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Research Article

Activation of Cholinergic Pathway via CCK-8 or Direct Vagal Stimulation Protects Hepatic Ischemia-Reperfusion Injury

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Abstract

Background: Cholecystokinin-8 (CCK-8) at physiological levels stimulates the release of acetylcholine (ACh) via cholinergic pathway. We previously reported ACh receptor agonists protected the liver from Ischemia-Reperfusion (IR) injury, suggesting a potential role of the cholinergic anti-inflammatory pathway. This study examined whether administration of CCK-8 or direct vagal stimulation would have a protective effect on hepatic-IR injury.

Methods: Adult male mice underwent 90min of partial liver ischemia followed by reperfusion. CCK-8 was administered i.p. prior to ischemia, followed by a second dose at reperfusion time. A second group of mice were subjected to bilateral vagotomy prior to CCK-8, while a third group were subjected to electrical vagal stimulation for 20min, followed by hepatic IR. Plasma alanine aminotransferase (ALT) levels and liver histopathology were assessed for liver injury. Plasma cytokine Tumor Necrosis Factor (TNF)-α, Macrophage Inflammatory Protein-2 (MIP-2), Monocyte Chemoattractant Protein-1 (MCP-1) and Interleukin-6 (IL-6) were measured.

Results: CCK-8 pretreated mice had significantly lower ALT levels (87%) and hepatic injury, as compared to saline- treated mice. This protective effect was absent in the vagotomized mice, indicating a role for an intact cholinergic pathway. Vagal stimulation confirmed this observation as ALT levels and liver injury were significantly decreased in these mice. The injury was associated with marked elevation of plasma cytokines in vagotomized mice and saline treated mice, which were significantly decreased in CCK-8/electrically stimulated groups.

Conclusion: Activation of the cholinergic pathway through humoral and electromechanical stimuli provides a protective effect on hepatic IR injury, which could present therapeutic means for the treatment of inflammatory diseases.

Keywords: Cholinergic pathway; Anti-Inflammatory; Tissue injury; Vagotomy

Abbreviations

ACh: Acetylcholine; AChR: Acetylcholine Receptor; CCK-8: Cholecystokinin-8; ELISA: Enzyme Linked Immunosorbent Assay; IR: Ischemia/Reperfusion; MIP-2: Macrophage Inflammatory Protein-2; MCP-1: Monocyte Chemoattractant Protein-1; TNF-α: Tumor Necrosis Factor-α

Introduction

The phenomenon, hepatic Ischemic-Reperfusion (IR) injury, is a feature of many clinically important events, including hepatic surgery, transplantation, trauma, and hemorrhagic shock [1]. The injury occurs in two different phases; the acute injury phase (early phase), is associated with kupffer cell activation, release of pro-inflammatory cytokines, and generation of reactive oxygen species, which is followed by a second phase (late phase) characterized by massive neutrophil infiltration and further production of the inflammatory mediators [2]. The Central Nervous System (CNS) regulates systemic inflammatory responses to various stimuli through humoral mechanisms. For example, pro- inflammatory mediators (e.g., TNF, IL-1) activate afferent pathways in the vagus nerve, which stimulates the release of the pituitary adrenocorticotropic hormone; the resultant increase in plasma corticosteroids suppresses cytokine release to prevent excessive inflammation [3]. In addition to this sensory function of the vagus nerve in systemic inflammation, there exists an efferent or motor vagus neural mechanism by which Acetylcholine (ACh) inhibits cytokine release from resident tissue macrophages [4]. Studies have shown that both pharmacological and electrical stimulation of the efferent vagus nerve significantly inhibited the development of inflammatory response to a wide range of stimuli including IR [5-7]. The inhibitory effect was associated with decreased levels of the pro-inflammatory cytokine, Tumor Necrosis Factor (TNF)-α. It appears that CNS-derived motor output through the cholinergic anti-inflammatory pathway regulates cytokine release to prevent potentially damaging inflammation and maintain

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homeostasis -- this neural reflex is referred to as the "inflammatory reflex" [8].

