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Suppression of Leukocyte-Enhanced Cold Ischemia/Reperfusion Injury of Liver Endothelium with the Benzoquinone Antioxidant Idebenone

EKKEHARD SCHÜTZ, EBERHARD WIELAND, ANDREA HENSEL, PAUL-DIETER NIEDMANN, ALEXANDRA DREISS, VICTOR WILLIAM ARMSTRONG, PETER SCHUFF-WERNER, and MICHAEL OELLERICH

Zentrum Innere Medizin, Abteilung Klinische Chemie, Georg-August-Universität, Robert-Koch-Str. 40, 37075 Göttingen, Germany

Objective: Despite the large body of evidence for a major role of neutrophils and oxidant stress, the exact pathogenesis of the early ischemia/reperfusion injury after cold preservation of the liver is not well understood. The potential benefit of an antioxidant on metabolic liver function during reperfusion has been demonstrated in several studies.

Materials and Methods: We describe a cold storage/reperfusion damage model with isolated perfused pig livers, where the effects of neutrophils and idebenone, a recently developed benzoquinone antioxidant were studied. The integrity of sinusoidal endothelial cells (SEC) was estimated by hyaluronic acid concentration in perfusate and the expression of endothelial constitutive nitric oxide synthase (ecNOS) after reperfusion and compared to lipid peroxidation and antioxidant content.

Results: Hyaluronic acid displayed the highest levels and ecNOS mRNA was most depressed in livers reperfused with neutrophils after 20 h cold storage; this was accompanied by an increase in lipid peroxidation (TBARS) and a breakdown of endogenous lipophilic antioxidants (α-tocopherol and coenzyme Q-10). These effects were attenuated, when neutrophils were excluded from reperfusion and almost completely abolished by the addition of 200 μmol/L idebenone.

Conclusions: These data suggest that a leukocyte-mediated damage based on reactive oxygen species markedly contributes to the reperfusion injury of SEC after cold preservation of the liver. Therefore, the presence of effective antioxidants in the early reperfusion phase may be beneficial for liver graft integrity.

KEY WORDS: pig liver model; reperfusion injury; neutrophils; oxidant stress; antioxidants; idebenone.

Correspondence: Dr. Ekkehard Schütz, Zentrum Innere Medizin, Abt. Klinische Chemie, Georg-August-Universität, Robert-Koch-Str 40, 37075 Göttingen, FRG. E-mail: eschuetz@med.uni-goettingen.de

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Introduction

Primary non function and dysfunction are still major problems in clinical liver transplantation (1). Although the causes of these complications are still not well understood, reperfusion injury after cold ischemic storage is thought to play a critical role (2,3). There is general agreement that reactive oxygen intermediates are of particular importance in initiation of this reoxygenation damage (4,5). The development of graft complications can be predicted early after revascularisation using biochemical parameters such as ketone-body ratio (6), hyaluronic acid (7,8), and the MEGX-test (8,9). It is, therefore, likely that the underlying pathogenic mechanisms take effect during the early reperfusion phase after cold preservation. Since hepatocytes are well equipped with antioxidative defense systems (5,10), a direct damage of these cells by reactive oxygen intermediates (ROI) can not be expected. The production of reactive oxygen species by Kupffer cells (KC) and sinusoidal endothelial cells (SEC), activated during cold storage (11), may initiate an inflammatory process involving cytokine-induced chemotaxis of polymorphonuclear neutrophils (PMN) (12,13) with induction of β_2-integrins leading to subsequent adherence and immigration of PMN (14). The sinusoidal endothelium is particularly susceptible for ischemic damage even under cold storage (10) and the free radical injury presumably occurs within the extracellular matrix when the endothelium is altered (15). Hyaluronic acid (HA) is a major glucosaminoglycan of this matrix and the glycocalix of SEC. The fact that SEC are the major cells clearing HA from plasma (16) may additionally explain the sensitivity of HA concentrations to graft endothelial damage (17). The usefulness of hyaluronic acid determination as an early predictor of graft function as well as of rejection has been under-

lined in several studies (8,18). Little is known about the effect of supplementing preservation solutions with a potent lipophilic antioxidant on reperfusion injury after cold storage of the liver. The aim of the present study was to assess the role of the potent benzoquinone antioxidant idebenone (19) in the suppression of preservation/reperfusion injury in an isolated pig liver perfusion model. Idebenone was given during organ harvesting and reperfusion, which was performed in the presence as well as the absence of polymorphonuclear leukocytes. To estimate the damage to SEC, hyaluronic acid was determined in perfusion medium during reperfusion after cold storage. In addition, the total arterial resistance was followed and expression of ecNOS was measured as additional independent markers of endothelial injury.

