Inflammation in ischemia and reperfusion: From mice to men
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SYSTEMIC LIDOCAINE DOES NOT ATTENUATE HEPATIC ISCHEMIA-REPERFUSION INJURY IN RATS

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ABSTRACT

BACKGROUND: Lidocaine has been shown to attenuate ischemia–reperfusion (I/R) injury in the heart, lung, and brain, potentially due to modulation of inflammatory responses and apoptotic signaling pathways. Because hepatic I/R injury after liver surgery still poses a significant risk for postoperative liver dysfunction or even failure, we investigated whether systemic lidocaine would also positively affect hepatocellular damage and overall liver function after hepatic I/R injury. In addition the potential underlying mechanisms of action were studied.

METHODS: A standardized rat model of 70% I/R injury was used to assess the effects of systemic lidocaine on hepatocellular damage after 60 minutes of ischemia and subsequent reperfusion. To better mimic the clinical situation, we combined 45 minutes of ischemia with partial hepatectomy in a second model. Systemic lidocaine was administered continuously, starting 30 minutes before the ischemic insult until 20 minutes of reperfusion. Hepatocellular function was assessed using different variables of liver synthesis, cellular integrity, and metabolism. Inflammation was evaluated by measuring leukocyte influx and apoptosis detected using TUNEL staining and a caspase-3 assay.

RESULTS: In both models, I/R injury resulted in a significant increase in biochemical and histological hepatocellular damage with comparable values in control and lidocaine-treated animals. Postoperative liver function was significantly impaired secondary to ischemia, yet no significant differences between control and lidocaine groups could be observed. Likewise, there was no significant difference between control and lidocaine-treated animals with respect to I/R injury–induced leukocyte influx, as a marker for inflammatory response.

CONCLUSION: Systemic lidocaine in therapeutic concentrations neither attenuated hepatocellular damage nor improved postoperative liver function after hepatic I/R injury.

INTRODUCTION

Liver dysfunction or even failure secondary to ischemia-reperfusion (I/R) injury remains a major clinical problem after partial hepatectomy (PHx) or transplantation surgery. Current vascular occlusion techniques for minimizing blood loss during surgery cause ischemic injury to the liver, exacerbated by oxidative stress and inflammatory mediators during reperfusion. Pharmacological strategies to attenuate the inflammatory response after I/R damage to the liver may thus improve postoperative hepatic function and patients’ outcomes. Local anesthetics (LA) are primarily known for inhibition of voltage-gated sodium channels, underlying their antinociceptive and antiarrhythmic effects. Yet growing evidence indicates a variety of additional therapeutic properties and with respect to I/R injury, modulation of the inflammatory response and attenuation of apoptosis are of special interest1,2. By preventing an overactive inflammatory response due to inhibition of polymorphonuclear leukocyte priming, the amide LA lidocaine was shown to reduce the release of toxic oxygen metabolites and to attenuate I/R injury in the heart, lung, and brain3-5. On the basis of these immunomodulatory properties of LA and their positive impact on I/R damage to other organ systems, we hypothesized that lidocaine may protect the liver from I/R injury during hepatic surgery. Using two different rat models of hepatic I/R injury, we thus investigated the effects of systemic lidocaine on hepatocellular damage and liver function and studied potential underlying mechanisms.

METHODS

Animals

All experimental animal protocols were approved by the institutional local Animal Ethics Committee. Male Wistar rats (250 to 300 g) were purchased from Harlan (Horst, The Netherlands) and housed in a climate-controlled institutional animal facility on a 12-hour light/dark cycle with access to standard chow and water ad libitum. Rats were acclimatized for 1 week and fasted overnight before surgery.