Cholecystokinin, a neuropeptide hormone that is released from the small intestinal endocrine cells upon dietary fat intake, can stimulate vagal sensory fibers and neural ACh release [9]. Cholecystokinin, and, in particular, the cleaved octapeptide Cholecystokinin-8s (CCK-8s) induces vagal-mediated gastric relaxation, increases pancreatic exocrine secretion, and induces short-term satiety [9-11]. Apart from its role in digestion and satiety, CCK-8 mediates the communication between nervous and immune systems, and is expressed in neurons, monocytes/macrophages and lymphocytes, where it regulates neurological and immune activities [12-15]. The anti-inflammatory activity of CCK-8 has been demonstrated in various disease models, including sepsis, endotoxic shock, hemorrhagic shock, and traumatic shock [16-20], suggesting that CCK-8 is a promising therapeutic drug candidate for inflammatory diseases. Studies have shown that the vagal-mediated effects of CCK-8 are almost exclusively due to CCK-8 paracrine action on peripheral capsaicin- sensitive vagal afferent fibers [21-23].

Borovikova *et al.* have reported that direct electrical stimulation of the peripheral vagus nerve *in vivo* during lethal endotoxemia in rats inhibited TNF synthesis in liver, attenuated peak serum TNF levels, and prevented the development of shock [4]. Furthermore, vagal activation inhibited cytokine release and improved the disease endpoints in experimental sepsis, ileus, hemorrhagic shock, pancreatitis, and a number of other inflammatory models [reviewed in 7]. The cholinergic anti-inflammatory pathway can be activated chemically or electrically, which effectively suppresses systemic TNF release and attenuates organ damage. In our previous study, we showed protection of early phase hepatic IR injury by pharmacological modulation of the cholinergic pathway [6]. The aim of our current study was to evaluate the protective effect of the endogenous cholinergic pathway stimulation on hepatic IR. Two different approaches were applied; 1) direct electrical stimulation of the vagus nerve and 2) CCK-8 induced vagal stimulation. To accomplish this, an *in-vivo* model of vagal stimulation and vagotomy in the mouse was developed. The inflammatory response and liver injury were determined by measurement of the plasma cytokines and ALT levels, and histopathology examination.

Materials and Methods

All chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO), unless otherwise noted. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1985). Experimental protocols were reviewed and approved by the Michigan State University Animal Use and Care Committee. Adult (8-10wk.) male C57BL/6 mice (Charles River Laboratories, Portage, MI) weighing between 24-29g were fed a standard diet and acclimated in the animal housing area for 1wk before experimentation. Three (3) sets of experiments were carried out. In the first set, mice were pretreated with vehicle (i.e. normal saline), or CCK-8 (i.p., 200µl 10min prior to surgery and 100µl at start of reperfusion). The test group underwent 90min hepatic ischemia and 3h reperfusion, while the sham group underwent the same surgical protocol but without vascular occlusion (see below for IR protocol). In the second set of experiment, the mice were subjected to bilateral vagotomy 24h prior to hepatic IR. In the third set of experiment, mice were subjected to vagal stimulation for 20min (5mV, 2ms, 5Hz) followed by 90min of ischemia and 3h of reperfusion.

