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Electron spin resonance and spin trapping technique provide direct evidence that edaravone prevents acute ischemia–reperfusion injury of the liver by limiting free radical-mediated tissue damage

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Abstract

A novel free radical scavenger, 3-methyl-1-phenyl-2-pyrazolin-5-one (edaravone), is used for the treatment of acute ischemic stroke and is protective in several animal models of organ injury. We tested whether edaravone is protective against acute liver warm ischemia/reperfusion injury in the rat by acting as a radical scavenger. When edaravone was administered prior to ischemia and at the time of initiation of the reperfusion, liver injury was markedly reduced. Production of oxidants in the liver in this model was assessed *in vivo* by spin-trapping/electron spin resonance (ESR) spectroscopy. Ischemia/reperfusion caused an increase in free radical adducts rapidly, an effect markedly blocked by edaravone. Furthermore, edaravone treatment blunted ischemia/reperfusion-induced elevation in pro-inflammatory cytokines, infiltration of leukocytes and lipid peroxidation in the liver. These results demonstrate that edaravone is an effective blocker of free radicals *in vivo* in the liver after ischemia/reperfusion, leading to prevention of organ injury by limiting the deleterious effects of free radicals.

Keywords: *Ischemia/reperfusion, electron spin resonance, reactive oxygen species, liver injury*

Abbreviations: *TNFα, tumor necrosis factor α; IL, interleukin; ALT, alanine aminotransferase; ESR, electron spin resonance; HA, hyaluronic acid; ROS, reactive oxygen species; EDA, edaravone; POBN, α-(4-pyridyl-1-oxide)-*N-tert-*butylnitrone*

Introduction

In the liver, ischemia/reperfusion injury may occur under many clinical conditions, such as liver transplantation, hepatic resection, and other types of abdominal surgery that require occlusion of the hepatic vasculature or general low-blood pressure status [1]. Depending on the type of ischemia, such as cold (liver graft storage with subsequent transplantation), warm (liver surgery, shock, or trauma), and rewarming (manipulation of the liver graft before or during transplantation), the mechanisms of liver injury upon reperfusion differ. All of these conditions cause an increase in production of oxidants, activation of Kupffer cells, disfunction or death of sinusoidal endothelial cells (SEC), and recruitment of neutrophils into the liver [2]. Warm ischemia is a common condition in surgical treatment of hepatocellular carcinoma and other diseases; however, it is poorly tolerated and is associated with severe liver damage and massive death of parenchymal cells [3].

Reactive oxygen species (ROS) produced upon reperfusion play a critical role in the injury caused by

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ischemia/reperfusion [4]. Accumulation of purine derivates due to ATP degradation and reduction of mitochondrial ubiquinone lead to production of superoxide radicals upon reoxygenation [4]. In addition, resident hepatic macrophages, Kupffer cells, and other inflammatory cells infiltrating into the liver shortly after the blood flow is restored, produce oxygen radicals [5,6]. ROS not only directly damage liver cells, they also trigger formation of toxic cytokines and increase adhesion molecules leading to an inflammatory cascade that exacerbates tissue injury in liver [7].

Due to a central role that oxidants play in a pathogenesis of liver injury in ischemia/reperfusion, therapies directed at alleviation of oxidative stress are needed to improve clinical outcomes of liver surgery. Several antioxidants and free radical scavengers were reported to be effective in protecting against warm ischemia [8]. While these data establish that such therapy may be beneficial in preventing liver damage, these agents are not suitable for clinical use yet. Interestingly, 3-methyl-1-phenyl-2-pyrazolin-5-one (edaravone, MCI-186, Radicut®) is a novel pharmaceutical approved for clinical use in stroke and is a potent scavenger of free radicals [9]. The beneficial effects of edaravone are thought to be due to protection of endothelial cells and neurons against lipid peroxidation [10]. Edaravone was shown to be protective in cardiovascular disease, cerebrovascular ischemia and cerebral edema in animals and humans [9,11]. It has been reported that edaravone is reacting with hydroxyl radicals with a rate constant near the diffusion limit [12] and as a result is converted to non-toxic 2-oxo-3-(phenyl hydrazono)-butanoic acid.

Recently, it was reported that edaravone is protective against ischemia/reperfusion injury in the rat liver [13–17]; however, the exact mechanism of this effect remains unclear. To elucidate whether edaravone is capable of scavenging free radicals produced in ischemia/reperfusion *in vivo*, electron spin resonance (ESR) technique was used. Our results demonstrate that edaravone may be an effective drug for acute warm ischemia/reperfusion as it effectively blocks the initial increase in oxidants, activation of Kupffer cells and considerably reduces signs of liver damage.

Materials and methods

Animals

Male Sprague–Dawley rats (200–250 g, Japan SLC Inc., Shizuoka, Japan) were used in these experiments. The experimental protocol was approved by the Institutional Review Board and followed the National Research Council's criteria for care and use of laboratory animals in research. All animals received humane care in compliance with institutional guidelines.

