The spatial distribution of apoptotic cells within the livers was examined by histochemical detection of DNA fragmentation using the in situ terminal deoxynucleotidyl transferase d- uridine triphosphate nick end labeling (TUNEL) assay [22], thus confirming the results on PARP cleavage. Significant positive TUNEL staining of sinusoidal lining cells (SLCs) was present in untreated livers, while in all groups, apoptotic DNA strand breaks were rarely detectable in hepatocytes (fig. 2). These results are in keeping with data reported by Gao and coworkers [14], who demonstrated apoptosis of SLCs upon postischemic reperfusion of rat livers by TUNEL staining and transmission electron microscopy. Moreover, they suggested SLC apoptosis to be a pivotal event determining the ultimate viability of the grafts. Interestingly, db-cAMP was effective in reducing these alterations to the SLCs (fig. 2B), an effect, which was completely antagonized by the AKAP-inhibitor HT31 (fig. 2C). TUNEL-positive SLCs in the respective groups were quantitatively analyzed in five high-power fields (×200) of at least three livers per group and revealed a mean of 3.3 ± 0.2 positive cells in untreated livers, which was significantly ($p < 0.05$) reduced to 1.9 ± 0.4 by db-cAMP, returning to 3.4 ± 0.3 upon additional admixture of HT31.

Given that an impairment of vascular homeostasis, e.g., by discontinuity in the endothelial lining [23] is generally acknowledged to be a major culprit of postischemic liver dysfunction, the effects of db-cAMP on vascular perfusion and recovery of functional integrity of the liver grafts should be further investigated.

TVR upon postischemic reperfusion showed constant values during the 2 h of observation in all livers but varied significantly between the groups (fig. 3). In untreated livers, TVR was found to be elevated to 1699 ± 268 Pa s/ml. Stimulation of the cAMP second-messenger pathway significantly reduced vascular resistance to 672 ± 62 Pa s/ml ($p < 0.05$), while this effect was completely abolished by addition of HT31 (2058 ± 193 Pa s/ml). For comparison, normal values of non-ischemic livers in this model are about 600 Pa s/ml [T. Minor, unpublished observations]. Since cAMP has most recently been shown to increase the production of the endogenous vasodilator NO in the heart [24], we examined levels of this mediator in the perfusates of the reperfused livers (fig. 4). Interestingly, low levels of NO were seen in the cAMP group, while significantly elevated amounts of NO were released in untreated livers. Thus, no correlation could be established between TVR and hepatic effluent concentrations of NO, suggesting that other mechanisms were impairing vascular conductivity in postischemic livers despite higher intravasal levels of NO. Thimgan and Yee [25] have shown that hepatic stellate cells (HSCs) may contract within minutes and that the magnitude and rate of HSC contraction or relaxation are capable of modulating sinusoidal resistance. The improvement in hepatovascular conductivity observed after stimulation with db-cAMP is hence in keeping with the observation that cAMP markedly reduces pathological contractions of isolated HSCs on collagen gels [26].

The increased quantities of NO generated in the postischemic livers may relate to an increased expression of iNOS in Kupffer cells, which are located in close proximity to the hepatovasculature. In fact, we detected transcriptional induction of iNOS in the reperfused livers. Real-time PCR analysis revealed mRNA concentrations