Experimental animal model of hepatic ischemia and reperfusion

The partial hepatic IR model used in this study induces severe ischemic insult to the liver without inducing hypertension and subsequent bacterial translocation into the portal venous blood [24]. The mice were anesthetized by an *i.p.* injection of 35mg/kg sodium pentobarbital (Abbott Laboratories, North Chicago, IL). A midline laparotomy was performed, the portal circulation to the median and left lateral lobes of the liver was carefully dissected, and an a traumatic vascular clip (Accurate Surgical and Scientific Instruments Corp. Westbury, NY) was placed on the vessels, interrupting the portal venous, and hepatic arterial blood supply to these lobes. Five drops of sterile saline were applied over the abdominal viscera to keep the organs moist and to compensate for the fluid loss. The abdomen was temporarily closed with sterile staple sutures to prevent dehydration and possible contamination. The animal was kept in the recovery room under close supervision. After 90min of partial hepatic ischemia, the clamp was removed and reperfusion was resumed. The abdomen was closed in a double layer using 5-O nylon, and 0.8ml sterile lactated Ringer's Solution (Abbott Laboratories, North Chicago, IL) was administered subcutaneously to compensate for operative fluid loss. During the reperfusion, the mice were kept in clean cages with controlled warm temperature (i.e., 23-25°C), with no further administration of anesthesia or analgesics. After reperfusion, mice were euthanized, and blood and tissue samples were collected as described below.

All the surgical procedures were performed under aseptic conditions. In this model, the caudate and right lateral lobes, as well as the papillary and quadrate processes retained an intact portal and arterial inflow and venous outflow to prevent intestinal venous congestion. This resulted in the induction of ischemia to approximately 70% of the liver. There was no to minimal bleeding during the surgical operation (bleeding <1+ of scale of 0-5+ considered minimal). If a small (2+) to moderate (3+) amount of bleeding occurred due to blood vessel puncture, the animal was excluded from the group and euthanized. The bleeding scale (0 – 5+) was arbitrarily based on saturation of each cotton swab that held about 150-200µl of blood. This was considered as 1+ bleeding. The liver IR model with 90min of ischemia induces a severe, isolated, and reproducible liver injury with very minimal animal mortality within one week (i.e. 0-5%).

Electric stimulation of the vagus nerve

Following anesthesia, a small midline incision was made on the ventral surface of the mouse neck, the vagus nerve (right) was dissected, isolated, and placed across a bipolar platinum stimulator electrodes connected to a stimulator module (Model SI USA, Grass S88 Stimulator). An optimal stimulatory condition was determined, and a constant voltage stimulus (5v, 2ms, 5Hz) was applied to the nerve for 20min to provide an isolated voltage stimulus to the nerve, followed by hepatic IR (90min of ischemia and 3h of reperfusion). Sham-operated animals underwent right vagus nerve dissection, isolation and exposure without stimulation.

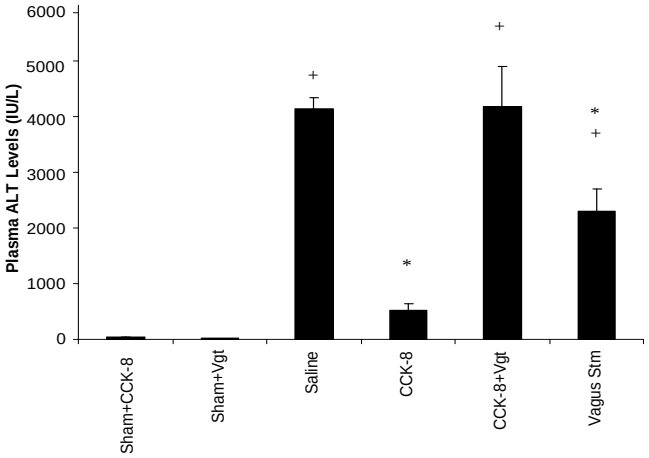

Figure 1: Effect of pretreatment with CCK-8 or vagotomy on ALT levels induced by hepatic IR in mice. Mice were pretreated with vehicle (i.e. normal saline), CCK-8 (50ug/kg b.w.), vagotomy ("vgt" surgery performed 24h before IR) or vagal stimulation ("vagus Stm" 20min of continuous electrical stimulation) prior to the onset of ischemia (90min), followed by 3h of reperfusion (IR). "Sham" animals received the same pretreatment, followed by sham operation (n=3 mice per group). Peripheral blood was collected, and plasma ALT was measured. Values are expressed as means ± SEM (IU/L) of n = 5 to 7 mice per IR group. $^{+}P<0.05$, IR group *vs*. sham-operated group. $^{*}P<0.05$, Saline-treated IR group vs. CCK-8-or Vagus Stm-treated IR group.