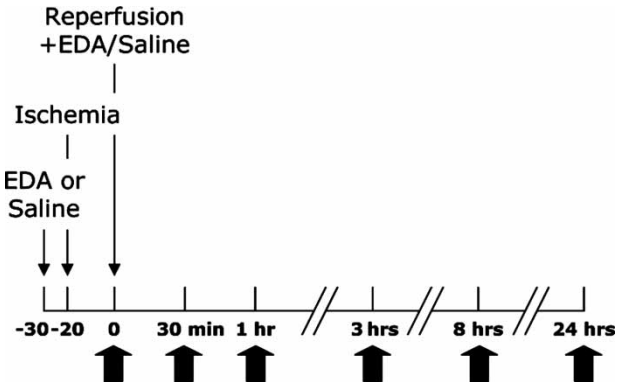
Experimental procedures

Rats ($n = 3–6$ in each treatment group) were anesthetized intraperitoneally with pentobarbital sodium 50 mg/kg, and the abdomen was opened by midline incision. Either edaravone (3 mg/kg, i.v., a kind gift from Mitsubishi Pharma Corporation, Tokyo, Japan), or saline vehicle (1 ml/kg, i.v.) were injected 10 min prior to ischemia whereby the portal vein and the hepatic artery were occluded for 20 min with an atraumatic vascular clamp (Scheme 1). During sham operation, the abdomen was opened and the liver was manipulated similarly except for clamping of the blood flow. At the end of ischemia, the blood flow to the organ was restored, edaravone or saline injected i.v. again (same dose as above), abdominal opening closed with atraumatic sutures, and the animals were allowed to recover for various time periods (see below). In a separate experiment, where bile was collected for ESR measurements, the abdominal opening was not closed to allow bile collection and the animals were sacrificed 3 h after reperfusion (see details below).

Histopathological and immunohistochemical evaluation of liver injury

Animals were sacrificed and liver and blood specimens were collected at 0.5, 1, 2, 3, 8, and 24 h after initiation of the reperfusion. A section of the liver was formalin-fixed, embedded in paraffin, and stained with hematoxylin–eosin (24 h time point) to assess inflammation and necrosis. Histopathological evaluation was performed by one of the authors and by an outside expert in rodent liver pathology in a blind manner. The number of neutrophils ($n = 6$ in each group) in the liver was expressed per 400 hepatocytes in five high-power (×400) fields per slide.

In addition, immunohistochemical detection of intracellular adhesion molecule-1 (ICAM-1, 0.5 and



Scheme 1. Experimental protocol. Time points for test article (edaravone, EDA; or saline) administration and surgical procedures applied in this study are shown. Full description of the protocol is provided in materials and methods section. Black arrows identify time points at which samples (blood and liver tissue) were collected.

2 h time points) and 4-hydroxynonenal (4-HNE, 8 h time point) was performed. Paraffin-embedded sections of liver tissue were deparaffinized, rehydrated, and incubated with polyclonal antibodies against ICAM-1 (R&D Systems, Minneapolis, MN), or 4-hne (Alpha Diagnostic International, San Antonio, TX) in Phosphate-Buffered Saline (PBS, PH 7.4) containing 1% Tween-20 and 1% bovine serum albumin. Peroxidase-linked secondary antibody and diaminobenzidine were used to detect specific binding. The slides were rinsed twice with PBS-0.1% Tween-20 between all incubations, and sections were counterstained with hematoxylin to control for non-specific binding of the secondary antibody, sections from the same animals were processed without the primary antibody, followed by the procedure detailed above. No positive staining was observed in these control experiments (data not shown).

Serum markers of liver injury

Hepatic parenchymal cell injury was estimated by an increase in activity of serum alanine aminotransferase (ALT) that was measured spectrophotometrically using a commercial kit (Wako Pure Chemical Industries, Tokyo, Japan). SEC function was estimated using a commercially available, enzyme-linked immunosorbent assay (ELISA) for hyaluronic acid (HA; Chugai Pharmaceutical Co. Ltd, Tokyo, Japan).

Determination of serum levels of TNFα and IL-6

For measurement of serum cytokine levels, blood samples were collected from aorta at 0.5, 1, 3, 8, and 24 h after reperfusion ($n = 6$ in each group). Samples were centrifuged at 1200*g* for 10 min at 4°C, and serum was stored at −80°C until assays. Measurements were performed using cytokine ELISA kits (Cosmo Bio Co., Tokyo, Japan).

Reverse-transcription polymerase chain reaction (RT-PCR) for the mRNA expression of TNFα

Liver tissue samples were collected from animals sacrificed at 0.5, 1, 3, 8, and 24 h after reperfusion. In additional experiments, Kupffer cells were isolated from liver (see [18] for detailed procedure) 0.5 h after reperfusion. Total RNA was isolated from frozen samples of liver tissue using RNA purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and used for PCR assay to detect mRNA expressions of TNFα as detailed in [19]. PCR primers for TNFα and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) contained following sequences:

TNFα sense (5′-ATGAGCACAGAAAGCATGATG-3′),
TNFα antisense (5′-TACAGGCTTGTCACTCGAATT-3′),
GAPDH sense (5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′),
GAPDH antisense (5′-CATGTGGGCCATGAGGTCCACCAC-3′).

After PCR, the amplified products were subjected to electrophoresis through 2% agarose gel for about 30 min at 100 V. Gels were stained with 0.5 mg/ml ethidium bromide Tris–borate–ethylene diaminetetraacetic acid buffer (ICN, Costa Mesa, CA) and photographed with type 55 Polaroid positive/negative film. Densitometric analysis of the captured image was performed on a Macintosh computer using NIH image 1.54 analysis software.

