Up-Regulation of Histamine H₄ Receptors Contributes to Splenic Apoptosis in Septic Mice: Counteraction of the Anti-Apoptotic Action of Nuclear Factor-κB

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ABBREVIATIONS: HDC, histidine decarboxylase; CLP, cecal ligation and puncture; NF-κB, nuclear factor-κB; ODN, oligodeoxynucleotide; HVJ, hemagglutinating virus of the Japan; siRNA, small interfering RNA; RT-PCR, reverse transcription polymerase chain reaction; TdT, terminal deoxynucleotidyl transferase; TUNEL, Tdt-mediated dUTP-biotin nick end labeling; FADD, Fas-associated death domain; FLIP, FADD-like interleukin-1-converting enzyme-inhibitory protein

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The histamine H₄ receptor is the most recently identified receptor and is considered to play a role in a variety of inflammatory diseases. Histamine levels in the plasma are known to be elevated in animal models of sepsis as well as in septic patients. The aim of this study was to test the hypothesis that the H₄ receptor may play a significant role in the pathophysiology of sepsis. Polymicrobial sepsis was induced by cecal ligation and puncture in BALB/c mice. Although the H₄ receptor gene was undetectable in normal peripheral key organs except the spleen, the expression levels of this gene were highly up-regulated in all those organs of septic mice. In vivo transfection of nuclear factor-κB (NF-κB) decoy oligodeoxynucleotide, but not of its scrambled form, resulted in a great inhibition of sepsis-induced overexpression of the H₄ receptor gene. In septic mice, marked increases in caspase-3 activation and follicular lymphocyte apoptosis in spleens were strongly suppressed by systemic treatment with synthetic small interfering RNA (siRNA) targeted to the H₄ receptor. This was associated with the up-regulation of a number of anti-apoptotic proteins. These anti-apoptotic effects of H₄ siRNA treatment were all inhibited by further application of NF-κB decoy oligonucleotide. Our results suggest that superinduction of the histamine H₄ receptor gene in peripheral key organs, including the spleen, promoted by sepsis is transcriptionally controlled by NF-κB, while stimulation of this receptor is involved in the development of sepsis-induced splenic apoptosis through counteraction of the anti-apoptotic action of NF-κB.
Introduction

Histamine has been established to be an important chemical mediator in various pathophysiological conditions, including allergy and inflammation. Histamine is synthesized from the basic amino acid L-histidine through the catalytic activity of the rate-limiting enzyme histidine decarboxylase (HDC) and serves as a versatile biogenic amine with multiple functions in peripheral and central tissues. The fundamental pleiotropic regulatory character of histamine in cellular events is attributed to its interaction with different subtypes of G-protein-coupled histamine receptors that are differentially expressed in a variety of cell types (Parsons and Ganellin, 2006).

The histamine H4 receptor is a novel histamine receptor that has been identified most recently, displaying distinct molecular and pharmacological properties from H1, H2, and H3 histamine receptor subtypes (Oda et al., 2000; Nguyen et al., 2001; Morse et al., 2001; Zhu et al., 2001). The H4 receptor shows little homology to the classical pro-inflammatory H1 receptor or the H2 receptor and about 35% homology with the H3 receptor. The H4 receptor is expressed by immunologically relevant tissues, such as spleen and thymus, and mast cells and leukocytes, such as eosinophils; therefore this novel histamine receptor is considered to exhibit immunomodulatory functions (Oda et al., 2000; Morse et al., 2001; Zhu et al., 2001; Lippert et al., 2004). Moreover, chemotaxis and calcium mobilization of mast cells have been indicated as important mechanisms for the action of the H4 receptor (Hofstra et al., 2003). In in vivo studies, the H4 receptor antagonist JNJ7777120 (1-[(5-chloro-1H-indol-2yl)-4-methylpiperazine]) has also been shown to be important in allergic inflammation (de Esch et al., 2005; Thurmond et al., 2008). Thus, the H4 receptor may play a role in chemotactic and inflammatory processes. In animal experimental studies, the H4 receptor has been implicated in inflammatory diseases,
including asthma and inflammatory bowel diseases (Dunford et al., 2006; Thurmond et al., 2004; 2008). In addition, the H₄ receptor can be detected in primary synovial culture from patients with rheumatoid arthritis (Ohki et al., 2007). Accordingly, this new histamine receptor is now regarded as a potential target for inflammatory disorders. However, the precise function of this receptor in regulating inflammation is not well understood.