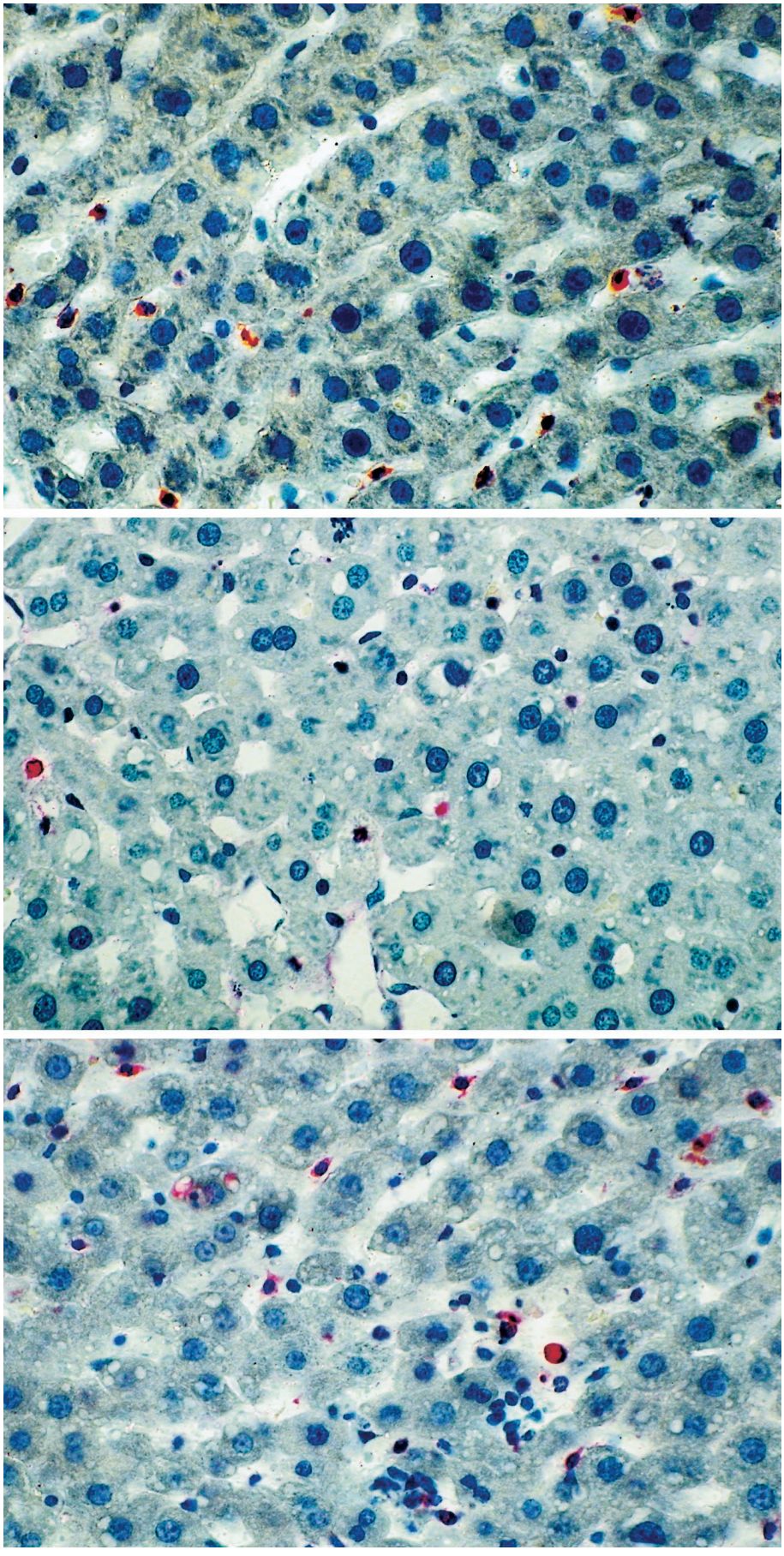

Figure 2. TUNEL staining of tissue sections from untreated livers preserved for 24 h in HTK solution and reperfused for 2 h with oxygenated, cell-free buffer. (× 200). Some positive-stained sinusoidal lining cells are present in untreated livers (*A*), but rareley, after addition of db-cAMP (2 µM) to the preservation fluid (*B*). Addition of HT31 (20 µM) prevented the anti-apoptotic effect of db-cAMP (*C*).

