Original article

Naloxone: a potent protective agent in ischemia – reperfusion – induced liver injury

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SUMMARY

Hepatic ischemia/reperfusion-induced injury is accompanied by free radical production, leading to endothelial cell destruction, adhesion of neutrophils and plugging of the hepatic sinusoids and thus to blood microcirculation flow reduction. Naloxone is a known opioid antagonist that has been shown to act by inhibiting the release of free radicals. The purpose of this study is to clarify the changes in hepatic microcirculation during liver ischemia and after reperfusion in the rat and the potential beneficial effects of Naloxone as pretreatment, in respect to microcirculation and oxidative stress.

One hundred and forty male Wistar rats, allocated to Naloxone [N] or Placebo [P] treatment, were subjected to either 30min or 60min normothermic ischemia [70% of total liver], followed by 60min reperfusion. Liver microcirculation was assessed by laser-Doppler flowmetry at baseline, at the end of the ischemia period [30min or 60min, respectively] and at the end of the reperfusion period, while oxidative stress was assessed by means of MDA at the same time periods.

Naloxone pretreatment seemed to protect liver parenchyma, since MDA levels were significantly decreased in relation to placebo treated rats, in both 30min ischemia/60min reperfusion and 60min ischemia/60min reperfusion groups. Similarly, Naloxone pretreatment was found to significantly improve liver microcirculation in relation to placebo treated rats, in both groups.

In conclusion, the results of this study indicate that Naloxone pretreatment protects the liver from ischemia/reperfusion hepatocellular injury.

Key words: Naloxone, ischemia/reperfusion injury, liver, laser-Doppler flowmetry, malondialdehyde

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INTRODUCTION

The liver is known to be very sensitive to oxygen deficiency and ischemic hepatocellular injury may easily occur during the vascular clamping manouvre in liver surgery or, more frequently, as a consequence of circulatory shock.[1,2] Upon reperfusion of a previously ischemic tissue a specific injury may occur - named reperfusion injury - which is assumed to be related to the production of free radicals upon tissue re-oxygenation. This reperfusion injury occurs in addition to any ischemic injury that may already exist. Since protecting the liver against oxygen deficiency and thus ischemic injury is a major concern in the area of hepatic injury and patient resuscitation after an ischemic insult, during recent years considerable research efforts have been directed towards the reduction of liver injury and the post-ischemic tissue alterations related to oxygenated reperfusion.[1,3]

Many pharmacological manipulations with compounds known for their antioxidant activity has been used over years in order achieve the elimination of the harmful effect of free radicals released by neutrophils, as well as by hepatocytes and Kuppfer cells.[4-7]

Naloxone, a well known opiate antagonist, has been

proposed as a liver cytoprotecting agent. It has been shown in many studies to act by inhibiting the release of free radicals[8-10] through an endogenous Naloxone-sensitive pathway, in which transforming growth factor [TGF-β] and interleukin-2 [IL-2] are involved.[11,12]

Thus, the purpose of this study is to clarify the changes in hepatic microcirculation during 70% liver ischemia and after reperfusion in the rat, and the potential beneficial effect of Naloxone, given as pretreatment, in respect to microcirculation and oxidative stress, as assessed by laser-Doppler flowmetry and malondyaldehyde [MDA] levels, respectively.

MATERIALS AND METHODS

1. Animals

One hundred and forty male Wistar rats, weighing 180-200g, were used in this study. The animals were housed together for a 1-week adaptation period, at a room temperature of 24°C and with an alternating 12h light dark cycle, and had free access to tap water and standard rat food.

All the experimental procedures were reviewed and approved by the Animal Research Committee, Veterinary Department of the Ministry of Agriculture, Greece.

2. Study design

Twelve hours prior to the experiment all rats were deprived of food but were allowed free access to water. Anesthesia was achieved by the intramuscular injection of ketamine [50mg/kg] and xylazine [10mg/kg], after which the rats were randomly allocated into Naloxone [N] treated and Placebo [P] groups. The N group was given 0.5ml Naloxone, [Narcan, Bristol-Myers Squibb, USA, 80 μg/kg] and the P group an equal volume of normal saline 0.9%, intravenously via the tail vein.