Sepsis is a subset of the systemic inflammatory response syndrome and is generally viewed as a disease that is accompanied by the inability to regulate the inflammatory response (Bochud and Calandra, 2003). Despite recent advances in antibiotics and critical care therapy, sepsis is still the leading cause of mortality in critically ill patients (Marshall et al., 2005). Its progression leads to septic shock and sequential multiple organ failure which correlate with poor outcome (Karima et al., 1999). Of note, elevated plasma levels of histamine during sepsis have been documented for a long time (see Matsuda et al., 2004). In our previous studies using endotoxemic rabbits and mice, the sustained elevation of plasma histamine was shown to be associated with the time-dependent increases in expression of HDC (Matsuda et al., 2002, 2004b). Moreover, we have found that endotoxemia causes superinduction of the H₁ receptor in cardiovascular and pulmonary tissues (Matsuda et al., 2002, 2003, 2004b). In light of a possible importance of the H₄ receptor in the inflammatory diseases, it would thus be reasonable to assume that this histamine receptor may play a role in the pathophysiology of sepsis syndrome.

In the present study, we initially examined alterations in expression of the H₄ receptor in different peripheral organs, including the spleen, under the septic condition. We used the cecal ligation and puncture (CLP) mouse model, regarded as a highly clinically relevant animal model of polymicrobial sepsis. Interestingly, we found that
expression of the H₄ receptor is highly up-regulated in the key organs of septic mice. It has been reported that binding sites for several cytokine-regulated transcription factors, such as nuclear factor-κB (NF-κB), are present upstream of the H₄ receptor gene (Cogé et al., 2001). We thus analyzed a regulatory role of NF-κB in the gene transcription and function of the H₄ receptor in spleen tissues of septic mice by means of in vivo transfer of NF-κB decoy oligodeoxynucleotides (ODNs). Finally, we were interested in investigating the impact of up-regulation of the H₄ receptor on splenic apoptosis in sepsis, because it has been highlighted that a major pathophysiological process in sepsis is apoptotic death of immune effector cells, including lymphocytes (Hotchkiss et al., 1999a, 1999b; Oberholzer et al., 2001; Wescher et al., 2005; Cheng et al., 2007).
Methods

Animal Preparation.

All animal works were conducted in accordance with the National Institute of Health guidelines on the use of laboratory animal and with approval of the Animal Care and Use Committee of University of Toyama. BALB/c mice, 8-12 weeks of age, were quarantined in quiet, humidified, light-cycled rooms for at least one week prior to use. Mice were allowed ad libitum access to food and water throughout quarantine. The surgical procedure to generate CLP-induced sepsis was carried out according to the method described in our previous studies with minor modification (Matsuda et al., 2005, 2007). In brief, mice were lightly anesthetized with gaseous diethyl ether, and a middle abdominal incision was made. The cecum was mobilized, ligated at 5 mm from its top, and then perforated in two locations with a 21-gauge needle, allowing expression of feces. The bowel was repositioned, and the abdomen was closed. Sham-operated control animals were subjected to the same surgical laparotomy, but the cecum was neither ligated nor punctured.

The CLP model, which causes peritonitis, leads to polymicrobial sepsis and represents an indirect insult similar to the pathogenesis of acute respiratory distress syndrome (Villar et al., 1994). Indeed, we have clearly demonstrated that mice 10 h after CLP exhibit marked hypoxemia, increased lung vascular permeability, and histological damage in lungs, including wall thickening, inflammatory infiltrate, and hemorrhage (Matsuda et al., 2005, 2009).

Preparation and Transfection of Decoy ODNs.

The NF-κB decoy ODN sequences are 5’-CCTTGAAGGGATTTCCTCC-3’ and 5’-GGAGGGAAATCCCTCAAGG-3’, whereas the scrambled decoy ODN
sequences are 5’-TTGCCGTACCTGACTTAGCC-3’ and 5’-GGCTAAGTCAGGTACGGCAA-3’. The hemagglutinating virus of the Japan envelope vector system (HVJ Envelope Vector Kit GENOMEONE-Neo; Ishihara Sangyo, Osaka, Japan) was used for in vivo gene transfer. Sterile saline containing the HVJ-liposome complex (100 μg of encapsulated ODN) was infused into the tail vein over 30 s at room temperature 10 h after sepsis induction. Further details of the transfer procedure have been published elsewhere (Matsuda et al., 2005). At indicated times after each treatment, tissue samples were carefully removed under induction of terminal anesthesia with pentobarbital and were immediately frozen with liquid nitrogen.

