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c-fos and c-jun mRNA Expression in a Pig Liver Model of Ischemia/Reperfusion: Effect of Extended Cold Storage and the Antioxidant Idebenone

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Objectives: Expression of immediate early genes has been reported during reperfusion after ischemia in rat livers due to oxygen radical formation. This study investigates in perfused pig livers the effect of the antioxidant idebenone and of cold ischemia time on the gene expression of c-fos and c-jun.
Design and methods: Livers were perfused for 210 min after 0.5 h or 20 h ischemic storage (4 °C). One group of pigs was fed idebenone (280 mg/day/7days) prior to organ harvesting. C-fos and c-jun mRNA were determined by RT-PCR at 3, 30, 60, 120 180, 210 min during reperfusion.
Results: Lipid peroxidation increased in liver tissue form 0.54 ± 0.21 to 1.09 ± 0.54 nmol MDA/mg protein during reperfusion after 20 h compared to 0.5 h cold storage. This was antagonized by idebenone (0.68 ± 0.20 nmol/MDA/mg protein). C-fos and c-jun were strongly induced in livers stored for 20 h, which was attenuated by idebenone ($p < 0.05$).
Conclusions: These findings suggest that cold ischemia time and oxygen radicals are critical for immediate early gene expression and that application of an effective antioxidant can attenuate this early stress reaction of the pig liver.

KEY WORDS: oxidative stress; lipid peroxidation; immediate early genes; benzoquinone antioxidant.

Introduction

During liver transplantation, the donor organ is subjected to a variable period of cold ischemia, which is then followed, by an additional period of warm ischemia before reperfusion of the graft is established. It has been postulated that oxygen-derived free radicals are involved in the pathophysiology of graft injury both during the period of ischemia as well as during reperfusion after engraftment (1). Possible sources of free radicals are the xanthine/xanthine oxidase system (2), neutrophils (3), and the respiratory chain of mitochondria (4). Oxygen radicals, particularly hydroxyl radicals are highly reactive and can react with the unsaturated lipids of cell membranes, resulting in lipid peroxidation and cell injury (5). Patients with chronic liver disease undergoing liver transplantation are known to have diminished antioxidant defenses, which may render them more susceptible to free radical damage during reperfusion (6).

Immediate early genes (IEG) such as HSP70, c-fos, and c-jun have been shown to be activated during reperfusion after ischemia of the rat liver (7). Both c-fos and c-jun belong to gene families whose expression is induced by a plethora of extracellular stimuli including oxidants and cytokines (8). Preferentially H_2O_2 has been shown to induce the expression of c-fos and c-jun (9). The protein products of the two proto-oncogenes fos and jun are known to form stable homo- or heterodimeric complexes known as nuclear transcription factor AP-1 which interacts with a common binding site found in the promotor of a variety of inducible genes including those of proinflammatory cytokines (10). Recently, it has been reported by Bradham *et al.* (11) in a rat liver transplantation model that reperfusion after 24 h cold storage in University of Wisconsin organ preservation solution (UW) activates mitogen-activated protein kinases (MAPKs). Mammalian MAPKs include the extracellular-signal-regulated kinase (ERK), which regulates transcription of c-fos, and the jun N-terminal kinase (JNK) also known as stress activated protein kinase (SAPK) regulating transcription of c-jun (12). Free radicals have been shown to mediate JNK and ERK activation (8) and N-acetyl cysteine, a free radical scavenger blocks JNK induction in kidneys during ischemia/reperfusion (13).

Besides being markers of stress to the liver, c-fos and c-jun expression have been suggested to be associated with liver function after ischemia/reperfusion. Several studies have demonstrated that c-fos and c-jun are involved in tissue repair (14) and/or

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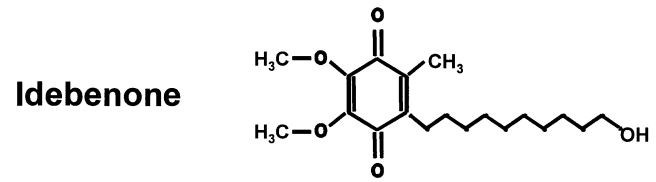

Figure 1 — Structural formula of idebenone ([6-(10-hydroxydecyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone]).

apoptosis (15,16). An association between c-fos and c-jun expression and the severity of ischemia/reperfusion related insults as well as subsequent graft failure has been shown in rat liver transplantation (17). In summary, there is good evidence that c-fos and c-jun are activated in rat livers by oxygen radicals generated during reperfusion after ischemia and can be interpreted as a marker of organ damage.