Material and methods

The reagents used were purchased from Sigma Chemical, Deisenhofen, FRG if not stated otherwise, and were of the highest purity. Idebenone was a gift of Takeda Chemical Co., Osaka, Japan.

Livers of 25 to 35 kg pigs (Deutsches Edelschwein) were harvested according to standard surgical techniques. Animals were fasted for 12 h with free access to water prior to operation. Anesthesia was performed by an inhalation technique with Halothane, supplemented with neuroleptic and relaxing agents; 3000 mL Histidine-Tryptophan-Ketoglutarate (Custodiol™, Köhler Chemie, Alsbach, FRG) solution was used for organ preservation at 4° C before reperfusion.

Isolated liver perfusion was performed at 38° C according to Neuhaus (20) in a chamber with alternating external pressure (0–25 cm H_2O 6/min). The pumping device consisted of four roller pumps which were microprocessor controlled (Möller Feinmechanik, Fulda, FRG). Arterial pressure was kept between 90 and 130 mm Hg, portal vein pressure at 15 cm H_2O. The pO_2 was 90–120 mm Hg in the art. hepatica and approximately 70 mm Hg in the portal vein. The arterial pH was kept between 7.35 and 7.45 by regulating CO_2 pressure in perfusate or adding $NaHCO_3$. Oxygenation was performed via a membrane oxygenator (Jostra, Hechingen, FRG) with a model 960 air mixer (Endema Siemens, Sweden). The perfusion medium consisted of HBSS/pig plasma (2 + 1) with 40 g/L albumin. Red blood cells (RBC) and platelets were added to give a final hematocrit of about 30% and a final concentration of 75×10^9/L, respectively. Homologous pig RBC were washed using a capillary plasma separator (B. Braun, Melsungen, FRG) with HBSS prior to perfusion. PMN and platelets were additionally isolated from buffy-coat by density centrifugation and washed in PBS before adding to the perfusion medium. PMN were added where indicated after 150 min reperfusion, to achieve a final concentration of 12×10^9/L (21). Tissue samples were taken after flushing the organ with HTK *in situ,* after cold storage and after a perfusion time of 210 min. Samples were immediately stored in liquid nitrogen and frozen at −90° C until analysis. Samples from the perfusate were taken at 15 min intervals during the perfusion period.

Study groups

1. Controls (n = 5). Livers were harvested and subsequently reperfused after a cold ischemia maximum time of 30 min. Polymorphonuclear neutrophils (PMN) were added to the perfusate after 150 min of the 210 min perfusion period.
2. Ischemia (n = 5). Livers were subjected to 20 h of cold ischemia in HTK solution and reperfused for 210 min without adding PMN.
3. Ischemia with PMN (n = 5). As described for group 2 with PMN added at 150 min of perfusion.
4. Idebenone (n = 5). As described for group 3, but including 200 μmol/L idebenone given over the art. hepatica 15 min prior to flushing the organ in the pig as well as 200 μmol/L idebenone given into the perfusate.

Hyaluronic acid was measured with an isotopic protein binding test (HA Test, Pharmacia, Uppsala, Sweden). α-Tocopherol (TOC) and coenzyme Q-10 (Q-10) were measured in liver tissue homogenates by HPLC with fluorescence and UV detection, respectively (22). TBARS were determined as malon dialdehyde equivalents as described elsewhere (23). Expression ecNOS mRNA was determined by competitive RT-PCR with translation elongation factor-2 (EF-2) as reference gene (24). Briefly liver tissue was homogenized in GTC-buffer and total RNA was extracted according to the method of Chomzcynski and Sacchi (25). First strand cDNA was synthesized from 2 μg total RNA using reverse transcriptase with oligo-(dT 12-18) primers (Superscript™, Preamplification System, Boehringer, Mannheim, FRG) in a total volume of 50 μL. One microliter cDNA solution and 1 μL of diluted competitor was used for PCR with 2.5 U Taq DNA polymerase in 50 μL TRIS-HCl buffer containing 1.5 mM MgCl and 0.2 nM dNTP (PCR Core Kit, Boehringer Mannheim, FRG).

Primers for the specific genes were designed from conserved coding sequences of EF-2 (5′-ACAACATGCGGGTGATGAAG-3′ for.; 5′-TTTGTCCAGGAAGTTGCCA-3′ rev.) and ecNOS (5′CAGGTTCTGTGTGTTCGGG-3′ for., corresponding to human exon 16; 5′-GGCTCAGCAGCGCCTC-3′ rev., corresponding to human exon 19) of rat, mouse, bovine and human genes (26). Since the porcine gene sequences are not known. PCR products were verified by partial sequencing of the amplicons.