A midline laparotomy was then performed in all rats and the liver was mobilized by dividing the triangular and the falciform ligaments and all peritoneal attachments. The hepatic hilus was exposed and the portal vein, the hepatic artery and the bile duct of the left lateral and median lobes were recognized and occluded by a small vascular clamp applied distal to the origin of the vessels supplying the omental [caudate] and right liver lobes. The blood supply to those lobes thus remained uninterrupted and the portal blood flow was maintained though them without evidence of vascular congestion in the alimentary tract. This procedure is considered to yield about 70% partial ischemia of the liver by weight.

After 30min or 60min of normothermic ischemia recirculation of the blood through the ischemic lobes was achieved by simply removing the clamp and the rats were allowed a 60min period for reperfusion.

Twenty rats [2 groups] of Naloxone-treated and 20 rats [2 groups] of Placebo-treated were used for microcirculation assessment. The time points of measurements was: baseline [0min], 30min ischemia [30min], and 60min reperfusion [90min] for the one group of each treatment and baseline [0min], 60min ischemia [60min], and 60min reperfusion [120min] for the second group of each treatment.

For the MDA, as well as for alanine aminotransferase [ALT] assessment 50 Naloxone treated rats [5 groups] and 50 [5 groups] Placebo treated rats were used, since the liver samples needed did not allow the animal to survive after tissue sampling. These sub-groups were as follows: baseline at 0min, just after laparotomy, after 30min of ischemia and after 60min of reperfusion, i.e. after 90min from baseline, as well as after 60min ischemia and after 60min reperfusion, i.e. after 120min from baseline measurement.

3. Hepatic tissue microcirculation

Hepatic tissue microcirculation was assessed non-invasively by the use of the laser-Doppler flowmetry technique.[13,14] The apparatus used in this study was the Periflux PF2B flowmeter in conjunction with the PF319:2L self-adhesive single fiber probe [Perimed, Jarfalla Sweden]. This specially designed probe comprises one optical fiber both transmitting and collecting the backscattered light and having a diameter of 0.5 mm; it was incorporated into a small latex sheet, which adheres to the moist surface of the liver and keeps the probe in place, thus reducing the impact of artifact movement and ensuring good optical coupling. The probe was calibrated by immersing its tip in a latex spheres suspension [Perimed, Sweden], where the Brownian motion of the latex particles provide the standard LDF signal. The signal from the liver, corresponding to the capsular perfusion,[14] expressed in arbitrary units of flow, was recorded with a sampling frequency of 16 Hz [1 sample/0.06 s] and a display frequency of 1Hz, over a 3-min period.

The flowmeter was serially connected to a multi-channel data aquisition system, through an A/D converter [DT2801 series Data Translation, Marlboro, MA] with a precision of 12 bits. The software program Perisoft [Perimed Sweden] was installed in a PC, to store and analyze the data.

4. Malondyaldehyde levels

The extent of liver damage, attributable to the free radicals produced by ischemia-reperfusion was assessed indirectly by measuring the malondialdehyde [MDA] levels, an intermediate product of lipid peroxidation.

The left lateral lobe was used as specimen under study. All samples were appropriately treated immediately upon sampling: they were weighed, minced and homogenized in 0.02m sodium phosphate buffer pH 7.4 [1:10wt/vol] using a smooth glass with a Teflon pestle hand homogenizer. The supernatant of the homogenate after centrifugation at 2800g for 5min was withdrawn and kept at –25°C until analyzed, by a technique described elsewhere.[15] Briefly, 1ml of 17.5% trichloric acid and 1ml of 0.6% thiobarbituric acid pH 2 were added to 1ml of the homogenate. This mixture was placed in a boiling waterbath for 15 min and then allowed to cool. A 1ml aliquot of 70% trichloric acid was added and the mixture allowed incubating for 20min. The sample was then centrifuged for 15min at 2000rpm and the optical density of the supernatant evaluated spectrophotometrically at 534nm against a reagent blank. The amount of MDA was expressed in nanomoles per milligram of protein, with the protein content determined by the Lowry method.