Preparation and Transfection of small interfering RNAs (siRNAs).

For silencing of gene expression of the H₄ receptor, siRNA oligonucleotides with the following sense and antisense sequences were designed: 5’-CGAGUGCCAACUUUAGUUUtt-3’ and 5’-AAACUAAAGUUGGCACUCGtg-3’. The Silencer Negative Control #1 siRNA (Ambion, Austin, TX) was used as a negative control. In vivo transfection of synthetic siRNAs was performed by using the HVJ-liposome method, by which 50 μg of each siRNA sequence was delivered into the tail vein at 10 h after surgery.

Reverse Transcription Polymerase Chain Reaction (RT-PCR).

The animals were trancardially perfused with phosphate-buffered saline, and then spleen, lung, liver, kidney, and heart tissues were harvested. Total RNA was extracted from the tissues with the use of a TRI reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacture’s instructions. RNA was reverse-transcribed to cDNA
and PCR was performed using a TAKARA RNA PCR kit (Takara Shuzo, Ohtsu, Japan) as described in the manufacture’s manual. Expression of the H$_4$ receptor gene was monitored by PCR with 5’-GAATCAGCTGCATCTCGTCA-3’ (sense) and 5’-GTGACCTGGCCTAGCTTCCTG-3’ (antisense). The PCR-amplified product was analyzed by agarose gel electrophoresis. The internal standard used was the ubiquitously expressed housekeeping gene β-actin, which was determined with the primer pair (sense, 5’-TGCGTGACATCAAGGGAGAG-3’; antisense, 5’-AAGGAAGGCTGGAAAGAGC-3’).

**Western Blot Analysis.**

The nuclear fractions were prepared from tissue homogenates using methods described previously (Matsuda et al., 2004b). Immunoblotting was performed as described in our previous reports (Matsuda et al., 2002, 2003, 2006). Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride filter membranes that were then incubated with the primary antibodies to NF-κB p65 (Acris Antibodies GmbH, Hiddenhausen, Germany), HDC (Acris Antibodies GmbH), Fas-associated death domain (FADD) (Acris Antibodies GmbH), FADD-like interleukin-1-converting enzyme-inhibitory protein (FLIP) (Novus Biologicals, Littleton, CO), Bcl-xL (Affinity BioReagents, Golden, CO), Bcl-2 (Affinity BioReagents), caspase-3 (GeneTex, San Antonio, TX), cleaved caspase-3 (Novus Biologicals), adaptin-α (Affinity BioReagents), lamin A (Novus Biologicals), and actin (GeneTex). The horseradish peroxidase-conjugated secondary antibody was anti-rabbit or anti-mouse IgG. Binding of the antibody was detected by enhanced chemiluminescence (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Levels of protein expression were quantitated by
densitometric scanning. To standardize between experiments, an arbitrary density of 1 was assigned to be the band obtained from sham control sample after the normalization with the indicated standard protein (Matsuda et al., 2006).

**Assessment of Apoptosis.**

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) was performed using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA) according to the manufacturer’s instructions. In brief, paraffin sections were digested with 20 μg/ml of proteinase K (Takara Shuzo) for 10-15 min at room temperature, and reacted with TdT enzyme for 60 min at 37°C. The 5 μm-thick sections were then incubated with anti-digoxigenin conjugate at room temperature for 30 min, followed by incubation with diaminobenzidine solution and counterstained with methyl-green. Apoptotic cells were observed in cross-section in randomly selected microscopic field at a final magnification of × 400.

**Plasma Histamine Measurement.**

As previously reported (Matsuda et al., 2002), a blood sample was rapidly collected into heparin tubes and centrifuged (4°C) to separate the plasma. The plasma was stored at -80°C until the day of assay. The plasma histamine concentration was determined by use of a Histamine ELISA kit IMMUNOTECH (MBL, Nagoya, Japan). This method showed little cross-reactivity with histamine metabolites, such as 1-methylhistamine (N2-methylhistamine) and 3-methylhistamine (Nββ-methylhistamine), which were all below 0.03%.

**Statistical Analysis.**
All data were presented in terms of means ± S.E. Statistical assessment of the data was made by one-way analysis of variance followed by Tukey’s multiple comparison test. Analyses were performed using PRISM software (Version 4; Graph Pad, San Diego, CA). A significant difference was assumed to exist if the $P$ value was less than 0.05.
Results

Changes in Histamine Synthesis in Sepsis.