For both genes, DNA competitor constructs with an internal deletion were produced by enzymatic amplification with appropriate primer pairs (27). The total length of the constructs was approximately 85% of the native amplificate. After cleaning from primers and redissolving in sterile water, the

amount of DNA in the stock solution was measured with a GeneQuant II DNA-photometer (Pharmacia, Uppsala, Sweden). Three appropriate concentrations of the competitors for each sample were used in ecNOS and EF-2 PCR as internal standard.

The PCR products were quantified by agarose gel electrophoresis with ethidium bromide staining followed by densitometry using a digitizing video image system (28), and the amount of native mRNA was calculated vs competior on a log scale basis as proposed elsewhere (29). Expression of ecNOS mRNA was normalized to the expression of EF-2 mRNA in the same cDNA preparation and expressed as ratio of mRNA copies per 1 μL cDNA.

This study was approved by the local animal Ethics Committee and by the Bezirksregierung Braunschweig. Statistical analyses were performed using BMDP and SAS statistical packages. For comparison of groups nonparametric and robust procedures were used and differences were assumed to be significant if the p value was less than 0.05. If appropriate adjustation for multiple comparisons has been used.

Results

In the nonischemic control group, hyaluronic acid concentrations in the perfusate remained low during the whole perfusion period and only a minor increase was seen over time. The concentrations were significantly lower than all HA concentrations observed in the groups, in which the livers were subjected to cold ischemia ($p < 0.001$). After cold ischemic storage HA concentrations were always higher, even at the beginning of the perfusion, than in the control group. A steady increase of HA concentration during perfusion was seen in these groups and this was additionally enhanced by adding PMN to the perfusion medium after 150 min of perfusion. This leukocyte mediated increase, however, was not detected in the presence of 200 μmol/L idebenone (Fig. 1). The damage to the SEC was strictly paralleled by an increase in the hepatic arterial resistance (HAR), which most likely reflects an impairment of the microcirculation after leukocyte mediated oxidative injury (Fig. 2). The expression of ecNOS was dramatically decreased in those livers which showed the highest HA release and the greatest increase in HAR, suggesting a severe SEC damage in the group reperfused with leukocytes after 20 h cold storage (Fig. 3). However, the addition of idebenone into perfusate preserved the ecNOS to values comparable to the control group. These effects were paralleled by a reduced formation of lipid peroxidation products (TBARS) measured in pig liver homogenates of the idebenone group at the end of reperfusion (Fig. 4). When PMN were not present in the perfusion medium, the increase of TBARS during reperfusion was lower than that seen with the PMN group, but still significant (*in situ* versus after reperfusion). No changes were seen in the control group without extended cold preservation time prior to perfusion. The endogenous lipophilic antioxidants α-tocopherol (TOC) and coenzyme Q-10 (Q10) were fairly stable during reperfusion in control organs without preceding prolonged cold ischemic storage in HTK. However, a dramatic decrease of both, TOC and Q-10 was seen after reperfusion when organs were subjected to cold storage, and this was significantly enhanced by adding PMN into the perfusate. As shown for TBARS formation, this decrease of endogenous antioxidants was completely prevented by the addition

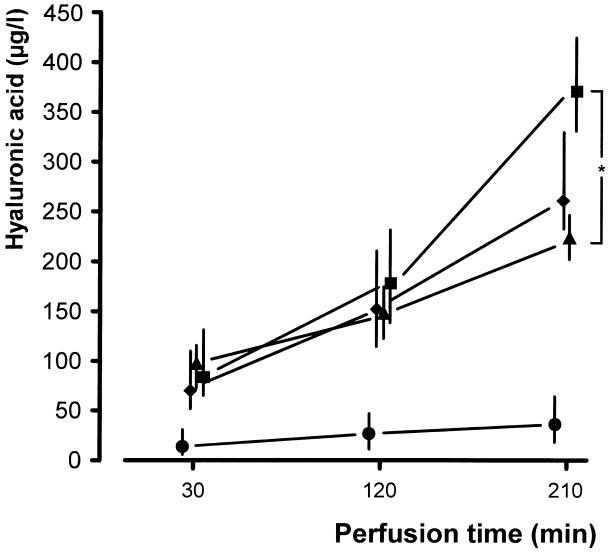

Figure 1 — Hyaluronic acid concentration in perfusate during reperfusion. Medians from 5 experiments. *p <0.05. Groups: (●) 0.5 h cold storage with PMN in perfusate; (◆) 20 h cold storage without PMN in perfusate; (■) 20 h cold storage with PMN in perfusate; (▲) 20h cold storage with PMN and 200 μmol idebenone in perfusate.