Experimental Procedures

Rodent Model of Hepatic I/R Injury

To assess whether systemic lidocaine positively affects I/R injury after liver surgery, we used a standardized 70% partial I/R injury model. Animals were randomized into 4 groups (n=8 per group) to receive either lidocaine or saline and subjected to 60 minutes of ischemia followed by 6 (I/R 6-hour group) or 24 (I/R 24-hour group) hours of reperfusion (Figure 1). Animals were anesthetized by inhalation of a mixture of air: O2 (1:1 v/v, 2 L/min) and 4% isoflurane (Florence, Abbott Laboratories, Queensborough, UK). After endotracheal intubation, their lungs were ventilated (Zovent, Instruvet, Amerongen, The Netherlands) with a mixture of air: O2 (1:1 v/v, 2 L/min) and 2%–3% isoflurane. Adequate ventilation was confirmed by continuous measurement of end-tidal CO2, assuring physiological pH during the entire procedure. Rectal temperature was maintained at 37.0°C (±0.2°C). A midline...
laparotomy, partial liver ischemia was induced by clamping the afferent vessels to the median and left lateral lobes with a microvascular clamp, as described previously. Sham operated animals (n=4), which did not undergo ischemia, were used as control. To better mimic the clinical situation, we combined hepatic I/R injury with a partial (30%) liver resection (PHx) of the nonischemic lobes (PHx+I/R injury model). Animals were again randomized for lidocaine or saline treatment and subsequently allocated to undergo 30% PHx only or 30% PHx in addition to 45 minutes of ischemia and 24 hours of reperfusion (PHx+I/R group). Surgery was performed as described above, followed by resection of all nonischemic liver lobes at the end of the ischemic period. Resection was completed within 1 minute before reperfusion to prevent splanchnic congestion. Each group consisted of 6 animals. A sham group undergoing no hepatic resection or ischemia was included as control (n=4). In both models, lidocaine was administered IV, starting 30 minutes before ischemia with an initial loading dose of 5.0 mg/kg (0.9 mg/mL) over 2 minutes, and followed by a continuous lidocaine infusion at a rate of 5.0 mg/kg/h (0.9 mg/mL) until 20 minutes of reperfusion (Figure1). Animals that underwent 30% PHx received a lower dose of continuous lidocaine (2.0 mg/kg/h) to compensate for the reduction in hepatic mass (Figure1). Saline-treated animals likewise received systemic saline. A continuous administration of the LA was used, because the modulatory effects of LA on inflammation and coagulation were shown to be time dependent. Plasma lidocaine concentrations were measured at the beginning of ischemia and after 20 minutes of reperfusion.

Plasma Variables of Hepatocellular Injury and Liver Function
Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured as markers of hepatocellular damage using routine laboratory activity assays. Blood samples were drawn before ischemia and at 20 minutes, 6 hours, and 24 hours of reperfusion from the caval vein, the tail-vein, and carotid artery, respectively. Twenty-four hours after reperfusion, liver function was assessed by prothrombin time (PT), antithrombin (AT), and total bilirubin using routine laboratory assays. PT and AT were used to determine synthetic function, whereas plasma bilirubin presented an indicator of uptake and excretory capacity of the liver.

ICG Clearance Test
To further quantify the hepatic uptake and elimination capacity, the indocyanine green clearance (ICG) test was used as a well-established dynamic quantitative liver function test, as has been described before. Briefly, after cannulation of the right carotid artery for blood sampling, freshly prepared ICG (PULSION Medical Systems, München, Germany) in a concentration of 2.5 mg/mL was injected into the penile vein (2.5 mg/kg). Blood samples were obtained before and 1, 2, 3, 4, 6, 8, and 10 minutes after ICG injection in control and 30% PHx animals. Because the decreased uptake of ICG in the ischemic groups, blood samples were drawn before and at 2, 4, 6, 8, 10, 14, and 16 minutes after ICG injection. Plasma ICG was determined spectrophotometrically at 805 nm (Uvikon 850, Kontron Instruments, München, Germany). The ICG disappearance constant (k) was derived from the slope of the semilogarithmic decay curve, and the ICG plasma disappearance rate (PDR, %/min) was calculated on the basis of the following formula: PDR = k x 100

Analysis of Histopathological Damage
After 24 hours of reperfusion rats were sacrificed. Biopsies from the ischemic liver lobes were taken and immediately frozen in liquid nitrogen or fixed in 4% buffered formaldehyde. Various sections (4 µm) were routinely stained with hematoxylin and eosin and scored for hepatocellular necrosis and vacuolization by 2 independent and blinded observers at 2.5x and 10x magnification. Necrosis and vacuolization are expressed as percentage of total surface area of the liver section.