Bilateral vagotomy

A ventral cervical incision was made and both vagus trunks were exposed. The vagus nerve was ligated at both cephalic and caudal ends using 4-0silk-suture and cut in half using a surgical scissors. In the sham-operated animals, both vagal trunks were dissected and exposed, but not ligated, and were left intact.

Drug administration

Animals received an *i.p.* injection of the CCK-8 (50µg/Kg b.w.); 200µl of CCK-8 at10min before the induction of ischemia and 100µl at start of reperfusion. Saline-treated mice received an equal volume of vehicle (saline).

Peripheral blood and tissue procurement

Following anesthesia, blood samples were obtained from the right ventricle via a left anterior thoracotomy at the conclusion of reperfusion. The blood was collected in a sterile syringe containing 50µl of heparin (100 USP Units/ml), and centrifuged to separate the plasma. The plasma samples were stored at -70^{0}C until use for cytokine and ALT assays. A portion of the ischemic and non-ischemic liver lobes were fixed in buffered 10% formalin, embedded in paraffin, and used for Hematoxylin and Eosin (H&E) staining.

Measurement of plasma alanine aminotransferase levels

The plasma ALT levels were determined spectrophotometrically, as previously described [6, 24]. The ALT values are expressed in International Units per Liter (IU/L).

Histopathology

H&E staining was performed on tissue sections prepared at 5-µm intervals from paraffin-embedded liver tissue. A pathologist, blinded to the experimental conditions, examined the liver tissue sections.

Plasma cytokine concentrations

Plasma TNF-α, IL-6, MCP-1 and MIP-2 were determined in a 96-well Nunc-Immuno microplate (VWR Scientific, Chicago, IL), using a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) technique, as previously described [6]. The capture antibody was a polyclonal anti-mouse TNF-α, IL-6, MCP-1 or MIP-2 specific goat IgG (R&D Systems, Minneapolis, MN) and the detection antibody was a biotinylated polyclonal anti-mouse TNF-α, IL-6, MCP-1 or MIP-2 specific goat IgG, (R&D Systems). All plasma samples were tested in duplicate. The minimal detectable protein concentration was 20 pg/ml.

Statistical analysis

The data is expressed as means ± standard error of the mean (Mean ± SEM). Comparisons between two groups were performed using an unpaired t-test. Comparisons between multiple groups and various time points were analyzed using ANOVA followed by a Fisher's PLSD post-hoc test. $P \leq 0.05$ was considered statistically significant. All data were analyzed using the *StatView version 5.0.1 software* for Windows.

Results

Effects of CCK-8 induced vagal stimulation on hepatocellular injury

Our previous published work has shown that 90min of hepatic ischemia followed by reperfusion resulted in hepatocellular injury in a time-dependent fashion, as demonstrated by plasma ALT level [6,24]. After 90min of ischemia followed by 3h of reperfusion, there was moderate to severe liver injury histologically that increased by 6h of reperfusion, and this injury was associated with significant plasma ALT elevation. The current study was focused on the effect of CCK-8 on the early phase of liver injury, i.e. 90min ischemia and 3h of reperfusion. The optimal dose that was determined for the study (50ug/Kg b.w.) did not produce any clinical signs (i.e., altered breathing, heart rate). Figure 1 depicts the changes in the plasma ALT level in the different animal groups studied. The results showed that CCK-8 pretreatment (10min before the induction of ischemia and at the start of reperfusion) significantly reduced plasma ALT levels (i.e., Mean ± SEM, Saline IR= 4422 ± 544 vs. CCK-8 IR= 456 ± 130 IU/L, $p < 0.0001$, ANOVA). The protective effect of CCK-8 on hepatic injury via the cholinergic pathway was confirmed by another group of mice, which underwent bilateral vagotomy 24h before subjected to CCK-8 pretreatment and hepatic IR (i.e., ALT levels, Mean + SEM, Saline IR= 4422 ± 544 and in vagotomized CCK-8 IR= 4846 ± 395 IU/L). The results showed that vagotomy completely inhibited the protective effect of CCK-8 pretreatment. Further, the sham group demonstrated that either the injection of CCK-8 alone or vagotomy did not cause any hepatic injury as judged by the ALT release (Figure 1).