5. Serum marker of reperfusion injury

Immediately after liver tissue sampling for MDA determination, blood samples were obtained from the inferior vena cava for alanine aminotransferase [ALT] activity. Blood stored in serum separator tubes and immediately centrifuged at 11.000rpm for 5min. A 10μl sample of serum was diluted in 0,9% saline and was analyzed using the serum multiple biochemical analyzer [Ektachem DTSCII, Johnson & Johnson, Rochester, NY] with appropriate standards.

STATISTICAL ANALYSIS

The results of liver microcirculation data were initially expressed as percentages of the baseline value for each animal and then were averaged [±SD] across the rats of the same group treatment [N or P] and the same ischemia-time [30 or 60min], for each of the 3 study points. These 3 time point values were examined by repeated-measure analysis of variance. Significant differences with respect to baseline within the same group were assessed by the paired *t*-test, and differences at matched time points between Naloxone and Placebo groups were assessed using the *t*-test for independent samples.

The MDA as well as ALT data were expressed as the means±SD. Then the same statistic al was used package, as that for the microcirculation.

All calculations were performed on a Macintosh PC, with the Statview (Brain Power,Berkeley, CA, USA) statistical package. A probability value of less than 0.05 was considered significant.

RESULTS

Microcirculation measurements revealed a 95% reduction during the ischemia period, both at 30min or 60min, in relation to baseline. Upon reperfusion, there was a noticeable sudden increase [reactive hyperaemia], but at the end of the 60min reperfusion period the microcirculatory flow levels were as high as 70% of the baseline in the placebo treated and 85% of the baseline in the Naloxone treated rats [p=0.001]. There was no statistical difference between groups of 30min or 60min ischemia for either treatment (Figure 1).

In respect to MDA measurements, 30min ischemia produced only a slight increase in both groups. Upon reperfusion a highly significant increase was observed in both groups in relation to baseline. However, N group exhibited significantly lower levels in relation to P group, at the same reperfusion period. In the 60min ischemia group, there was a highly significant increase in both P and N groups in relation to baseline, as well as in the 30min ischemia same treatment groups. Sixty min reperfusion led to a further increase of MDA levels in relation both to baseline and the 60min ischemia period and a high statistical difference within the same time-period between P and N groups (Table 1, Figure 2).

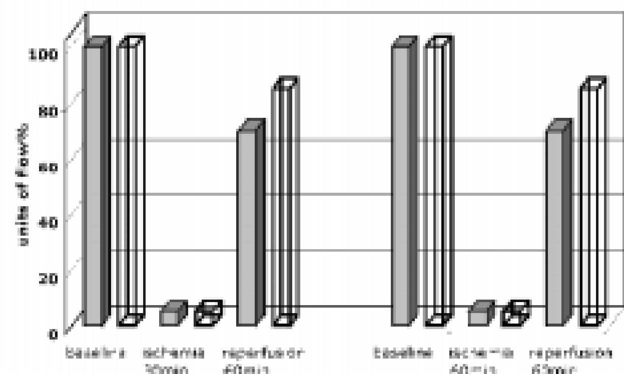

Fig. 1. Naloxone pretreatment [open bars] led to a restoration of liver microcirculation to the levels of 85% the baseline values in relation to placebo treatment [closed bars] which reached only the level of 70% [p=0.001].

Serum activities of ALT remained low during the ischemic period of 30 or 60min [32±10 versus 38±7, respectively]. A significant increase was seen only during reperfusion, the severity of the representing liver damage correlated well with the length of the ischemic period, i.e. a significant difference [p=0.01] was found between 30min ischemia - 60min reperfusion and 60min ischemia - 60min reperfusion groups, both in P and N treated groups. However, N group exhibited significantly lower levels [p=0.01] in relation to P group at the same time-periods, respectively (Table 2).