We initially ascertained whether histamine synthesis is altered in mice with CLP-induced sepsis. The basal concentration of histamine in the mouse plasma was 1.84 ± 0.33 μM (n = 5). When polymicrobial sepsis was induced by CLP in mice, the plasma concentration of histamine significantly increased within 1 h (Fig. 1A). At 24 h after CLP, the plasma histamine level remained significantly higher than that before surgery. The sepsis-induced increase in the plasma histamine concentration was evidently inhibited when NF-κB decoy ODN, but not scrambled decoy ODN, was transfected with the HVJ-liposome method at the time point of 10 h after CLP. In mammalian tissues, histamine is synthesized from L-histidine by HDC. Immunoblot analysis indicated the presence of two forms of HDC with different molecular sizes, 53 and 74 kDa, in the mouse spleen (Fig. 1B), as previously demonstrated in rabbit cardiovascular tissues (Matsuda et al., 2002, 2003) and in mouse lungs (Matsuda et al., 2004b). It is believed that the 74-kDa form of HDC is the cytosolic enzyme and the 53-kDa form is the particulate enzyme recognized as an active form (Tanaka et al., 1998). Thus, when the relative levels of the 53-kDa band were quantitatively analyzed, an about 3-fold increase in the active form of HDC was found after sepsis induction with CLP. Transfection of NF-κB decoy ODN, but not of its scrambled form, greatly inhibited the increase in HDC protein expression in spleen tissues of septic mice.

Changes in H4 receptor mRNA Expression in Sepsis.

Distribution of the H4 receptor transcripts was investigated in various peripheral organs of mice by RT-PCR analysis (Fig. 2A). No signal was observed in the lung, heart, liver, and kidney. The H4 receptor mRNA was predominantly detected in the...
spleen. On the other hand, 24 h after CLP, the H₄ receptor mRNA was expressed in all peripheral organs. A quantitative control (β-actin) showed the highest expression of the H₄ receptor mRNA in the spleen. Transfection of NF-κB decoy ODN, but not of scrambled decoy ODN, at 10 h after CLP, resulted in a strong elimination of CLP-induced expression of the H₄ receptor gene in all peripheral organs. In spleens, treatment with NF-κB decoy ODN nearly completely suppressed both constitutively and sepsis inductively up-regulated expression of the H₄ receptor mRNA (Fig. 2B).

Changes in NF-κB Activation in Sepsis.

To examine the activation of NF-κB in mouse peripheral tissues, we measured NF-κB translocation to the nucleus with the antibody to an NF-κB subunit of p65. As depicted in Fig. 3A, sepsis induction with CLP led to an increase in translocation of NF-κB in all peripheral tissues without changing the amounts of total NF-κB p65 protein. Interestingly, the nuclear fraction of spleen exhibited strong immunoreactivity to NF-κB p65 even in the sham-operated animal, suggesting that the basal level of NF-κB activation in the spleen is much higher than other peripheral tissues. When NF-κB decoy ODN was transfected via intravenous injection, the nuclear level of NF-κB p65 in septic mouse spleen tissues was markedly reduced (Fig. 3B). This effect was not seen in the case of transfection of scrambled decoy ODN.

When mice were rendered septic by CLP and siRNA targeted to the H₄ receptor was systemically transfected 10 h later, gene expression of this receptor was successfully silenced in the lung, heart, liver, kidney, and spleen at 24 h after CLP (Fig. 4A). Translocation of NF-κB to the nucleus in spleen tissues following CLP-induced sepsis was dramatically up-regulated by systemic delivery of H₄ receptor siRNA, but not of nonsense siRNA (Fig. 4B). Thus, stimulation of the H₄ receptor appears to
display a masking effect on NF-κB activation during sepsis.