Effects of electrically induced vagal stimulation on hepatocellular injury

Mice pretreated with electrical stimulation of the vagus nerve had significantly lower plasma ALT level than their counterparts who did not receive vagal stimulation (i.e., Saline IR= 4422 ± 544 *vs*. vagus nerve stimulation IR= 2620 ± 451 IU/L, $p = 0.0057$, ANOVA).

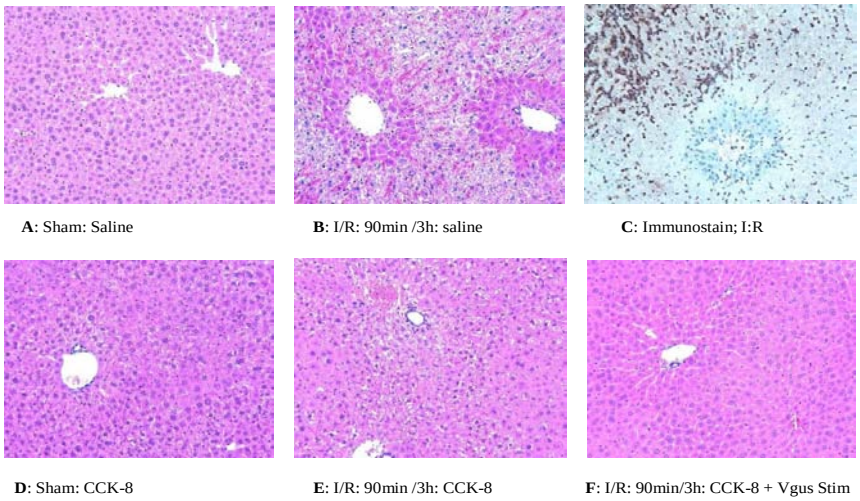

Figure 2: Hepatic histopathology following IR. Mice were pretreated with vehicle (i.e. saline), CCK (50 ug/kg b.w.), or vagal stimulation ("Vgus Stm" 20min of continuous electrical stimulation) prior to the onset of ischemia (90 min), followed by 3h of reperfusion (IR). The ischemic liver sections were prepared and stained with H&E or immunohistochemical staining. "**A**" sham mice (saline treated) shows normal hepatic histology, "**B**" the ischemic liver section of saline-treated mice subjected to IR (90min/3h), "**C**" Immunohistochemical staining of liver neutrophils of mice subjected to IR (90min/3h). Neutrophils are presented by a dark brown stain, "**D**" sham mice (CCK-8 treated), "**E**" CCK-8 pretreated mice subjected to IR (90min/3h), and "**F**" CCK-8 plus vagus nerve stimulation of mice subjected to IR (90min/3h). In image "B" a pattern of reperfusion damage is evident by necrosis of hepatocytes in the pericentral and midzonal regions, with relative sparing of the periportal areas. Note the presence of neutrophils in the midzonal region around the central vein. Pretreatment of mice with CCk-8 and vagus stimulation show significant inhibition of liver damage ("**E**", and "**F**"). Images are representative of n = 6 mice per saline or CCK-8, and n = 3 per CCK-8 plus vagus nerve stimulation group.