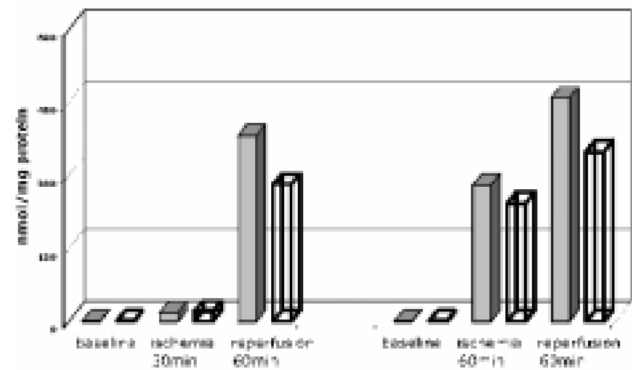

Fig. 2. Naloxone pretreatment [open bars] led to significantly lower levels of MDA in relation to Placebo-treated group [closed bars], at the same time points, throughout the study period.

DISCUSSION

During liver ischemia, the lack of metabolite substrates induces important biochemical changes such as degradation of ATP and other high energy metabolites, activation of phospholipases and other degradative enzyme systems and substantial alterations in the intracellular milieu.[16] Reperfusion of a previously ischemic tissue leads to an aggravation of the ischemia-induced injury. There has been increasing evidence from animal studies over the years that severe damage can occur in the heart, stomach,[17] and liver[4,16,18-21] during reperfusion time following ischemia, the oxygen free radicals hydrogen peroxide [H_2O_2], hypochlorous acid [HOCl], super-

TABLE 1.

	PLACEBO	NALOXONE
Baseline	0.88±0.1	0.91±0.1
Ischemia 30 min	17.39±3.5 #	18.8±2.9 #
Reperfusion 60min	382.84±97.6 #	279.53±41.4 *#
Baseline	0.88±0.1	0.91±0.1
Ischemia 60min	279.52±69.4 #	241.66±23.4 *#
Reperfusion 60min	458.96±90.4 #	347.33±49.7 *#

Hepatic tissue MDA levels in naloxone and placebo treated rats. MDA is expressed in nmol/mg tissue protein. [*] represents a p value of 0.001 in relation to placebo treated rats, within the same-time period. [#] represents a p value of 0.001 in relation both to ischemia and baseline measurements, within the same treatment group.

TABLE 2.

	PLACEBO	NALOXONE
Baseline	30±9	29±10
Ischemia 30 min	32±10	30±7
Reperfusion 60min	102±12 *	85±9 #
Baseline	30±7	31±9
Ischemia 60min	38±7	36±8
Reperfusion 60min	135±28 *	98±7 #

Serum ALT activities in naloxone and placebo treated rats. ALT is expressed in U/L. [*] represents a p value of 0.01 between the two ischemia periods subjected rats [30min or 60min], within the same treatment group. [#] represents a p value of 0.01 in relation to placebo treated rats, within the same time period, respectively.

Thus, a session of plasma exchange was performed in all patients 48-72 hours after admission. In 3/7 (43%) a significant reduction of serum TG and pancreatic enzymes was noted, within a few hours from the procedure. In the remaining patients, the same results were noted after a

oxide anion [O_2^-], as well as nitric oxide [NO], playing the dominant role in the pathogenesis of cell injury. In most experimental systems, increased formation of reactive oxygen species has been documented to derive from biochemical reactions with essential target molecules such as lipids and proteins, and especially those of the biological membranes.[2] The potent activity of free radicals against cellular target molecules is not the same, but the less reactive can easily be converted to highly reactive species.