Collection of bile and free radical detection by ESR

The rat was anesthetized with pentobarbital sodium (75 mg/kg), the abdomen was opened, and the spin trap α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN, 1 g/kg) was administered intravenously through the tail vein. The proximal bile duct was cannulated with a small length of PE-10 tubing, and bile samples were collected at 30 min intervals for up to 3 h into 50 μl of transition metal chelator (30 mM dipyridyl and 30 mM bathocuproine) to prevent *ex vivo* radical formation. About 30 min after POBN administration and bile duct cannulation, edaravone or saline vehicle were administered and ischemia/reperfusion was applied followed by a second dose of edaravone (or vehicle) as detailed in the experimental procedure section above. Samples were stored at −80°C until analysis of free radical adducts by ESR spectrometer [20]. Bile samples were thawed, placed in an ESR flat cell and bubbled with oxygen in the presence of an ascorbate oxidase paddle to oxidize ascorbate to ascorbate dione, which is no longer a free radical. Free radical adducts were detected with a Bruker EMX ESR spectrometer. Instrument conditions were as follows: 20 mW microwave power, 1.0 G modulation amplitude, 80 G scan width, 1.3 s conversion time, and 1.3 s time constant. Spectra were recorded on a desktop computer interfaced with the spectrophotometer and were analyzed for ESR hyperfine coupling constants by computer simulation [20]. Quantification of free radical adducts was achieved by measuring the peak-to-peak amplitude to bile volume collection in 1 h.

Determination of ROS formation in the liver after ischemia–reperfusion

Ischemia/reperfusion injury of the liver was induced, and rats administered edaravone or saline as described above. At various times (30 min, 1 and 2 h) after reperfusion, livers were perfused through the portal

vein with Krebs–Henseleit bicarbonate buffer (KHB) containing 0.05% nitro blue tetrazolium (NBT; Sigma-Aldrich Co., St Louis, MO) for 10 min as detailed in [21]. Then, livers were fixed by infusion of 10% formalin, embedded in paraffin, sectioned, and stained with nuclear fast red. The degree of formazan deposition, which was formed by reaction of NBT with ROS, was evaluated by light microscopy in liver sections counterstained with eosin (magnification of 400×).

Statistical analysis

Data are expressed as mean ± SEM. Either ANOVA with Bonferroni's *post hoc* test, or Student's *t*-test were used for the determination of significance as appropriate. A p value less than 0.05 was selected before the study as the level of significance.

Results

Edaravone is protective against acute ischemia–reperfusion injury in the rat liver

In order to investigate whether edaravone is protective in a rat model of acute ischemia/reperfusion injury to the liver, the hepatic artery and portal vein were clamped for 20 min followed by a restoration of blood flow. In sham-operated rats, where blood flow to the liver was not interrupted, that were administered vehicle (saline), or edaravone (EDA), no histopathological signs of liver injury were observed (Figure 1A,B). However, 24 h after ischemia/reperfusion, severe liver injury, as evident from the histopathological signs of liver necrosis and marked elevation in serum ALT levels, was observed in rats that received only vehicle (Figure 1C and E). When edaravone was administered prior to ischemia and again at the time of initiation of the reperfusion,

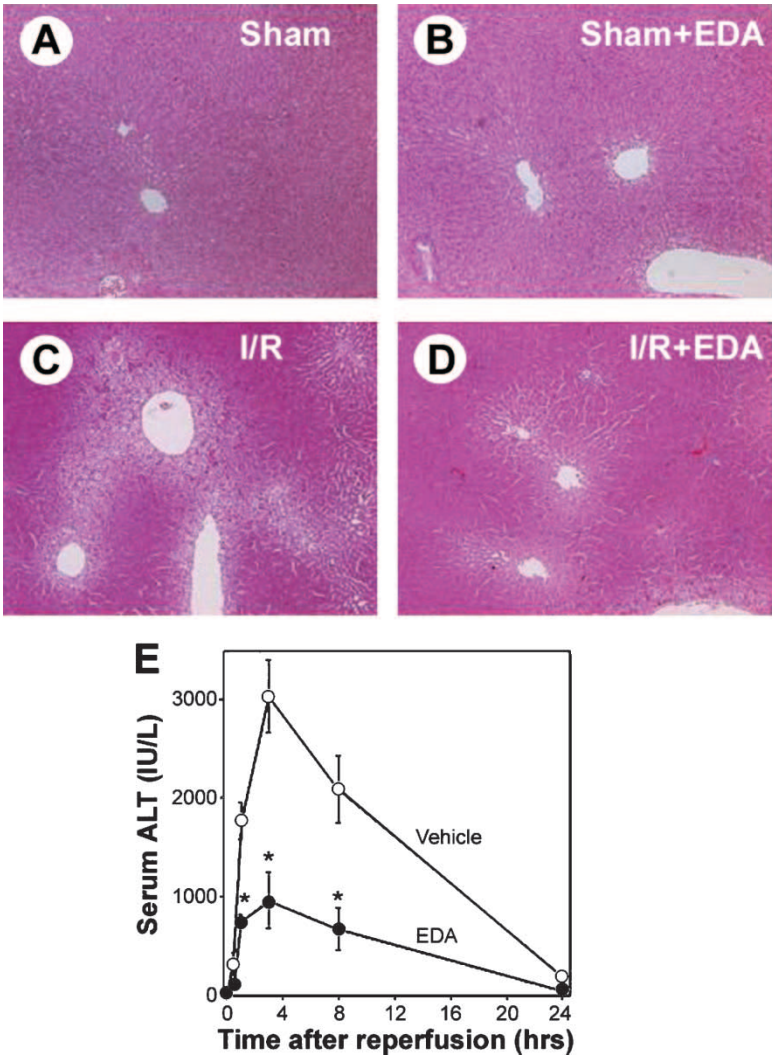


Figure 1. Edaravone alleviates liver injury due to warm ischemia–reperfusion. A–D, Hematoxilin/eosin-stained sections of the liver collected 24 h after rats were subjected to the sham operation and treated with saline (A), or edaravone (EDA, B); or underwent the ischemia–reperfusion (I/R) and were treated with saline (C) or EDA (D). Original magnification, × 200. Representative photomicrographs from six animals/group. E, Blood samples were collected after sham operation, or ischemia/reperfusion at the time points shown and the activity of ALT was measured as detailed in materials and methods. Vehicle, saline vehicle (open circles); and EDA, edaravone (closed circles). Values are mean ± SEM ($n = 6$). *, Statistically different ($p < 0.05$) from the vehicle control group by Student's *t*-test.

liver injury was significantly diminished (Figure 1D and E).