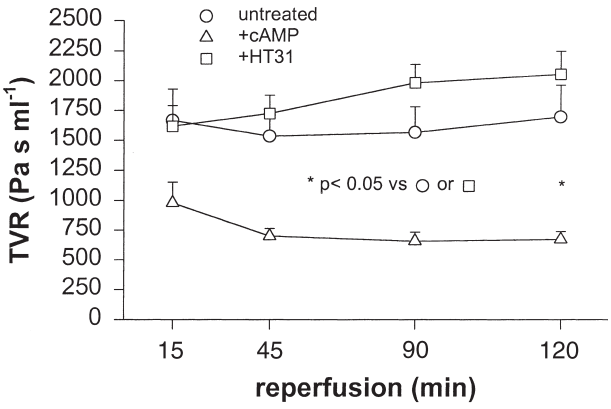

Figure 3. Total vascular resistance (TVR) upon postischemic reperfusion after cold preservation of untreated livers, after addition of 2 µM db-cAMP to the preservation fluid (+cAMP) or addition of db-cAMP (2 µM) and HT31 (20 µM) (+HT31).

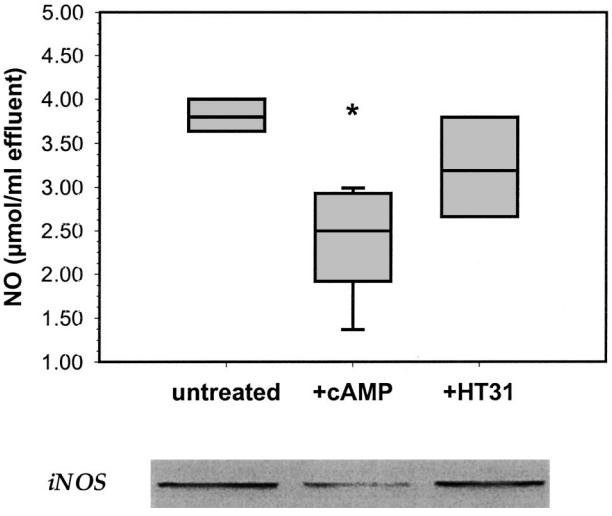

Figure 4. Upper panel: NO in hepatic venous effluent at the end of the experiment. Data are shown as a box plot (median ± 95% confidence interval; * $p < 0.05$ vs untreated by ANOVA and SNK test). Lower panel: Western analysis of iNOS in corresponding tissue homogenates.

of iNOS relative to the respective GAPDH signal of 3.7 ± 0.6‰ in untreated livers, while significantly reduced iNOS transcription was observed after stimulation of the cAMP messenger pathway (1.6 ± 0.3, $p < 0.05$) but not upon blockade of AKAP by simultaneous addition of HT31 (2.7 ± 0.2).

Accordingly, protein expression of iNOS was evident in homogenates of untreated livers (fig. 4) as well as after inhibition of PKA, but marked depression of iNOS induction occurred in the presence cAMP. Thus, iNOS expression in our study correlated closely with the increased amounts of NO released into the hepatic effluent, but the inhibitory effect of cAMP on hepatic production of inducible NO was in contrast to recent observations on cardiac fibroblasts, where iNOS was induced by β-adrenergic stimulation [27]. However, effects of cAMP appear to differ among cell types, and in isolated Kupffer cells, expression of iNOS has previously been shown to decreases upon exposure to cAMP-elevating agents [28]. Taken together, we conjecture that db-cAMP in our study alleviated postischemic contraction of HSCs, thus normalizing vascular perfusion characteristics and, further, preventing pathological elevation of induced NO production by Kupffer cells.

NO generated by iNOS is known to cause injury in the liver [29], where it may initiate pro-inflammatory signal responses and negatively affect hepatic mitochondrial function [30]. Functional recovery of the livers upon postischemic reperfusion is summarised in table 1. Supplementation of the preservation fluid with db-cAMP significantly enhanced metabolic activity of the reperfused livers and resulted, furthermore, in a significant improvement in secretory function as shown by cumulative bile production. This salutary effect was obviously through cAMP-mediated activation of PKA, since the addition of HT31 completely abolished the metabolic influences of db-cAMP agonism on hepatic oxygen utilization and bile juice excretion.

In close corroboration with these metabolic data, stimulation of the cAMP-PKA pathway significantly reduced parenchymal enzyme leakage of ALT to levels about half those observed in untreated livers, while ALT release upon reperfusion was comparable to untreated livers when cAMP-mediated signal transduction was counteracted by addition of HT31.