In respect to the liver injury, it has been found that besides the circulating neutrophils, both the hepatocytes and the Kupffer cells are involved in reactive species production, the extent of their participation depending on the duration of ischemia and on the time lapse after reperfusion. The duration of ischemia appears to be different between in-vitro and in-vivo systems and may be significantly shorter in-vivo. Evidence from experimental systems indicates that hepatocytes begin releasing free radicals immediately after re-oxygenation and continue for hours while Kupffer cells become activated as a result of cell injury due to hypoxia and reperfusion. Immediately following reperfusion, neutrophils become stimulated and this activation favours the expression of neutrophil adhesion molecules,[2,21,22] leading, through a complex sequence of events, to the neutrophils adhesion and plugging of liver sinusoids. Hepatic sinusoids being plugged by the adherent neutrophils cause reduction of blood flow through them or total obstruction, thus prolonging the ischemia period.[1,2,16,21,22]

Liver ischemia is an unavoidable condition, either provoced, as during the vascular clamping manouver in liver surgery, or, more frequently, as a consequence of circulatory shock of any cause. Although the living cell systems contain their own endogenous-derived scavenging systems, including superoxide dismutase, catalase, peroxidase and glutathione reductase, these finally appear insufficient to counteract with a continuous attack.[4,10,21] Thus, maintaining liver cell viability against both ischemia and reperfusion injuries is of major concern and a large number of free radical scavengers - allopurinol, adenosine, corticosteroids, methylurea - have been tested over the years as potential cytoprotective agents.[4,10]

In the present study we evaluated the possible cytoprotective role of naloxone against liver ischemia-reperfusion-injury. Naloxone is a well known opiate antagonist of all three, μ, ϰ, and δ, opioid receptor subclasses, which is unable to cross the blood-brain barrier.[23] In animal models, blockade of opioid receptors using the antagonist naloxone, results in increased survival time, restoration of blood pressure to normal levels, and increased perfusion of organs.[24-27] Very recent data have shown that synthetic opioid peptides may have beneficial effects, e.g. in cardiac ischemic preconditioning and infarction,[28,29] while in-vitro and in-vivo experiments have also shown that opioids could alter the functional activity of neutrophils.[30,31] Although, the exact mechanism[s] by which the opioid receptor antagonists work has not yet been fully elucidated, there are several possible ones that could account for their ability to inhibit systemic TNF-α production, including: a) indirect inhibition by binding to μ, ϰ, or δ opioid receptors in the central nervous system, thus preventing the binding of endogenous opioids which may regulate TNF-α production directly or through induction of unknown factors, b) may induce unknown intermediates which function to directly inhibit macrophage-derived TNF-α, or c) may induce the production of anti-inflammatory cytokines, such as IL-10, IL-4, and IL-13, which suppress TNF-α production.[32] On the other hand, it is now well accepted that the non-parenchymal liver cells – including endothelial cells and Kupffer cells - express opioid receptors[33,34] thus naloxone would intervene in these cells, too.

The present study was conducted to clarify the potential beneficial effect of Naloxone in preventing reperfusion injury of the liver. The model of a 70% partial hepatic ischemia was chosen to avoid splanchnic congestion during the ischemia period, which might interfere with the specific hepatic phenomena under investigation. Liver injury was assessed by means of microcirculation measurements as a direct index of the extent of capillary plugging by adhered neutrophils during reperfusion. To the best of our knowledge, no other study has used microcirculation for the evaluation of the protective effect of Naloxone, and the laser-Doppler flowmetry technique is a well-established, non-invasive method for liver perfusion.[14,35]

The findings of the present study support the current opinion that Naloxone protects from reperfusion injury. The restoration of microcirculation at the percentage of 85% after 60min reperfusion in relation to the baseline measurement reflects a highly acceptable percentage of capillary patency. On the other hand, placebo treated rats reveal an improvement of only 70% in relation to the baseline. This protection, occurring in both groups, i.e. after both 30 and 60min of ischemia, is absolutely attributable to Naloxone pretreatment, since ALT levels, used as a conventional marker of liver damage, as well as the MDA levels, used as an index of oxidative stress, confirm its effect against reperfusion injury.

It therefore seems clear that Naloxone pretreatment exerts a beneficial effect on hepatic tissue subjected to ischemia-reperfusion through a mechanism of prohibiting leukocytes adhesion in the sinusoidal epithelium. Based on the findings of the present study and since Naloxone is a commonly used drug with no side-effects in humans, it's use could be advised in any case of suspected hepatic reperfusion injury, although further studies are needed on this topic.

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