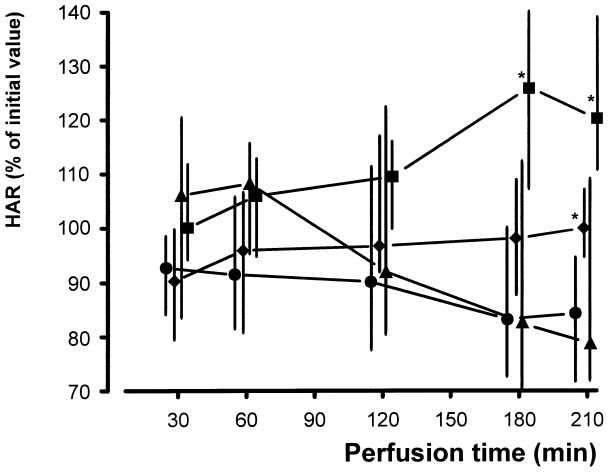

Figure 2 — Hepatic arterial resistance (HAR) during reperfusion. Medians from 5 experiments. *$p < 0.05$ versus 0.5 h control group. Groups: (●) 0.5 h cold storage with PMN in perfusate; (◆) 20 h cold storage without PMN in perfusate; (■) 20 h cold storage with PMN in perfusate; (▲) 20 h cold storage with PMN and 200 μmol idebenone in perfusate.

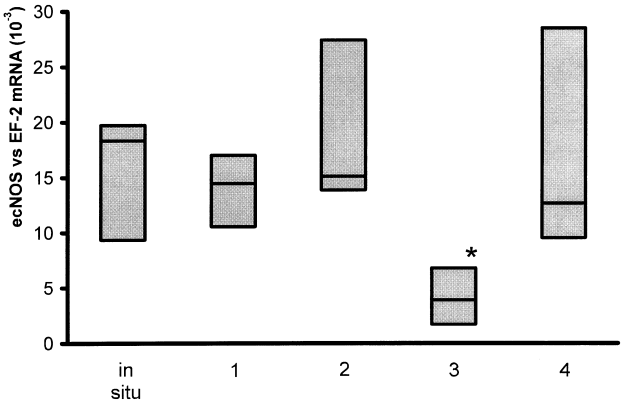

Figure 3 — Expression of endothelial constitutive nitric oxide synthase (ecNOS) mRNA in pig liver homogenates determined with competitive RT-PCR. Medians from five experiments each are displayed with 16th and 84th percentiles. Data are given as ratio of ecNOS (copies/μL cDNA) and EF-2 (copies/μL cDNA). Groups: (1–4) at the end of 210 min perfusion; (1) 0.5 h cold storage with PMN in perfusate; (2) 20 h cold storage without PMN in perfusate; (3) 20 h cold storage with PMN in perfusate; (4) 20 h cold storage with PMN and 200 μmol idebenone in perfusate.

of 200 μmol/L idebenone into perfusion medium (Fig. 5).

Discussion

Reestablishing blood flow after ischemia causes major damage described as ischemia/reperfusion injury (2,30). The nature of the reperfusion injury after cold preservation is not yet well understood. In particular, the contribution of liver cells and circulating blood cells needs to be clarified (2). It seems unlikely that hepatocytes can be directly affected by reactive oxygen intermediates, since these cells are well equipped with antioxidant defense systems (10). However, SEC may be much more vulnerable

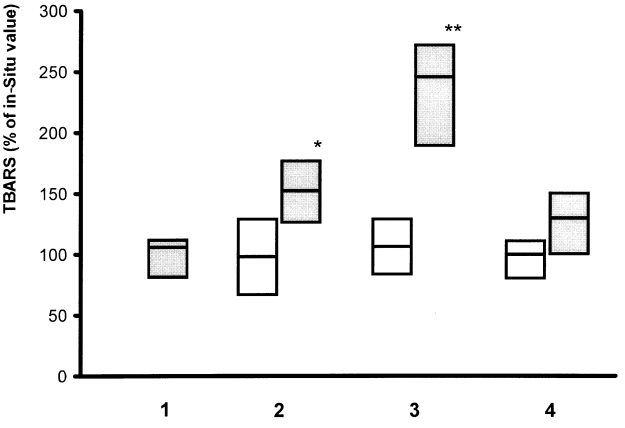

Figure 4 — Lipid peroxidation as assessed by TBARS formation in pig liver homogenates. Medians from five experiments each are displayed with 16th and 84th percentiles. $*p < 0.05$, $**p < 0.01$ versus 0.5 h control group. Open boxes: after cold storage; shaded boxes after 210 min perfusion. Groups: (1–4) as described in Figure 3.