In search of suitable antioxidants to improve liver quality after reperfusion, we have investigated the effect of benzoquinones. Benzoquinones, of which coenzyme Q-10 is an intrinsic component of lipid membranes and the respiratory chain, seemed promising candidates since they combine antioxidative and energy conserving effects (18). Pretreatment of rats with coenzyme Q-10 suppressed lipid peroxide levels and accelerated ATP re-synthesis in livers exposed to ischemia and reperfusion (19). A coenzyme Q-10 derivative idebenone has been synthesized by modifying the prenyl side chain of the benzoquinone molecule (Figure 1) leading to an even more effective antioxidant which has conserved its electron transferring activity (20). We have shown in a rat liver microsomal model subjected to oxidative stress by NADPH/ADP/Fe^{3+} that idebenone is an about 100 times more effective antioxidant as compared to coenzyme Q-10 (21). In addition, its antioxidative capacity is not restricted to the reduced from of the molecule (22). Idebenone is non-toxic to humans (23) and has been successfully used for the therapy of Alzheimer's disease (24). We have recently reported that oral pretreatment of donor pigs with idebenone improves liver quality during reperfusion as shown by inhibition of HSP70 mRNA expression and suppression of injury to sinusoidal endothelium (25,26).

In the present study, we investigated in this large animal model, relevant for human liver transplantation, whether extended cold ischemia is a prerequisite for c-fos and c-jun induction in liver tissue during reperfusion. In addition, we were interested to elucidate whether the antioxidant idebenone would suppress c-fos and c-jun mRNA expression.

Materials and Methods

Organ harvesting and cold storage

Pigs (Deutsches Edelschwein, 25–35 kg body weight) of either gender were kept under 12 h dark and light periods, were fed a standard chow (HEMO X100, HEMO KG, Scheden, Germany) ad libitum, and had free access to water. The experimental protocol was approved by District Government (504.42502/01-0-01.93) and, therefore, met with national standards for the humane care of animals. Porcine livers were harvested according to the protocol commonly used for human liver procurement. Starved (12 h) animals were anesthetized with 10 mg/kg azaperone, 4.5 mg/kg metomidate, 0.25 mg/kg pitiramide and maintained with 0.8–1.5% halothane per inhalation with auxiliary breathing under circulation- and temperature-controlled conditions. Systolic blood pressure was maintained above 80 mm Hg. A midline laparatomy was made and the abdomen was entered. The truncus coeliacus was cannulated and the liver was flushed using 5.0 l of cold (4 °C) Histidine-Tryptophan-Ketoglutarate (HTK) organ protection solution (Dr. Franz Köhler Chemie, Alsbach, Germany). The vena cava superior was isolated, ligated and the arteria hepatica and vena portae were dissected.

After the liver has been detached from all surrounding ligaments it was removed and stored for either 0.5 h or 20 h in HTK at 4 °C. To one group of animals the antioxidant idebenone (Mnesis, Takeda, Catonia, Italy) was supplemented in the diet such that each animal received 280 mg/day over a period of 7 days before explantation of the liver. Livers from these animals were stored for 20 h at 4 °C in HTK organ preservation solution.

Isolated pig liver perfusion

Livers were perfused (210 min) in a closed water bath (38 °C), which subjects the liver to fluctuating pressure (25 cm H_2O, 6/min). The perfusion medium was composed of HBSS/pig plasma (2+1), homologous washed pig erythrocytes (hematocrit 25–30%), and washed platelets (75×10^9/l) and 40 g/l albumine. Homologous porcine leukocytes (12×10^3/l) were added after 150 min of perfusion. Leukocytes were isolated by separation of pig blood into buffy-coat, plasma and red cells followed by density centrifugation of the buffy coat (25). Perfusion was through arteria hepatica (150–220 mL/min, 100 mm Hg pressure, 90–120 mm Hg pO_2), vena portae (350–475 mL/min, 25 cm H_2O and 50 mm Hg pO_2), and back flow was via vena cava. The perfusate was oxygenated with a plate oxygenator (ME-10, Jostra, Hechingen, Germany) and an air mixer (Siemens-Endema, Sweden). Perfusion was achieved by a custom made pump system consisting of four computer controlled roller pumps (Möller Feinmechanik, Fulda, Germany). Porcine blood was provided by the local slaughterhouse. Liver biopsies were taken 3, 30, 60, 120, 180, and 210 min after the start of perfusion.