Inflammation
Myeloperoxidase (MPO) is stored within the azurophilic granules of leukocytes and is mainly found within neutrophils, but also in monocytes as well as in some tissue macrophages, such as Kupffer cells. A fluorometric MPO assay was used to quantify the accumulation of these inflammatory cells within the hepatic tissue, as has been described. Hepatic edema was assessed by calculating the percentage of tissue water. Biopsies from the median

Figure 1. Experimental design. Lidocaine groups of both models (70% partial ischemia–reperfusion [I/R] injury model and partial hepatectomy [PHx] + I/R injury model) are shown. Control animals were treated similarly with normal saline.
(ischemic) liver lobe were weighed and subsequently stored at 60°C. Dry weight was determined by the same scales until weight remained unchanged (approximately 21 days later). Percentage of tissue water was calculated as follows: (wet weight – dry weight) x 100/Wet weight.

**Apoptosis**

Apoptosis was detected using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, ApopTaq detection kit; Chemicon) according to manufacturer’s instruction and immunohistochemistry using cleaved caspase-3 antibodies, as described previously. Immunolabeled and hematoxylin-positive nuclei were quantified with ImageJ software (K-67 plugin, National Institutes of Health, Bethesda, MD). Apoptotic cells are expressed as percentage of total number of hepatocytes averaged over 6 visual fields per slide (20x magnification).

**Statistical Analysis**

Statistical analysis was performed with GraphPad Prism (GraphPad Software, San Diego, CA) and Statistical Package for Social Sciences (SPSS 12.02, Chicago, IL). Continuous data were compared using the Kruskal–Wallis test or a repeated-measures analysis of variance (ANOVA) for measurements over time. A 1-way ANOVA using ranked values and corrected with a Tukey post hoc test was performed for comparing 2 groups. Data are expressed as mean ± SD. All statistical tests were 2 tailed, and differences were considered significant at a *p* value ≤ 0.05.

**RESULTS**

**Lidocaine Concentration**

Preischemic plasma concentrations of lidocaine were similar in groups of both models, ranging from 2.59 ± 0.54 mg/L (70% partial I/R injury model) to 2.91 ± 0.58 mg/L (PHx+I/R injury model) (Figure 2). Twenty minutes after reperfusion, lidocaine concentrations increased to 4.90 ± 0.54 mg/L in the I/R groups of the 70% I/R injury model, and were even more pronounced up to 5.49 ± 0.66 mg/L in the PHx+I/R groups (*p* < 0.0001). Clamping 70% of the liver results in a major reduction of liver tissue available for lidocaine metabolism, leading to the observed increase of lidocaine plasma concentration. However, because systemic lidocaine was shown to exert its therapeutic effects in plasma concentrations between 1.5 mg/L and 6 mg/L, lidocaine concentrations were within the therapeutic range.