Evaluation of hepatic histopathology

Figure 2 depicts the hepatic histopathology following IR. Liver histopathology was evaluated based on sinusoidal congestion, cytoplasmic vacuolization, hepatocellular necrosis, and neutrophil infiltration. Sham-operated mice did not show any hepatocellular injury. This result correlates with negligible plasma ALT and cytokine levels (Figures 1 and 3). Reperfusion of the ischemic liver caused hepatocellular necrosis and sinusoidal congestion by 3h of reperfusion in saline- treated mice (Figure 2B). There was mostly sparing of the periportal areas with progressive injury in the midzonal and pericentral areas. In contrast, in CCK-8 treated mice, there were only patchy spots of mild necrosis in various areas of the liver tissue (Figure 2F). Most areas of the liver tissue from CCK-8 treated mice exhibited normal structure similar to those of the sham group. The hepatocellular damage and hepatic necrosis in CCK-8 treated mice appeared less severe than those of the saline treated IR mice. The vagotomized CCK-8 treated animals showed severe hepatocellular necrosis and sinusoidal congestion by 3h of reperfusion and failed to show the protective effect of CCK-8 pretreatment. Further, the hepatic injury was significantly less in the mice subjected to both CCK-8 treatment and the electrical vagal stimulation, which correlated with the plasma ALT levels (Figure 2F).

Effects of CCK-8 or electrical vagal stimulation on plasma TNF-α, IL-6, MCP-1 and MIP-2 levels

Inflammatory cytokines such as TNF-α and IL-6 have shown to play key roles in pathophysiology of hepatic IR injury [25-28]. In saline-treated mice, plasma concentrations of TNF-α and IL-6 were significantly increased after 90min of ischemia followed by 3h of reperfusion (Figure 3). The sham operation induced a slight negligible cytokine response, and plasma TNF-α and IL-6 in sham-operated animals remained low compared to the corresponding IR mice. Vagal stimulation through CCK-8 or electrical stimulation significantly reduced plasma levels of TNF-α and IL-6 in mice subjected to 90min of ischemia and 3h of reperfusion. MIP-2 and MCP-1 are chemokines involved in neutrophil and monocyte recruitment respectively and are implicated in hepatic IR injury [25-27]. Similar to the other above noted cytokines, plasma chemokine MIP-2 and MCP-1 levels significantly increased in saline treated mice after ischemia and 3h of reperfusion (Figure 3). Vagal stimulation significantly reduced the release of MIP-2 and MCP-1, which were caused by liver IR. The inhibition of cytokine production was removed when mice were subjected to bilateral vagotomy.

Discussion

The study presented here has shown that activation of the cholinergic pathway through humoral (i.e., CCK-8) and electromechanical stimuli provided a protective effect on the early phase of hepatic IR injury. The findings showed that administration of CCK-8 or electrical vagal stimulation significantly attenuated the release of cytokines/chemokine at the early phase of hepatic injury induced by hepatic IR in mice.

The present data indicates an association between decreased cytokine production and hepatocellular injury by cholinergic agonist treatment as well as electrical vagal stimulation. This finding supports our previously published studies [6] that administration of nicotinic ACh agonists, DMPP (1,1-dimethyl-4-phenyl-L-pioperazinium-iodide) and nicotine, significantly decreased the release of cytokines/chemokine, and attenuated the hepatic injury caused by liver IR in mice. The protective effect of pharmacological stimulation of the cholinergic anti-inflammatory pathway at the early phase of I/R injury is well in agreement with the previously reported studies [4, 5].