Analytical methods

Total RNA was extracted from biopsies using the method of Chomzinsky and Sacchi (27). Frozen (−80

°C) liver tissue (100 mg) was homogenized in guanosine-thiocyanate-chloroform (GTC)-buffer on ice and total RNA was extracted with chloroform/phenol. The kinetics of c-fos and c-jun mRNA expression during reperfusion were determined by differential reverse transcriptase polymerase chain reaction (RT-PCR) and calculated by densitometric analysis of PCR products after electrophoretic separation and ethidium bromide staining vs. transcription elongation factor-2 (EF-2) as house keeping gene. Primers for the specific genes were designed from conserved coding sequences of EF-2 (5′-ACAACATGCGGGTGATGAAAG-3′ forward; 5′-TTTGTCCAGGAAGTTGCCA-3′reverse), c-fos (5′-AAGGAGAATCCGAAGGGAAAGGAATAAGATGGCT-3′ forward; 5′-AGACGAAGGAAGACGTGTAAGCAGTGCAGCT-3′ reverse), and c-jun (5′-GCATGAGGAACCGCATCGCTGCCTCCAAGT-3′ forward; 5′-GCGACCAAGTCCTTCCCACTCGTCCACACT-3′ reverse) of rat, mouse, bovine, and human genes. Reverse transcription was achieved by using the Superscript preamplification system (Roche Diagnostics, Mannheim, Germany) with oligo(dt)$_{12–18}$ primers and 2 μg of total RNA. One microliter of cDNA solution was used for PCR with 2.5 U Taq DNA polymerase in 50 μl of 10 mmol/l TRIS-HCL, 50 mmol/l KCl, 1.5 mmol/l MgCl, and 0.2 mmol/l dNTP (pH 8.3) (PCR core kit, Roche Diagnostics, Mannheim, Germany). Amplification conditions for c-fos and c-jun were identical and as follows: denaturation for 5 min at 95 °C, followed by 30 cycles of denaturation at 95 °C for 90 s, annealing at 50 °C for 45 s, elongation at 72 °C for 60 s, with 5 min final elongation at 72 °C. EF-2 was amplified essential as described here with the exceptions that 25 cycles were performed and an annealing temperature of 55 °C was used. Since the porcine gene sequences were not known, PCR products were verified by partial sequencing of the amplicons. Computing of sequence homology between species and primer construction were performed using GCG package version 8.1-UNIX (Genetics Computer Group, Madison, WI, USA). Lipid peroxidation in liver tissue was assessed by the TBA method as previously described (21).

STATISTICAL ANALYSIS

Data were analyzed using the Mann-Whitney U-test and INSTAT computer software (San Diego, CA, USA). A two-sided p value below 0.05 was considered significant.

Results

Reperfusion of pig livers for 210 min after 20 h cold storage in organ preservation solution resulted in a significant formation of lipid peroxidation products in liver tissue when compared to livers, which were perfused after short (0.5 h) cold storage (Table 1). In contrast, when donor pigs were orally pretreated with 280 mg/day idebenone for 7 days prior to organ harvesting, lipid peroxidation was markedly and significantly reduced in liver tissue even though organs had been stored for 20 h at 4 °C in HTK organ preservation solution before perfusion (Table 1).

TABLE 1
Lipid peroxidation products in liver tissue after 210 min perfusion

Condition	n	TBARS (nmol MDA/mg protein)
20 h cold storage	5	1.09 ± 0.54
20 h cold storage fed 280 mg idebenone per day over 7 days before organ harvesting	5	0.68 ± 0.20[a]
0.5 h cold storage	5	0.54 ± 0.21[a]

Data are given as mean (SD).
[a]$p < 0.05$ vs. 20 h cold storage.