**Changes in Cell Apoptotic Regulators in Sepsis.**

Our recent study showed that sepsis induction with CLP resulted in a time-dependent increase in protein expression of FADD, an adaptor molecule to recruit procaspase-8 into the death-inducing signal complex, in lung tissues (Matsuda et al., 2009). As shown in Fig. 5, protein expression of FADD in spleen tissues was strikingly up-regulated at 24 h after CLP-induced sepsis. Either systemically administered H4 receptor siRNA or NF-κB decoy ODN was without effect on the increased level of FADD protein in septic spleens. In contrast to FADD, FLIP, an endogenous inhibitor of death receptor-induced apoptosis through the caspase-8 pathway, was significantly down-regulated at a protein level after CLP induction of sepsis. Furthermore, this down-regulation of FLIP was significantly reversed by systemic application of H4 receptor siRNA, and in vivo transfection of NF-κB decoy ODN strongly attenuated FLIP expression in spleen tissues of septic mice. The two anti-apoptotic proteins Bcl-xL and Bcl-2 levels were substantially unchanged in septic spleens, but were greatly up-regulated by H4 receptor siRNA treatment following CLP. When NF-κB decoy ODN was further treated, splenic expression levels of these anti-apoptotic proteins were markedly reduced.

Expression and activation of caspase-3, an effector protease in the apoptosis cascade, in spleen tissues were evaluated with the two different antibodies that react with pro- and cleaved forms of the enzyme. On immunoblots, inactive procaspase-3 of 32 kDa was marginally affected by sepsis, while a significant increase in a catalytically active caspase-3 of 17 kDa was observed at 24 h after CLP (Fig. 6). The increase in the active form of caspase-3 was eliminated by systemic H4 receptor treatment. The
level of cleaved caspase-3 in spleens became more pronounced when septic mice were further given NF-κB decoy ODN.

**Apoptosis in Septic Spleens.**

To detect splenic apoptosis, the tissues were labeled with an in situ TUNEL assay. Physiologic TUNEL-positive cells, morphologically identical to lymphocytes (Matsuda et al., 2009), were sporadically present in the sham control spleen (Fig. 7A). In the spleen 24 h following septic insult, marked apoptosis of follicular lymphocytes was observed (Fig. 7B). Most apoptotic lymphocytes were located in the white pulp of the spleen. TUNEL-positive lymphocytes in spleen follicles were greatly reduced when H₄ receptor siRNA was given after CLP (Fig. 7C). Further administration of NF-κB decoy ODN reversed TUNEL positivity to the same level as CLP alone (Fig. 7D).
Discussion

Since the addition of the H_4 receptor to the list of the histamine receptor family (Oda et al., 2000; Nguyen et al., 2001; Morse et al., 2001; Zhu et al., 2001), attention has been focused on this new histamine receptor subtype that may play a pathophysiological role in various allergic and inflammatory conditions (de Esch et al., 2005; Thurmond et al., 2008). We showed here that gene expression of the H_4 receptor was up-regulated in the key organs when mice were subjected to polymicrobial sepsis induced by CLP. Notably, the H_4 receptor gene was poorly expressed in normal key organs except the spleen. Plasma histamine levels were significantly increased in mice after induction of sepsis with CLP. This elevation of the circulating level of histamine was associated with increased tissue expression of HDC, an enzyme that only forms histamine in mammals, during sepsis. Sepsis-induced increases in circulating histamine and tissue HDC expression levels are consistent with our previous studies in which animals were rendered endotoxemic by lipopolysaccharide (Matsuda et al., 2002, 2003, 2004b). Taken together, these data reveal a possible role of the up-regulated H_4 receptors through stimulation with overproduction of histamine in the development of sepsis syndrome.

Activation of NF-κB is a central event leading to the transcriptional regulation of many of the immunomodulatory mediators involved in septic pathophysiology (Liu and Malik, 2006). We have previously demonstrated on the gel mobility shift assay that the binding activity of NF-κB is markedly increased in tissues from mice with CLP-induced polymicrobial sepsis (Matsuda et al., 2005). The present study showed that translocation of NF-κB to the nucleus was increased in the mouse key organs, including the spleen, after CLP, indicating that NF-κB activity is systemically activated during CLP-induced sepsis. Furthermore, the validity of our NF-κB decoy strategy
was confirmed by the finding that in vivo transfer of NF-κB decoy ODN greatly reduced NF-κB nuclear translocation in septic spleen tissues. This can be also corroborated by our previous assessment with the use of the gel mobility shift assay (Matsuda et al., 2005). Importantly, when NF-κB decoy ODN was introduced to septic mice, overexpression of the H₄ receptor was strongly suppressed in all the peripheral organ tissues employed in this study. We thus suggest that superinduction of the H₄ receptor gene promoted by sepsis may be transcriptionally controlled by NF-κB. In agreement with our suggestion, Cogé et al. (2001) have reported that the 5′-flanking region of the H₄ receptor gene contains several cis-acting binding sites including NF-κB.