Inflammatory responses activated by acute ischemia/reperfusion in rat liver are blunted by edaravone

Ischemia/reperfusion liver injury is mediated, to the large degree, by a biphasic inflammatory response that involves activation of Kupffer cells, the resident hepatic macrophages, and later infiltration of activated neutrophils [5]. In order to evaluate whether edaravone is protective against inflammatory reaction to ischemia/reperfusion, various steps of this pathophysiological response were assessed.

Hepatic SEC dysfunction is known to occur in liver during ischemia/reperfusion and an increase in serum HA concentration is commonly used as a biomarker of altered SEC function [22]. In our studies, serum HA levels increased dramatically in rats treated with saline as early as 30 min after ischemia/reperfusion and were elevated above control levels for up to 24 h (Figure 2). This increase was largely blunted by treatment with edaravone.

The activation of Kupffer cells was assessed by measuring liver mRNA levels for tumor necrosis factor (TNF)α and serum levels of TNFα and IL-6, pro-inflammatory cytokines that play a key role in ischemia/reperfusion-induced inflammation and are known to be produced by activated Kupffer cells. The mRNA expression of TNFα in liver was significantly induced by ischemia/reperfusion within hours, an effect that was diminished by edaravone (Figure 3A). Serum levels of TNFα and IL-6 were also markedly elevated after reperfusion in the vehicle group, but this effect was significantly blunted by edaravone (Figure 3B and C). Interestingly, Kupffer cells, resident hepatic macrophages, were shown to be the source of TNFα induction in liver following ischemia/reperfusion and edaravone significantly reduced induction of mRNA for this pro-inflammatory cytokine (Figure 4).

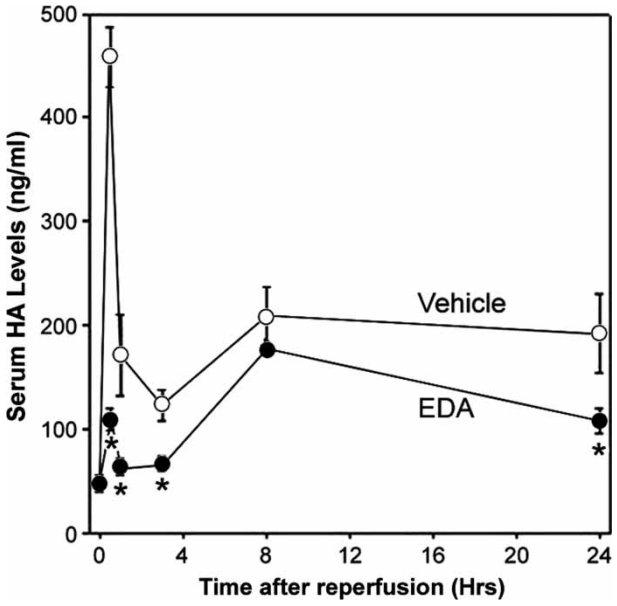


Figure 2. Edaravone blocks activation of hepatic stellate cells caused by ischemia/reperfusion. Hyaluronic acid (HA) levels were measured in blood samples collected at various time points for up to 24 h after ischemia/reperfusion as detailed in materials and methods. Vehicle, saline vehicle (open circles); and EDA, edaravone (closed circles). Values are mean ± SEM ($n = 3$–6). *, Statistically different ($p < 0.05$) from the vehicle control ischemia/reperfusion group by Student's *t*-test.