Cholecystokinin octapeptide (CCK-8) is an 8-amino acid

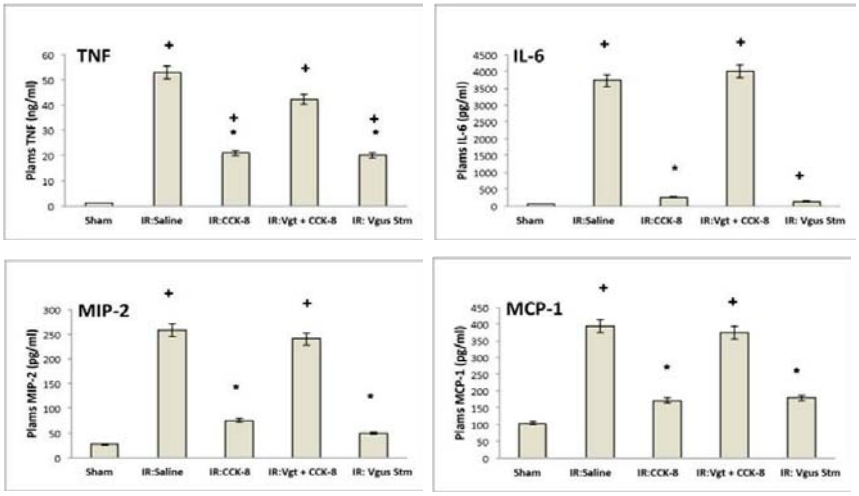

Figure 3: Cholinergic pathway stimulation effect on plasma cytokine induced by hepatic IR in mice. Mice were pretreated with vehicle (i.e., normal saline), CCK-8 (50 ug/kg b.w.), vagotomy (vgt), or vagal stimulation ("Vgus Stm" 20min of continuous electrical stimulation) prior to the onset of ischemia (90 min), followed by 3h of reperfusion (IR). "Sham" animals received the same pretreatment as the "IR" group, followed by sham operation (n = 3 mice per group). Peripheral blood was collected, and plasma cytokines were measured using an ELISA. Values are expressed as means ±SEM (pg/ml) of n = 4 to 8 mice per group. (+) $P < 0.05$, IR group vs. sham group. (*) $P < 0.05$, saline- treated group vs. CCK-8-or vagal stimulation treated group.

long neuropeptide produced by the intracellular cleavage of the procholecystokinin and is the biologically predominant active form among the CCK family of peptides [10,11]. The anti-inflammatory activity of CCK-8 has been demonstrated in various diseases models, including endotoxic shock, hemorrhagic shock, and traumatic shock [16-18], suggesting that CCK-8 is a promising therapeutic drug candidate for inflammatory diseases. The cellular signaling transduction mediated through the CCK receptor was shown to be cell specific [12-15]. For example, CCK was found to activate pancreatic acinar cells, whereas opposite effects were observed in mouse lymphocytes, neutrophil, and peritoneal macrophages [12-14]. Meng et al. [22] reported that CCK-8 inhibits TNF-α production in endotoxin shock rats, and later on it was found that the inhibition of NF-kB by CCK-8 is mediated through the inhibition of p^{38} kinase [20]. The results of our study are in accordance with these previously published studies with immune cells, including peritoneal macrophages, neutrophils and lymphocytes.

The pro-inflammatory mediators (eg., LPS, TNF-α, IL-6) activate afferent neural pathways through vagus nerve that stimulate release of pituitary adrenocorticotrophic hormones, the resultant increase in serum corticosteroids suppresses cytokine release to prevent excessive inflammation [3,23]. In addition to this sensory function of the vagus nerve in systemic inflammation, Borovikova et al. described an efferent or motor vagus neural mechanism by which acetylcholine, the principal vagus nerve neurotransmitter, inhibits cytokine release from resident tissue macrophages, termed the "cholinergic anti-inflammatory pathway" [4,8]. In our experiments presented here vagus nerve stimulation, either pharmacologically or electrically, resulted in decreased cytokine release, whereas vagotomy resulted in an increase in cytokine release. Previous study has shown that the vagus nerve down regulates inflammation by decreasing the release of Tumor Necrosis Factor (TNF)- α by macrophages [4].

Our finding that the direct electrical stimulation of vagus nerve decreased the plasma TNF levels at the early hepatic IR is in accordance with the previous studies that direct electrical stimulation of the efferent vagus nerve resulted in significant inhibition of endotoxin-induced shock and aortic clamping-induced inflammatory response during IR [4]. In hepatic IR, TNF is involved in the production of chemokines. Previous studies by Colletti et al. [25] and Lentsch et al. [26] have shown that neutralization of TNF-α or MIP-2 by administration of antibodies significantly attenuated hepatocellular injury induced by hepatic IR. Further, our laboratory has previously identified the Kupffer cells as the major source of the cytokines and chemokines in liver IR, and inhibition of Kupffer cell activity significantly attenuated the liver IR injury [27]. Further, other studies have shown that vagotomy renders animals sensitive to endotoxin; vagotomized animals produced significantly more TNF and were significantly more sensitive to endotoxin-induced hypotension [4]. All together, our current data and previous published data indicate that vagus nerve play a role in activation of macrophages, regulation of cytokine production and tissue injury.