**Hepatocellular Damage**

Plasma AST and ALT were assessed as established markers of hepatocellular damage. In the 70% partial I/R injury model, plasma ALT/AST levels were significantly elevated during the entire reperfusion phase compared to preischemic values (Figure 3, A and B). There were no significant differences between the saline and lidocaine groups during the reperfusion phase at all time points. Although the ischemic period was reduced to 45 minutes in the PHx+I/R model, AST/ALT levels were almost twice the values of the 70% partial I/R injury model at 20 minutes (ALT: 6581 ± 1353 vs 3099 ± 1517) and 6 hours (ALT: 6948 ± 1822 vs...
3457 ± 1563) of reperfusion (Figure 3, C and D). After 24 hours reperfusion, however, plasma levels of ALT decreased to almost 50% of levels observed in the 70% partial I/R injury model (ALT: 1313 ± 477 vs 2266 ± 1616), demonstrating a difference in the dynamics of hepatocellular damage between both models. In the PHx+I/R groups, ALT/AST levels were significantly elevated compared to sham-operated animals throughout the entire reperfusion (P<0.0001 and P<0.0001, respectively), with no significant difference between saline- and lidocaine-treated animals at any time point (Figure 3, C and D). PHx alone was also associated with significantly elevated plasma ALT and AST levels compared to sham surgery (P<0.05) at all time points during reperfusion, without significant differences between saline- and lidocaine-treated animals. Histopathological evaluation revealed necrosis of approximately 16% of total surface area of the liver sections in all groups of the 70% partial I/R injury model after 6 hours and 24 hours of reperfusion, with comparable values for lidocaine- and saline-treated animals (P=0.99) (Figure 4A). Accordingly, the extent of necrosis after 24 hours of reperfusion was similar for animals of the PHx+I/R model without any differences between the lidocaine and saline groups (16.5 ± 8.9% versus 17.5 ± 10.7, respectively [P=0.99]). In the 70% PHx model, vacuolization, as an indicator of severe (intra-)cellular damage, was only observed in a small percentage after 6 hours of reperfusion, with a substantial increase after 24 hours of reperfusion (Figure 4A). Likewise, there was no difference between lidocaine- and saline-treated animals after 6 hours (1.61% ± 0.74% versus 1.88% ± 0.79%) and 24 hours (51.7% ± 17.3% versus 41.2% ± 14.0% [P=0.82]) of reperfusion; (Figure 4A). Vacuolization at 24 hours of reperfusion was only 1.3% ± 1.0% in the PHx+I/R saline group and 3.5% ± 2.6% in the lidocaine group, which is substantially less than in the 70% partial I/R model. There were no differences, however, between saline- and lidocaine-treated animals (P=0.65). Sham operated or 30% PHx animals did not show any necrosis or vacuolization after 24 hours of reperfusion (Figure 4B).

Liver Function

Postoperative liver function was assessed by monitoring various indicators of synthesis, hepatic uptake, and excretory capacity. Blood levels of PT, used as a marker for hepatic protein synthesis, were significantly prolonged during reperfusion in animals suffering from 70% partial I/R injury as well as in animals undergoing additional PHx. However, no significant differences between saline and lidocaine-treated groups could be observed at 6 hours (25.5 ± 1.3 seconds versus 24.1 ± 1.3 seconds, P = 0.54) and 24 hours of reperfusion (27.1 ± 5.3 seconds versus 23.7 ± 3.8 seconds, P = 0.38). In the PHx+I/R model PT remained unaffected in 30% PHx and sham-operated animals but was substantially prolonged after 24 hours of reperfusion in the PHx+I/R groups (Figure 5A). There were no significant differences between lidocaine- and saline-treated animals (Figure 5A). Plasma levels of AT, used as a second indicator for liver synthesis, significantly decreased by approximately 20% 24 hours after 30% PHx with no significant differences between the lidocaine and control groups.
groups. AT was found to be further reduced (by >50%) when 30% PHx was combined with an additional ischemic insult. Again, results obtained were comparable in lidocaine- and saline-treated animals (Figure 5B). Plasma bilirubin levels, assessed for evaluation of hepatic uptake and excretory capacity, significantly increased from 23.6 µmol/L at 6 hours of reperfusion to 38.5 µmol/L after 24 hours of reperfusion in saline treated animals of the PHx+I/R group (Figure 5C). In combination with the prolonged PT, this suggests the development of profound liver insufficiency. Plasma bilirubin levels did not differ between lidocaine- and saline-treated animals at any time point after reperfusion (P=0.55 and P=0.90, respectively). For further assessment of hepatocellular function, the ICG clearance test was used as a quantitative liver function test. The calculated PDR of ICG was significantly decreased by approximately 75% in the PHx + I/R group (P<0.001 versus sham-operated animals). Again, no difference was found between lidocaine- and salinetreated animals (Figure 5D).