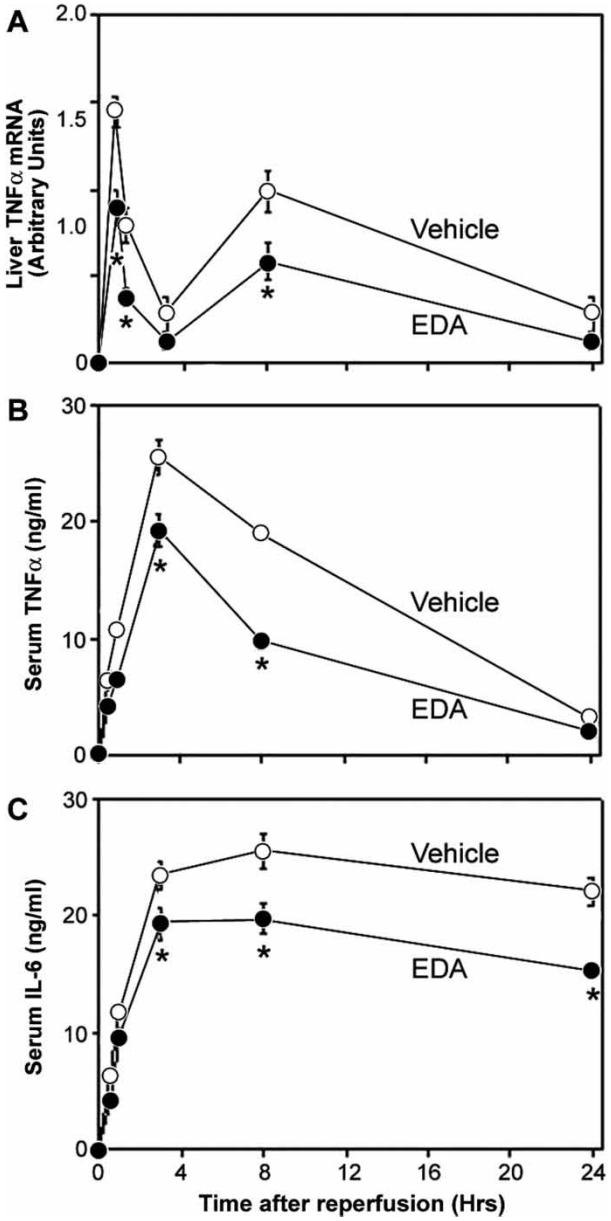


Figure 3. Ischemia/reperfusion-induced cytokine release is blunted by edaravone treatment. Liver mRNA levels of TNFα (A), and serum levels of TNFα (B) and IL-6 (C) were assessed at various time points for up to 24 h after ischemia/reperfusion in rats that received saline vehicle (open circles), or edaravone (EDA, closed circles). TNFα mRNA levels are expressed as a ratio to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Values are mean ± SEM ($n = 4$–6). *, Statistically different ($p < 0.05$) from the vehicle control ischemia/reperfusion group by Student's *t*-test.

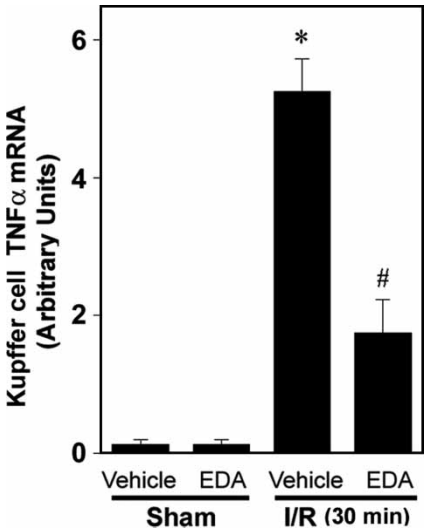


Figure 4. Ischemia/reperfusion-induced cytokine release by Kupffer cells is blunted by edaravone treatment. mRNA levels of TNFα were assessed 30 min after ischemia/reperfusion in rats that received saline vehicle, or edaravone (EDA). TNFα mRNA levels are expressed as a ratio to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Values are mean ± SEM ($n = 4–6$). ⋆, Statistically different ($p < 0.05$) from the vehicle control ischemia/reperfusion group by Student's *t*-test.

Neutrophil recruitment is thought to be responsible for a second wave of liver inflammation after ischemia/reperfusion and is mediated by a variety of adhesion molecules and SEC dysfunction [23,24]. In liver sections taken 30 min after reperfusion, expression of intercellular adhesion molecule (ICAM)-1 was minimal and not different between vehicle and edaravone groups (Figure 5A and C). The expression of ICAM-1 markedly increased 2 h after reperfusion in the vehicle-treated group (Figure 5B), but not in the edaravone-treated group (Figure 5D). The number of neutrophils in liver (Figure 5E) was minimal in the sham-operated animals, but increased sharply 24 h after ischemia–reperfusion in the vehicle-treated group. This increase was partially but significantly blunted by edaravone.

Edaravone blocks formation of free radicals after ischemia/reperfusion

To determine formation of superoxide in this model, livers were reperfused in presence of NBT. NBT reacts with superoxide radicals to form insoluble blue formazan. Formazan deposition occurs principally on Kupffer cells [25,26]. In sham-operated vehicle- or edaravone-treated livers formazan depositions were not detectable (Figure 6A and B). After ischemia–reperfusion, formazan deposition was detected and occurred in both non-parenchymal and parenchymal cells (Figure 6C). First, 30 min after reperfusion the formazan-positive cells were morphologically identified as Kupffer cells. At later times, deposition