As demonstrated in our previous study using lipopolysaccharide-induced septic mice (Matsuda et al., 2004b), in vivo transfection of NF-κB decoy ODN caused a marked decrease in tissue expression of HDC in mice with CLP-induced polymicrobial sepsis, leading to a strong inhibition of elevated circulating histamine to the nonseptic control level. This would result in a reduction in excessive stimulation of the H₄ receptor. Combined with the suppressive effect of NF-κB decoy ODN on the up-regulation of H₄ receptor expression, NF-κB decoy ODN treatment could provide an effective suppression of the H₄ receptor activation that may play a role in the pathophysiology of sepsis.

Recently, cell apoptosis has been implicated to play a critical role in the development of organ failure and mortality associated with sepsis (Oberholzer et al., 2001). Parenchymal cells, including intestinal and lung epithelial cells, and vascular endothelial cells have increased apoptotic cell death in animal models of septic shock (Hotchkiss et al., 1997; Zhou et al., 2004; Matsuda et al., 2007). Notably, lymphocyte apoptosis may a central pathological process in sepsis. Studies using transgenic and
gene knockout mice, providing protection against apoptotic cell death, have revealed that prevention of lymphocyte apoptosis improves survival in sepsis (Hotchkiss et al., 1999b; Chang et al., 2007). Moreover, clinical studies with septic patients have shown that the degree of apoptosis of circulating lymphocytes correlates with sepsis severity (Le Tulzo et al., 2002). In the present study, TUNEL revealed that CLP-induced polymicrobial sepsis caused marked apoptosis of follicular lymphocytes in the spleen tissue. Very interestingly, splenic cell apoptosis was strikingly declined when septic mice were systemically treated with H4 receptor siRNA. We interpret this finding to indicate a specific effectiveness of gene silencing of the H4 receptor with siRNA in splenic apoptosis during polymicrobial sepsis, because systemic injection of scrambled siRNA has been found to show exacerbation of tissue histopathology of septic mice with increased apoptotic cells, implying that inappropriate siRNA therapy for sepsis could be counterproductive to performance (Matsuda et al., 2009). It would be thus reasonable to suggest that activation of the H4 receptor may be involved in the induction of apoptosis in lymphocytes during sepsis. Furthermore, superinduction of the H4 receptor gene in the key organs, such as lungs, of septic mice may lead to its significant role in the development of apoptosis in parenchymal cells and vascular endothelial cells.

Activation of the two apoptotic death pathways, the extrinsic death receptor pathway and the intrinsic mitochondrial-mediated pathway, appear to occur in sepsis (Chang et al., 2007). In our recent work, surface expression of death receptors has been shown to be highly up-regulated in lung tissues of CLP-induced septic mice (Matsuda et al., 2009). On ligand binding to death receptors, the adaptor protein FADD is recruited to death receptors via death effector domain-mediated homophilic interactions. Subsequently, FADD can recruit procaspase-8 into the death-inducing signaling complex, thereby causing its activation (Lavrik et al., 2005). FLIP is an
anti-apoptotic protein with significant homology to caspase-8, but a substitution of two amino acids in the region of FLIP that corresponds to the catalytic active site of caspase-8 renders it incapable of proteolysis (Imler et al., 1997). As demonstrated in lungs (Matsuda et al., 2009), CLP induction of sepsis resulted in a significant up-regulation of FADD expression in spleens. In contrast, expression of FLIP was significantly down-regulated in septic spleens. Importantly, systemic application of H₄ receptor siRNA restored FLIP expression to the nonseptic control level without affecting FADD expression. Moreover, H₄ receptor siRNA treatment of septic mice led to overexpression of the anti-apoptotic proteins Bcl-xL and Bcl-2, both of which inhibit the mitochondrial pathway of apoptosis. As a result, the sepsis-induced increase in activation of caspase-3 was strongly suppressed by systemic treatment with H₄ receptor siRNA. Caspase-3 is a downstream effector component of the apoptotic death processes that can be triggered not only by death receptor activation but also by mitochondrial dysfunction (Green and Kroemer, 2004). Thus, our present data suggest that activation of the H₄ receptor down-regulates expression of both death receptor-associated and mitochondrial related anti-apoptotic proteins, thereby promoting sepsis-induced apoptotic cell death.