**Inflammatory Response**

Animals undergoing 30% PHx did not show a significantly increased influx of inflammatory cells, as determined by MPO activity in both saline and lidocaine groups compared to sham-operated animals (Figure 6A). In combination with 45 minutes of ischemia, hepatic MPO activity increased significantly, yet without significant differences between saline- and lidocaine-treated animals. The wet/dry ratios, as assessed for potential hepatic edema, showed a similar pattern (Figure 6B); however, no significant differences between control and lidocaine treatment could be observed.

**Apoptosis**

TUNEL staining demonstrated few immunopositive cells within the necrotic areas and almost no apoptotic cells within the relatively intact liver parenchyma (<0.1%) in both saline and lidocaine groups. Immunohistochemistry, using cleaved caspase-3, confirmed the almost complete absence of apoptosis, suggesting apoptosis as underlying mechanisms of loss of liver tissue to be negligible within the PHx + I/R model.
Our study aimed to identify potential effects of systemic lidocaine on hepatocellular damage and postoperative liver function after an ischemic insult, using two different in vivo models of hepatic I/R injury. Although protective effects of lidocaine in I/R injury have been described in several organ systems such as heart, lung, and brain - we could not demonstrate a potential benefit of lidocaine administration on overall postoperative liver function in hepatic I/R injury. Studies investigating hepatic I/R injury mainly use a standard 70% I/R injury model, although the remaining 30% nonischemic liver tissue virtually excludes an accurate functional assessment of the postschismic liver parenchyma. Therefore, to better compare current data but also to mimic the clinical situation more closely, we used two different animal models in this study: the standard 70% I/R injury model and the PHx+I/R injury model, in which the ischemic insult is accompanied by partial liver resection. The dynamics of hepatocellular damage apparently differ between the two models: resection of liver tissue (PHx) increases bloodflow through the postschismic remnant liver, thereby augmenting hepatocellular injury, resulting in a more rapid release of liver transaminases into the systemic circulation, demonstrated by substantially higher ALT and AST in the PHx + I/R model 6 hours after reperfusion. For the 70% I/R injury model, however, preferential shunting to the nonischemic lobes occurs, bypassing the damaged liver parenchyma. Therapeutic concentrations of IV lidocaine, however, did not have any effect with respect to hepatocellular damage or postoperative liver function in both models. Why the LA is effective in the heart, brain, and lung but not in the liver remains unclear. Lidocaine is considered to benefit I/R injury by modulation of the inflammatory response and/or attenuation of apoptotic cell death. Underlying mechanisms of these anti-inflammatory effects have not been fully elucidated, yet lidocaine was suggested to attenuate signaling of inflammatory mediators by modulation of their respective G-protein coupled receptors. Inhibition of leukocyte migration was shown to be one major anti-inflammatory effect of LA; however, current literature is conflicting. Various in vitro studies demonstrated that lidocaine inhibits release of chemoattractants as well as expression of adhesion molecules, thereby potentially attenuating the migration of leukocytes to the site of inflammation. Because lidocaine concentrations used were far beyond toxic plasma levels of 10 µg/mL, these data cannot be easily translated into the clinical setting. Yet, few in vivo studies confirmed a decrease in adhesion and accumulation of leukocytes when lidocaine was administered in therapeutic concentrations. On the other hand, one study neither showed a reduction in leukocytes recruited to ischemic heart tissue in vivo, nor an alteration of endothelial–leukocyte interaction in vitro. Lidocaine, in clinically relevant concentrations, was further reported to inhibit priming of human neutrophils for release of toxic oxygen metabolites in vitro, potentially resulting in attenuation of I/R injury. However, our results did not show any effect of lidocaine on hepatocellular damage in the PHx+I/R injury animals or, in particular, on MPO activity, especially used as a marker for neutrophil influx. Because MPO is a peroxidase enzyme that is abundantly present in the azurophilic granules of neutrophilic granulocytes