In conclusion, enhancement of the cAMP-PKA second messenger pathway represents a useful approach to improve the resistance of the liver to cold preservation-induced injury. Besides a possible metabolic effect through reduction of iNOS induction in cold-stored livers, a prominent impact of cAMP seems to be vascular protection through inhibition of mitochondria-initiated apoptotic degeneration of SLCs and normalization of vascular perfusion characteristics.

Table 1. Hepatic viability upon reperfusion after 24 h of cold storage at 4 °C.

	VO_2 (µl/g per minute)	Bile (µl/ g per hour)	ALT_{max} (U/l)
Untreated	21.8 ± 1.4	10.7 ± 1.7	42 ± 17
cAMP	28.7 ± 1.3*	16.5 ± 1.3*	22 ± 6*
HT31	21.0 ± 3.0	8.5 ± 0.8	47 ± 11

Oxygen consumption (VO_2), cumulative bile production, and maximal leakage of alanine aminotransferase (ALT) of untreated livers, after addition of 2 µM db-cAMP to the preservation fluid (cAMP), and after combined admixture of cAMP and the PKA-anchoring inhibitor HT31 (20 µM). Values are given as the mean ± SE of $n \geq 5$ experiments per group (* $p < 0.05$ vs untreated or HT31 by ANOVA and SNK test).