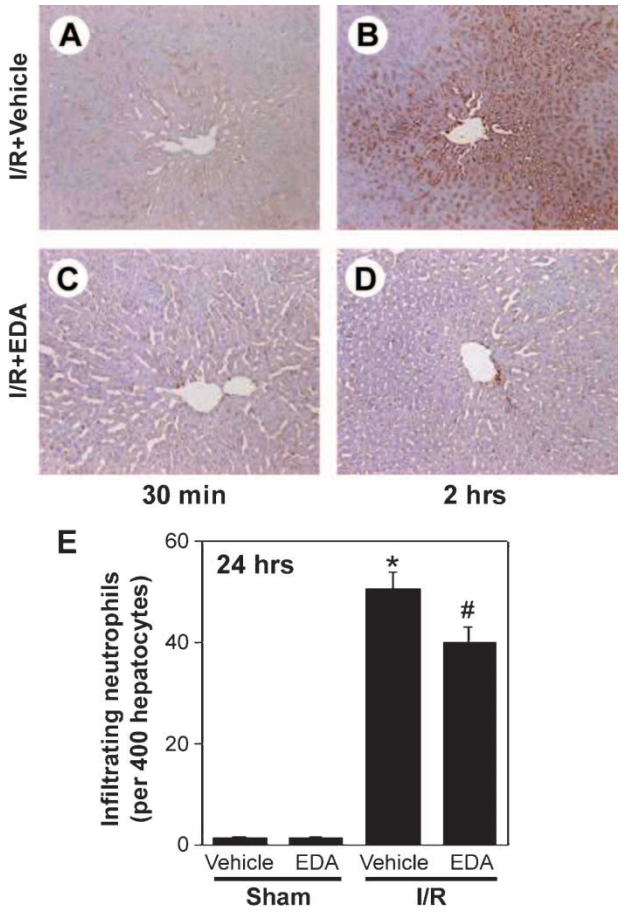


Figure 5. Ischemia/reperfusion-induced expression of the intercellular adhesion molecule (ICAM)-1 and neutrophil recruitment to the liver are blocked by edaravone. A–D, Expression of ICAM-1 in liver was determined by immunohistochemistry using tissue sections taken 30 min or 2 h after ischemia/reperfusion (I/R) in vehicle- (A and B), or edaravone- (EDA, C and D) treated animals as detailed in materials and methods. Original magnification, ×200. Representative photomicrographs are shown from six animals per group. E, The number of infiltrating neutrophils was counted in hematoxilin/eosin-stained liver sections taken 24 h after sham operation, or ischemia/reperfusion (I/R). VEH, saline vehicle- and EDA, edaravone-treated groups. Data are mean ± SEM ($n = 6$). Asterisks (⋆ and #) depict statistical significant ($p < 0.05$) differences from sham-operated and I/R vehicle-treated groups, respectively.

was detected also in the parenchymal and SECs. Treatment with edaravone resulted in blunted response whereby formazan formation was less pronounced than in vehicle-treated rats and was delayed (Figure 6D).

To test whether EDA also protects against lipid peroxidation in the liver after warm ischemia/reperfusion, 4-HNE-adducted proteins were measured by immunohistochemistry (Figure 6E and F). A massive accumulation of 4-HNE (brown staining) was observed in the ischemia/reperfusion vehicle-treated group 8 h after reperfusion (Figure 6E), as compared to sham-operated controls (data not shown). Accumulation of 4-HNE was considerably less in

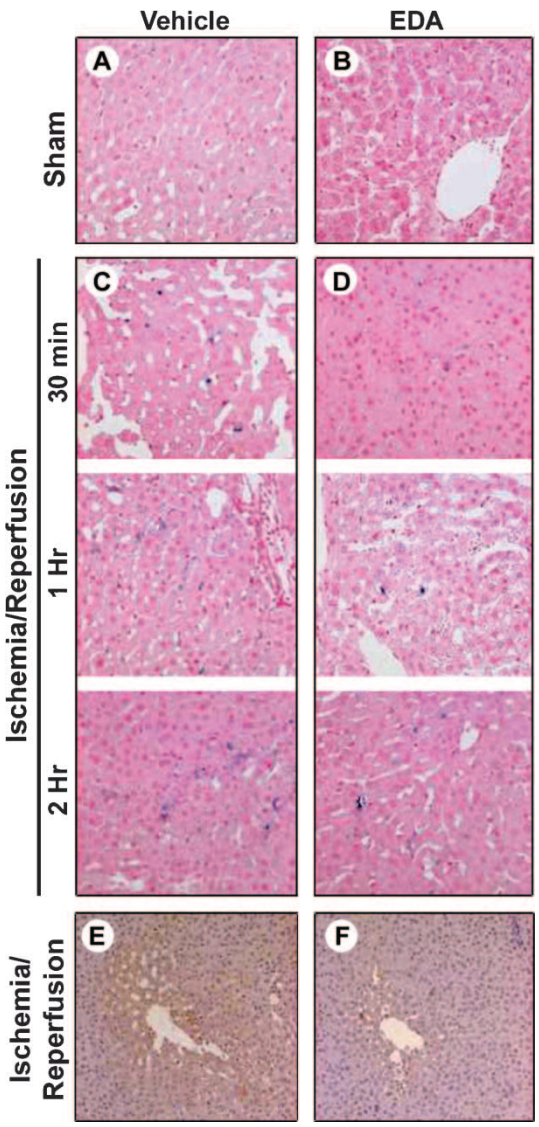


Figure 6. Edaravone blocks histopathological features of increased production of reactive oxygen and nitrogen species after warm ischemia/reperfusion in rat liver. A–D, Sham operated (A and B), or ischemia/reperfusion (C and D) groups were treated with Vehicle (A and C) or edaravone (EDA, B and D). At each time point shown, livers were perfused with Krebs-Henseleit bicarbonate buffer containing 0.05% NBT as described in "Materials and Methods". Liver sections were counter-stained with eosin. Original magnification, ×400. Representative photomicrographs are shown from four animals per group. E–F, 4-HNE-adducted proteins were measured by immunohistochemistry in vehicle (E) or edaravone (F)-treated animals subjected to ischemia/reperfusion for 8 h. Original magnification, ×200. Representative photomicrographs are shown from six animals per group.

rats whose livers were exposed to ischemia/reperfusion and treated with edaravone (Figure 6F).