Time course studies on the expression of c-fos mRNA in liver tissue revealed a sustained induction during reperfusion of the organs after 20 h cold storage, which reached a maximum after 120 min and continued for the next 90 min (Figure 2). C-fos gene expression during perfusion was lower in livers from pigs fed idebenone prior to organ harvesting even though these livers had also been preserved at 4 °C for 20 h (Figure 2). The difference in gene expression reached statistical significance after 210 min perfusion time. As already observed with lipid peroxidation products (Table 1), almost no c-fos mRNA induction was caused by reperfusion of livers, which were only subjected for 0.5 h to cold ischemia (Figure 2).

Perfusion of pig livers stored for 20 h at 4 °C in HTK organ preservation solution resulted in a

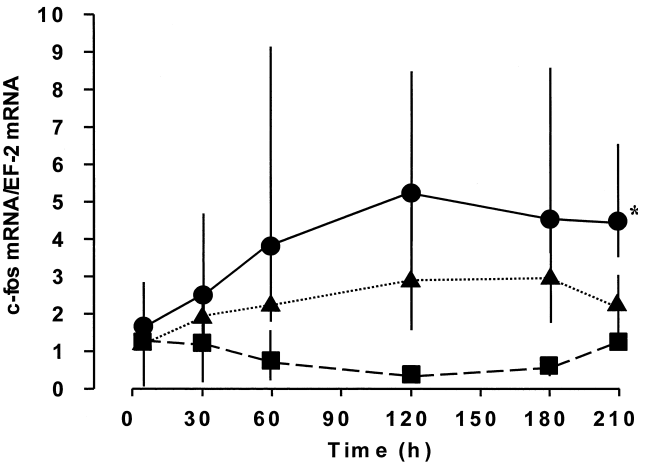

Figure 2 — Time course of c-fos mRNA in liver tissue during 210 min of perfusion after cold storage of organs ($n = 5$) for 20 h at 4 °C in HTK organ preservation solution (●), after cold storage of organs from donor pigs fed with 280 mg/day idebenone over 7 days before organ harvesting ($n = 5$) for 20 h at 4 °C in HTK organ preservation solution (▲), after cold storage of organs ($n = 2$) for 0.5 h at 4 °C in HTK organ preservation solution (■). Data for 20 h cold stored organs are displayed as medians with 16th to 84th percentile, data for 0.5 h stored organs are mean and range. *$p < 0.05$ vs. livers from the group fed idebenone.

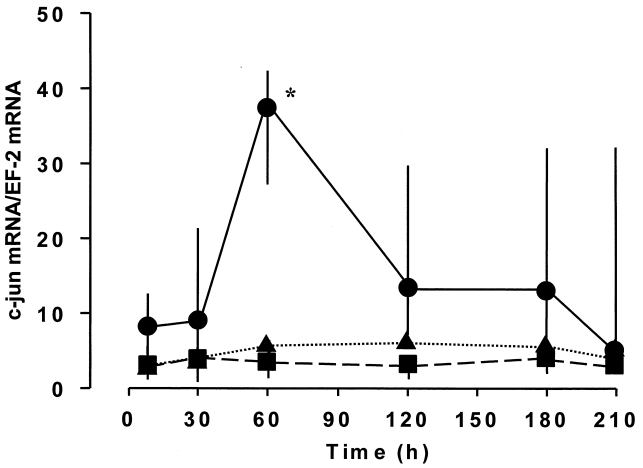

Figure 3 — Time course of c-jun mRNA in liver tissue during 210 min of perfusion after cold storage of organs ($n = 5$) for 20 h at 4 °C in HTK organ preservation solution (●), after cold storage of organs from donor pigs fed with 280 mg/day idebenone over 7 days before organ harvesting ($n = 5$) for 20 h at 4 °C in HTK organ preservation solution (▲), after cold storage of organs ($n = 2$) for 0.5 h at 4 °C in HTK organ preservation solution (■). Data for 20 h cold stored organs are displayed as medians with 16th to 84th percentile, data for 0.5 h stored organs are mean and range. *$p < 0.05$ vs. livers from the group fed idebenone.

strong and significant c-jun mRNA induction after 60 min, which declined afterwards to initial values. This steep increase of c-jun mRNA was completely inhibited by the intervention with idebenone (Figure 3). As already observed with c-fos, negligible c-jun mRNA expression was observed during perfusion after 0.5 h cold storage of livers (Figure 3).