1 Kootstra G., Kievit J. K. and Heineman E. (1997) The non heart-beating donor. Br. Med. Bull. **53:** 844–853
2 Cho Y. W., Terasaki P. I., Cecka J. M. and Gjertson D. W. (1998) Transplantation of kidneys from donors whose hearts have stopped beating. N. Engl. J. Med. **338:** 221–225
3 Casavilla A., Ramirez C., Shapiro R., Nghiem D., Miracle K., Bronsther O. et al. (1995) Experience with liver and kidney allografts from non-heart- beating donors. Transplantation **59:** 197–203
4 D'Alessandro A. M., Hoffmann R. M., Knechtle S. J., Odorico J. S., Becker Y. T., Musat A. et al. (2000) Liver transplantation from controlled non-heart-beating donors. Surgery **128:** 579–588
5 Schön M. R., Schrem H., Lemmens H. P., Wolf S and Neuhaus P. (1997) Erfolgreiche Transplantation von Lebern nicht herzschlagender Spender nach normothermer extrakorporaler Leberperfusion im Schweinemodell. Langenbecks Arch. Chir. Suppl. Kongressbd. **114:** 725–731
6 Minor T., Saad S., Nagelschmidt M., Kötting, M. Fu Z., Paul A. et al. (1998) Successful transplantation of porcine livers after warm ischemic insult in situ and cold preservation including postconditioning with gaseous oxygen. Transplantation **65**: 1262–1264
7 Kötting M. and Minor T. (2000) Biochemical effects and cyclic-amp second messenger signal upon venous oxygen persufflation of ischemically preserved livers. Transplant. Proc. **32**: 11–13
8 Adkins W. K., Barnard J. W., May S., Seibert A. F., Haynes J. and Taylor A. E. (1992) Compounds that increase cAMP prevent ischemia-reperfusion pulmonary capillary injury. J. Appl. Physiol. **72**: 492–497
9 Pinsky D., Oz M., Liao H., Morris S., Brett J., Sciacca R. et al. (1993) Restoration of the cAMP second messenger pathway enhances cardiac preservation for transplantation in a heterotopic rat model. J. Clin. Invest. **92:** 2994–3002
10 Chang H. S., Jeon K. W., Kim Y. H., Chung I. Y. and Park C. S. (2000) Role of cAMP-dependent pathway in eosinophil apoptosis and survival. Cell Immunol. **203**: 29–38
11 Li J., Yang S. and Billiar T. R. (2000) Cyclic nucleotides suppress tumor necrosis factor alpha-mediated apoptosis by inhibiting caspase activation and cytochrome c release in primary hepatocytes via a mechanism independent of Akt activation. J. Biol. Chem. **275**: 13026–13034
12 Araki M., Hasegawa K., Iwai-Kanai E., Fujita M., Sawamura T. et al. (2000) Endothelin-1 as a protective factor against beta-adrenergic agonist-induced apoptosis in cardiac myocytes. J. Am. Coll. Cardiol. **36**: 1411–1418
13 Kuo P. C., Drachenberg C. I., Torre A. de la, Bartlett S. T., Lim J. W., Plotkin J. S. et al. (1998) Apoptosis and hepatic allograft reperfusion injury. Clin. Transplant. **12**: 219–223
14 Gao W., Bentley R. C., Madden J. F. and Clavien P. A. (1998) Apoptosis of sinusoidal endothelial cells is a critical mechanism of preservation injury in rat liver transplantation. Hepatology **27**: 1652–1660
15 Harada H., Becknell B., Wilm M., Mann M., Huang L. J., Taylor S. S. et al. (1999) Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. Mol. Cell **3:** 413–422
16 Minor T., Akbar S. and Yamamoto Y. (2000) Adenosine A2 receptor stimulation protects the predamaged liver from cold preservation through activation of cyclic adenosine monophosphate-protein kinase A pathway. Liver Transplant. **6**: 196–200
17 Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248–254
18 Minor T. and Kötting M. (2000) Gaseous oxygen for hypothermic preservation of predamaged liver grafts: fuel to cellular homeostasis or radical tissue alteration? Cryobiology **40**: 182–186
19 Minor T., Akbar S., Tolba R. and Dombrowski F. (2000) Cold preservation of fatty liver grafts: prevention of functional and ultrastructural impairments by venous oxygen persufflation. J. Hepatol. **32**: 105–111
20 Evans T., Carpenter A., Kinderman H. and Cohen J. (1993) Evidence of increased nitric oxide production in patients with the sepsis syndrome. Circ. Shock **41**: 77–81
21 Gottlieb R. A. (2000) Mitochondria: execution central. FEBS Lett. **482**: 6–12
22 Sgonc R., Boeck G., Dietrich H., Gruber J., Recheis H. and Wick G. (1994) Simultaneous determination of cell surface antigens and apoptosis. Trends. Genet. **10**: 41–42
23 McKeown C. M. B., Edwards V., Philips M. J., Harvey P. R. C, Petrunka C. N. and Strasberg S. M. (1988) Sinusoidal lining cell damage: the critical injury in cold preservation of liver allografts on the rat. Transplantation **46**: 178–191
24 Wang C. Y., Aronson I., Takuma S., Homma S., Naka Y., Alshafie T. et al. (2000) cAMP pulse during preservation inhibits the late development of cardiac isograft and allograft vasculopathy. Circ. Res. **86**: 982–988
25 Thimgan M. S. and Yee H. F. J. (1999) Quantitation of rat hepatic stellate cell contraction: stellate cells' contribution to sinusoidal resistance. Am. J. Physiol. **277**: G137–G143
26 Kawada N., Harada K., Ikeda K. and Kaneda K. (1996) Morphological study of endothelin-1-induced contraction of cultured hepatic stellate cells on hydrated collagen gels. Cell Tissue Res. **286**: 477–486
27 Gustafsson A. B. and Brunton L. L. (2000) Beta-adrenergic stimulation of rat cardiac fibroblasts enhances induction of nitric-oxide synthase by interleukin-1beta via message stabilization. Mol. Pharmacol. **58**: 1470–1478
28 Mustafa S. B. and Olson M. S. (1998) Expression of nitric-oxide synthase in rat Kupffer cells is regulated by cAMP. J. Biol. Chem. **273**: 5073–5080
29 Hierholzer C., Harbrecht B., Menezes J. M., Kane J., MacMicking J., Nathan C. F. et al. (1998) Essential role of induced nitric oxide in the initiation of the inflammatory response after hemorrhagic shock. J. Exp. Med. **187**: 917–928
30 Brown G. C., Foxwell N. and Moncada S. (1998) Transcellular regulation of cell respiration by nitric oxide generated by activated macrophages. FEBS Lett. **439**: 321–324