A direct association between CXC chemokines, neutrophil recruitment, and liver injury has been reported by previous studies. It has been shown that neutralization of CXC chemokines significantly attenuated neutrophil infiltration and liver injury in rat and mouse models of warm hepatic IR [25, 26]. Our present study showed that decreased production of cytokine/chemokine (TNF-α, IL-6, MCP-1 and MIP-2) correlated with lesser degree of liver injury (plasma ALT level) during the early phase of hepatic IR. The effect of CCK-8 or electrical vagal stimulation on the late phase of the liver IR was not examined in our current study. However, our previous published study indicated that during the late phase of hepatic IR, cholinergic agonists significantly inhibited CXC chemokine production (i.e., MIP-2), which did not correlate with neutrophil infiltration and the intensity of liver IR injury. One explanation might be that the cholinergic agonist treatment did not sufficiently abolish CXC chemokine (i.e., MIP-2) production and that the small concentration of the generated MIP-2 chemokine was sufficient enough to trigger neutrophil

recruitment and liver injury during the late phase of IR. Another argument is that during the late phase of liver IR, cytokine/chemokine role may be of limited relevance for neutrophil infiltration and liver injury, suggesting participation of other more potent inflammatory factors. In favor of the latter hypothesis, Dorman *et al.* have shown that neutrophil infiltration and liver injury occurred independent of CXC chemokine production in response to apoptotic cell injury in mice subjected to endotoximia [28]. Of course, one should keep in mind a potential role of other factors such as reduced neutrophil deformability [29], sinusoidal cell swelling [30], and vasoconstriction of the sinusoids [31], which can facilitate sinusoidal neutrophil trapping. Other studies have underscored the role of complement factors as a mediator of IR injury [32,33]. Another potential argument may involve cholinergic receptor desensitization and/or recycling. Further, a lack of sufficient concentration of the agonists, due to their clearance from the body, and therefore, resulting in a diminished protective effect of the cholinergic pathway. Furthermore, the delay in hepatic injury may be due to the effect of cholinergic agonists on the hepatic microcirculation. The cholinergic receptors are present in hepatic microcirculation and have shown a role in vasodilatory function [34,35]. The study has shown the involvement of vagus nerve in lack of blood reflow into sinusoids after hepatic ischemia that might cause the trapping of the neutrophils within the liver and subsequent hepatocellular injury [35].

In conclusion, our data have showed that CCK-8 and/or electrical stimulation of vagal nerve provided a protective effect on early hepatic IR injury. Further, our study highlighted on the crucial anti-inflammatory role of the endogenous cholinergic pathway on hepatic IR injury as supported by the loss of the protective effect of CCK-8 pretreatment in the vagotomized mice. Thus, activation of cholinergic pathway through humoral and electromechanical stimuli presents a potential therapeutic tool for inflammation-related diseases. Future studies will be aimed at different time points (early and late phase) after hepatic IR, which is necessary to understand the molecular mechanism of the function of cholinergic agonists (either pharmacological or electrical stimulation) on modulation of hepatic IR injury. Whether the cholinergic receptor agonist's act on endothelial cell functions in hepatic IR remains to be elucidated.

Authors' Contributions

EC conceived the study, participated in its design and coordination, carried out the surgical procedures and ELISA, data analysis and preparation of the manuscript. SD carried out analysis for ALT and cytokine measurements, and assisted in surgical procedures and collection of the tissue samples. KN assisted with the review and analysis of the data and preparation of the manuscript. All authors read and approved the final manuscript.

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