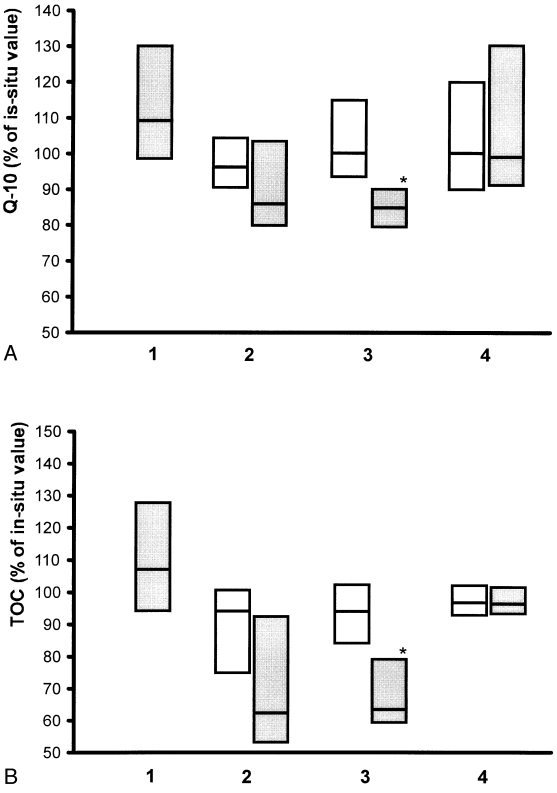

Figure 5 — Endogenous antioxidants coenzyme Q-10 (**A**) and α-tocopherol (**B**) in pig liver homogenates. Medians from five experiments are displayed with 16th and 84th percentiles. $*p < 0.05$ versus 0.5 h control group. Open boxes: after cold storage; shaded boxes after 210 min perfusion. Groups: (1–4) as described in Figure 3.

and are able to induce adhesion of PMN (3). Whether PMN mediated reperfusion injury is initiated by an oxygen radical dependent process is unclear, as is the role of PMN themselves (13,14,31). In a rat liver transplant model, it has been shown, that Q-10 is reduced during reperfusion after cold storage, despite the use of an antioxidant containing preservation solution (32). The advantage of the use of isolated organs from a large animal is the possibility to differentiate the effect of circulating cells and to identify the target cells within the organ by using appropriate study conditions. Thus, we were able to show substantial damage to SEC, when PMN were present during reperfusion after cold storage, indicating a major role of these cells in a rapid inflammatory process during reperfusion of cold stored livers (3,12). Reactive oxygen species may contribute to this PMN dependent injury, by the initiation of this inflammatory process (31). As demonstrated in this investigation a significant production of ROI occurred as shown by the significant increase of TBARS in the PMN group. Additional support for the role of ROI comes from the breakdown of the endogenous lipophilic antioxidants, α-tocopherol and Q-10, which showed the greatest decrease when PMN were present. Despite this decrease in α-tocopherol and Q-10, these antioxi-

dants were still abundant in liver homogenates, presumably due to the high relative mass of hepatocytes with their high concentration of these substances (10). The fact, that a substantial increase in lipid peroxidation was observed in the liver during reperfusion with PMN, is likely to reflect the local loss of endogenous antioxidants in nonparenchymal cells, such as SEC, leading to lipid peroxidation in these cells.

Most importantly, our data show that the supplementation with idebenone, which is about 100-fold more effective in its antioxidative capacity than coenzyme Q-10 (19), almost completely suppressed both SEC damage as assessed by HA concentration and ecNOS mRNA expression, as well as the breakdown of the endogenous antioxidants TOC and Q-10 with consecutive lipid peroxidation.

Since SEC damage is associated with microcirculatory disturbances (11,33,34), a secondary injury of hepatocytes exacerbated by the inflammatory reaction with the consequence of a deterioration of organ function in the later reperfusion phase must be expected.

Our data strongly suggest, that an inflammatory reaction targeted to SEC, which is initiated by PMN and mediated by reactive oxygen species significantly contributes to the reperfusion injury after cold storage. Supplementation of the organ preservation solution with idebenone and administration of the drug during early reoxygenation might, therefore, ameliorate reperfusion injury leading to a better graft quality.

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