but is also stored within monocytes and resident tissue macrophages, an effect of the LA on these cells also cannot be excluded. Yet, the anti-inflammatory effects of lidocaine on granulocytes and monocytes are well described and were not in the scope of this study. Furthermore, because there was no difference in MPO activity of lidocaine- and saline-treated animals, a potential effect on cells other than neutrophils cannot be discounted. Attenuation of apoptosis is a second mechanism by which lidocaine may exert its beneficial effect in I/R injury. Alongside their antiapoptotic properties, cell culture studies suggested that apoptosis was induced by lidocaine, particularly when high concentrations were used. Apoptosis, induced by I/R injury or lidocaine itself, was not a major contributor to tissue loss in our model. However, as apoptosis was only assessed after 24 hours of reperfusion, interpretation of these data is certainly limited. Hepatocytes, that were only sublethally damaged by ischemia, may have been triggered to undergo apoptosis even later, and thus the presence of delayed apoptosis and potential beneficial effects of lidocaine cannot be excluded. The lack of an effect of lidocaine on hepatic I/R injury therefore remains unsolved. Kupffer cells, the liver-specific macrophages, are ascribed a crucial role in hepatic I/R injury, whereas it is still not known if LA are able to modulate Kupffer cell responses. Moreover, lidocaine is almost exclusively metabolized by the liver, unlike in those organs in which lidocaine has proven to be effective. Additionally, administration of lidocaine has been demonstrated to stimulate metabolism and increase oxygen consumption of the liver. Cell metabolism and ischemic damage are closely linked as shown by the protective effects of hypothermia, in which cell metabolism and oxygen consumption are reduced, thus diminishing hepatocellular damage. Conversely, continuous administration of lidocaine increases oxygen demand and therefore renders parenchymal cells more vulnerable to oxygen deprivation. In our study, systemic lidocaine was started 30 minutes before ischemia for up to 20 minutes of reperfusion, possibly increasing the requirement of oxygen during the ischemic period and early reperfusion, thereby potentially counteracting the beneficial anti-inflammatory effects of lidocaine. The majority of studies published suggests a protective effect of lidocaine on I/R injury; however, one study reported that lidocaine, in clinically relevant concentrations, enhanced tissue damage by aggravating necrosis, apoptosis, and inflammation in a murine model of renal I/R injury. The beneficial effects of lidocaine in I/R injury may thus indeed be organ specific. With respect to hepatic I/R injury, two studies described positive effects of lidocaine treatment. Chen et al. injected lidocaine into the hepatoduodenal ligament 10 minutes before 40 minutes of ischemia and subsequently analyzed hepatocellular damage throughout 40 minutes of reperfusion. By local administration of the LA, the authors aimed to block hepatic innervations, thereby improving hepatic bloodflow and inhibiting recruitment of neutrophils, yet neither liver bloodflow nor neutrophil influx were determined in this study. Local administration of lidocaine most probably results in lower plasma levels of the LA in afferent blood vessels, thus resulting in less impairment of liver metabolism than after systemic administration. Hence, effects of local and systemic application of lidocaine are difficult to compare. Using isolated perfused rat livers as an ex vivo model, Tomori et al. demonstrated decreased neutrophil influx in conjunction with an attenuated liver damage after systemic lidocaine administration. Even though plasma levels of lidocaine were not determined, an infusion rate of 2.0 mg/kg/min as compared to 5.0 mg/kg/h in our study suggests considerably higher plasma levels than 10 mg/L, which is clinically associated with significant adverse effects. Using these high concentrations, the reduced influx of neutrophils may have outweighed the increased metabolic demand.
CONCLUSION

Taken together, systemic lidocaine in clinically relevant concentrations does neither attenuate hepatocellular damage nor improve postoperative liver function after hepatic I/R injury, with or without partial liver resection. Why lidocaine lacks effect in the liver when effective in cerebral, pulmonal, or cardiac I/R injury remains open to debate and requires further research.
REFERENCES