Discussion

In this study, we examined the effect of a short (0.5 h) versus prolonged (20 h) cold storage time in organ preservation solution on the expression of the IEG c-fos and c-jun in the pig liver during early reperfusion. In addition, the effect of the antioxidant idebenone on c-fos and c-jun mRNA expression was investigated. Previous rat models of ischemia/reperfusion have demonstrated that liver injury is associated with both acute and subacute responses such as an early direct ischemic damage as well as a late inflammatory response (1). These studies have implicated free radical formation in mechanisms of acute cellular damage and subsequent inflammatory responses mediated by cytokines. Recently two distinct pattern of c-jun and c-fos expression were observed after ischemia reperfusion in mouse liver during the acute (1–3 h) and subacute (6–20 h) phase after ischemia/reperfusion. Co-expression of c-jun and c-fos mRNA within damaged liver tissue during the acute postreperfusion phase was followed by a decline in c-fos expression with sustained high levels of c-jun expression in the subacute postreperfusion phase (28). It has been suggested that c-fos and c-jun are involved in both tissue repair as well as tissue damage in the form of programmed cell death and there is evidence that the time course of c-jun and c-fos expression may determine whether these molecules act to facilitate regeneration or apoptotic processes. In neuronal cells, persistent expression of c-jun and c-fos leads to cell death (15,29), whereas transient expression in the rat liver appears to be associated with regeneration (14,17). In the present investigation, we have observed a transient expression of c-jun mRNA in liver tissue during reperfusion after 20 h cold storage which reached its maximum after 60 min. In contrast, c-fos mRNA was induced more slowly and moderately and reached a steady-state expression after 120 min at which it continued until the end of the experiment (210 min). This pattern of c-jun and c-fos expression has not been reported before, but may be associated with both apoptotic and regenerative processes occurring in parenchymal and non-parenchymal liver cells. However, these findings are in line with a stress reaction of the pig liver caused by ischemia/reperfusion and reconfirm findings of Bradham *et al.* (11) in a rat liver transplantation model. These authors observed after 60 min of reperfusion a significant induction of c-fos and c-jun mRNA in liver tissue as well as activation of the mitogen-activated protein kinases ERK and JNK, which transactivate the c-fos and c-jun promotor, respectively.

Pretreatment of pigs with the antioxidant idebenone attenuated lipid peroxidation and induction of both IEGs during 210 min of perfusion. This is in accordance with a previous report from our group where we have shown in the same model that HSP70 gene expression was antagonized by idebenone pretreatment (25). In contrast to HSP70 gene expression, which has been associated with ATP shortage, c-jun and c-fos mRNA may not increase due to a lack of ATP in liver tissue during reperfusion since ATP has been shown to induce both c-fos and c-jun genes (30). The inhibition of c-fos and c-jun mRNA expression by idebenone may therefore not be related to the better energetic situation in liver tissue after idebenone treatment but rather due to radical processes. This is supported by results from several cell culture studies, which have shown a direct role of oxygen radicals in c-jun and c-fos gene induction (31,32).

Even though the large animal model and the complicated experimental set up limited the number of experiments, statistical significance of the differences between the intervention group (20 h cold storage after idebenone supplementation plus perfusion) and the group with most damage (20 h cold storage plus perfusion) was achieved after 60 min reperfusion for c-jun and after 210 min for c-fos. Statistical comparisons were not made to the group of livers, which were stored for 0.5 hours since in this group only two livers were investigated. Nevertheless, it becomes evident that short cold storage may not be associated with severe induction of IEGs. This is line with HSP70 gene expression for which

extended cold storage (20 h) was also a prerequisite (25). These findings point to the importance of cold ischemia time for reperfusion damage. It has been suggested by Clavien *et al.* (1) that during the period of cold preservation changes in the liver endothelium as well as activation of Kupffer cells occur which are predisposing for the severity of damage observed during reperfusion.

In conclusion, our data emphasize in a model more closely related to human liver transplantation than previous experiments with small laboratory animals that cold ischemia time and oxygen radicals are critical for IEG expression during reperfusion and that application of an effective antioxidant such as idebenone attenuates this early stress reaction of the liver. In addition, c-fos and c-jun mRNA may also be useful as indicators of liver injury during the early post-transplantation period in humans.

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