In vivo spin trapping linked with ESR spectroscopy is a useful experimental tool because it allows direct detection and characterization of oxidants in tissues and body fluids [27]. This technique was applied in this study in order to assess whether edaravone is capable of inhibiting the production of ischemia/reperfusion-induced free radicals in liver. A spin trapping agent POBN was administered before ischemia and bile samples were collected at 30 min intervals for up to 3.5 h thereafter. Edaravone had no effect on bile production in liver over the time of the experiment (data not shown). Representative ESR spectra are shown in Figure 6. In the sham-operated animals, a typical small POBN-radical adducts were detectable in either vehicle- or edaravone-treated groups (Figure 7A and B, respectively). The intensity of this radical adduct was constant over the time of the experiment (Figure 8, open and dotted bars). Ischemia–reperfusion caused a time-dependent increase in production of ROS in liver as evident from the increase in the amplitude of the POBN-radical signal (Figures 7C and 8, filled bars). Computer simulation (Figure 7D) of the ischemia/reperfusion-induced ESR spectra determined presence of three radical species with hyperfine coupling constants of (I) $a^N = 15.30$ G, $a\beta^H = 2.66$ G, (II) $a^N = 16.04$ G, $a\beta^H = 2.60$ G, and (III) $a\beta^H = 1.91$ G. Such coupling constants are

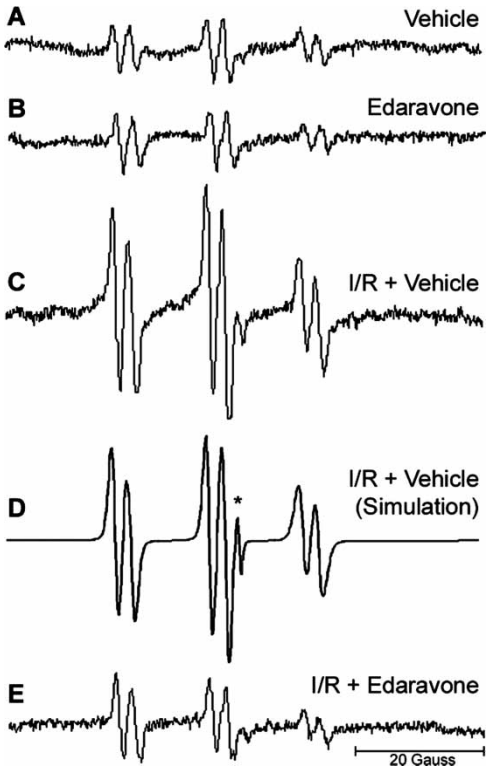


Figure 7. *In vivo* detection of radical production in rat liver using spin trap/electron spin resonance techniques shows a protective effect of edaravone. The spin-trapping reagent α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN; 1 g/kg, i.v.) was injected into the animals that were treated with vehicle (A and C) or edaravone (B and E) and underwent a sham operation (A and B), or were subjected to warm ischemia/reperfusion (I/R, C and E) of the liver for 60–90 min. Bile samples were collected and radical measurements performed as detailed in materials and methods. Typical ESR spectra are shown from 4 to 6 animals per group. D, a computer simulation of the spectrum depicted in panel C is shown. Hyperfine coupling constants for the species are (I) $a^N = 15.30$ G, $a\beta^H = 2.66$ G, (II) $a^N = 16.04$ G, $a\beta^H = 2.60$ G, (III, marked with ⋆) $a\beta^H = 1.91$ G.

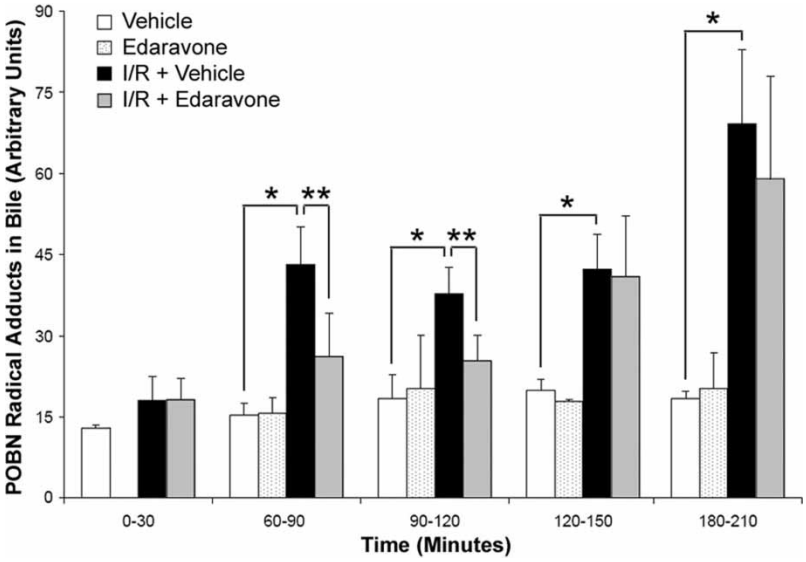


Figure 8. Edaravone blocks ischemia/reperfusion-induced free radical formation early after initiation of the reperfusion. Rats were administered POBN and either saline vehicle or edaravone and subjected to sham operation or ischemia/reperfusion (I/R). Free radical adducts were quantified in 30 min bile samples by double integration of ESR spectra as described in materials and methods. Data shown are mean ± SEM from 4 to 6 animals per group. Asterisks (*, **) show statistically significant ($p < 0.01$) difference as compared with the sham + vehicle, or I/R + edaravone groups, respectively, by Student's *t*-test.

characteristic of carbon-centered 4-POBN radical adducts and match values ($a^{N} = 15.63$ G; $a\beta^{H} = 2.73$ G) obtained from bile of rats given spin trap and oxidized polyunsaturated fatty acids [28]. The third radical species (marked with *) is likely an ascorbate derived radical [29].

The increase in ischemia/reperfusion-induced POBN-radical adducts was blunted effectively by treatment with edaravone for up to 2 h after reperfusion (Figures 7E and 8, grey bars). Interestingly, the initial increase in radical production in liver after acute ischemia/reperfusion was followed by a plateau for about 2 h. After 2 h the radical production was further increased. This observation is consistent with a well-accepted biphasic response to ischemia/reperfusion where the initial increase in radical production detected with ESR is most likely due to Kupffer cell activation and the second increase is likely due to additional radicals produced by the infiltrating neutrophils.

Discussion

This study aimed to understand if edaravone (MCI-186, Radicut®), a novel pharmaceutical approved for use in patients with stroke [9], could also be effective in preventing liver damage caused by a temporary obstruction of the blood flow to the liver, so-called warm ischemia/reperfusion, a procedure that is often used in liver surgery. Edaravone is a potent antioxidant pharmaceutical and was shown to be protective in several clinical conditions where an overt increase in ROS plays a key mechanistic role in tissue damage [9,11]. While it was recently reported that edaravone is protective against ischemia/reperfusion injury in rat liver [13–17], the exact mechanism of this effect remains unclear. This study used ESR technique coupled with spin trapping *in vivo* to directly assess if edaravone can block production of oxidants caused by ischemia/reperfusion. Furthermore, we measured other endpoints indicative of the mechanism of tissue damage under these conditions. Our data demonstrates that by effectively preventing oxidant generation within first 2 h of reperfusion, edaravone exerts a strong overall protective effect against liver damage by limiting oxidant-mediated deleterious events such as production of cytokines and recruitment of neutrophils.

Warm ischemia–reperfusion is damaging and leads to severe liver injury and death of hepatocytes [3]. Some of the early cellular changes in liver upon reperfusion include swelling and death of SECs, events that occur in parallel and are dependent upon activation of Kupffer cells that produce ROS [30]. We show that edaravone was very effective in limiting the effects of the early oxidative burst of Kupffer cells, as evident from the absence of NBT-positive hepatic macrophages, no increase in lipid peroxidation, and no increase in POBN-radical adducts in first 2 h in rats that were subjected to ischemia/reperfusion and received the drug. This overall protection against Kupffer cell-produced oxidants also led to blunted release of inflammatory cytokines, such as TNFα and IL-6, that are known to be regulated through oxidant-sensitive transcription factor NF-κB, as well as dramatically lower serum levels of hyaluronic acid,

a marker for endothelial cell damage, in edaravone-treated rats.

The loss of sinusoidal cells leads to microcirculatory disturbance and facilitates hepatocyte damage [31]. The pro-inflammatory cytokines produced by Kupffer cells not only can directly induce death signaling in neighboring hepatocytes [32], but they also can recruit neutrophils into the sinusoids [33]. The sinusoids that lost protective endothelial cell cover are also an attraction to inflammatory cells [34]. Polymorphonuclear cells attached to denuded hepatic sinusoids produce large amounts of reactive intermediates that include superoxide anion released by NADPH oxidase, tissue proteases, and hypochlorous acid [2]. Our observations of acute changes in liver after ischemia/reperfusion are in accordance with these previous reports. Specifically, expression of ICAM-1 increased on the SECs, and the number of infiltrating neutrophils was significantly elevated in the liver following ischemia/reperfusion. While administration of edaravone resulted in some protection against these later pathobiological changes caused by ischemia/reperfusion, the magnitude of such effect was much smaller when compared to that at earlier time points. Conversely, our direct measurements of ROS production in liver after ischemia/reperfusion showed that oxidant generation reached a plateau after first hour of reperfusion for about 90 min, but then increased substantially at a later time point. While it is not possible to collect additional samples from animals in these experiments due to technical limitations, this result is consistent with the hypothesis that the initial increase in free radicals is Kupffer cell-mediated and the subsequent larger increase is due to infiltrating neutrophils [5]. Edaravone blocked the initial increase in POBN-radical adducts, but was not effective 2 h after last injection, most likely due to the rapid decline in the effective concentration of the drug because of its metabolism and excretion (see below).

In addition to an encouraging significant overall protective effect of edaravone on histopathological signs of liver injury and serum ALT after warm ischemia/reperfusion, we observed that edaravone was most effective in preventing early molecular steps that facilitate liver damage. Edaravone exerts beneficial free radical scavenging and antioxidant characteristics both *in vitro* and *in vivo* [9,19]. Edaravone is a lipophilic, readily transferable to tissues compound that has effective tissue levels that are achieved shortly after injection [35]. It is rather quickly metabolized and excreted when administered *in vivo*. In pharmacokinetic studies, the plasma concentration of edaravone reached maximum levels 40 min after an intravenous continuous infusion at concentrations of 0.2–1.5 mg/kg and then decreased rapidly [36]. Edaravone is metabolized in the liver and excreted rapidly in the urine within 24 h after the beginning of infusion. Irrespective of the dose and infusion time, the urinary excretion rates of edaravone and its conjugates are very similar. Since free radicals are thought to be generated for a prolonged period of time after ischemia/reperfusion, the short half-life of the drug that possesses beneficial radical scavenging properties is a somewhat limiting factor. Still, several *in vivo* studies where edaravone was administered prior to, together with, and shortly after the insult to the liver, such as endotoxin injection, partial hepatectomy, or ischemia/reperfusion, showed positive results on animal survival, tissue damage and other markers of injury [13,19,37].

Conclusions

The data presented here are the first direct evidence that edaravone blocks production of free radicals in liver *in vivo* and prevents subsequent liver injury caused by ischemia/reperfusion. Since edaravone is currently being used in clinical practice without serious side effects, it may provide a new, effective therapy against organ injury due to ischemia/reperfusion that is unavoidable during gastro-intestinal surgery. While our results in the animal model of warm ischemia/reperfusion liver injury are encouraging and provide a mechanistic rationale for the potential clinical use of edaravone, further investigations are needed to elucidate the details of the appropriate dose and infusion methods.

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