NF-κB activation is associated with a predominantly anti-apoptotic role through its ability to up-regulate expression of gene products that are anti-apoptotic (LaCasse et al., 1998). The up-regulatory changes in splenic expression of FLIP, Bcl-xL, and Bcl-2 under treatment with H₄ receptor siRNA were completely inhibited by transfection of NF-κB decoy ODN. Eventually, the inhibitory effects of H₄ receptor siRNA on caspase-3 activity and cell apoptosis in septic spleens were largely prevented when NF-κB decoy ODN was introduced. Thus, activation of the H₄ receptor appears to play a potentially destructive role in counteracting the anti-apoptotic action of NF-κB.
during sepsis. Our present finding that treatment with H$_4$ receptor siRNA greatly enhanced nuclear translocation of NF-κB raises the possibility that stimulation of the H$_4$ receptor may down-regulate sepsis-induced NF-κB activation. However, whether such a regulatory effect, if any, can affect the central role of NF-κB in initiating inflammation awaits further study.

Limitations.

In the present study, we used BALB/c mice, because they are susceptible to sepsis mortality induced by CLP. Several studies have documented a shift from Th1 to a Th2 response in sepsis, which results in a marked suppression of cell-mediated immunity, and is characterized by alterations in monocyte/macrophage and lymphocyte function with an increased susceptibility to infection (Ayala et al., 1994). C57BL/6 and BALB/c mice are regarded as a Th1- and Th2-dominant mouse strain, respectively. Since the H$_4$ receptor itself could affect Th1/Th2 polarization by shifting the balance to Th2, the data obtained in BALB/c mice may not simply apply to other mouse strains such as C57BL/6 mice. Furthermore, the importance of the H$_4$ receptor activation in the Th1/Th2 balance in septic patients remains the subject of ongoing studies.

Conclusions.

When mice were subjected to CLP-induced sepsis, expression of the H$_4$ receptor gene was greatly up-regulated in peripheral key organs, including the spleen. This H$_4$ receptor superinduction was associated with activation of NF-κB. In the meantime, activation of the H$_4$ receptor appeared to induce splenic cell apoptosis during sepsis through counteraction of the anti-apoptotic action of NF-κB. The present study is the first to indicate that the histamine H$_4$ receptor plays a significant role in the
pathophysiology of sepsis syndrome.
References


Footnotes

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Legends for Figures

**Fig. 1.** Effect of NF-κB decoy ODN transfection on histamine synthesis in mice with CLP-induced sepsis.  

A, Changes in plasma concentrations of histamine in mice after CLP. When NF-κB decoy ODN or its scrambled form was given, their transfection was performed 10 h after CLP. Values are means ± S.E. (n = 5). *, P<0.05 compared with the value before CLP. #, P<0.05 compared with the corresponding value obtained in CLP alone.  

B, Immunoblot analysis of HDC protein in spleen tissues. Mice underwent sham procedure, CLP, CLP and NF-κB decoy ODN administration, or CLP and scrambled decoy ODN administration, and tissues were harvested 24 h after surgery. In the top trace, typical Western blots are shown. Actin served as loading control. In the bottom trace, the summary of quantification of measurement as ratio of the 53-kDa form of HDC relative to actin is presented. Values are means ± S.E. (n = 4). *, P<0.05 compared with sham control. #, P<0.05 compared with CLP alone.

**Fig. 2.** Effect of NF-κB decoy ODN transfection on tissue expression of H₄ receptor mRNA in mice with CLP-induced sepsis.  

A, RT-PCR analysis showing gene expression of the H₄ receptor in the lung, heart, liver, kidney, and spleen. Mice underwent sham procedure, CLP, CLP and NF-κB decoy ODN administration, or CLP and scrambled decoy ODN administration, and tissues were harvested 24 h after surgery. β-Actin was used as internal control.  

B, RT-PCR analysis of H₄ receptor mRNA expression in spleen tissues of mice that were subjected to sham-operation or sepsis (24 h after CLP) with or without NF-κB decoy ODN transfection. In the top trace, representative RT-PCR showing the strong inhibition by NF-κB decoy ODN transfection of the H₄ receptor mRNA expression is depicted. Note that there is no apparent difference in β-actin mRNA among groups. In the bottom trace, the summary
of quantification of densitometric measurement as ratio of $H_4$ receptor mRNA relative to $\beta$-actin is presented. Values are means ± S.E. ($n = 4$). *, $P < 0.05$ compared with sham control alone. #, $P < 0.05$ compared with CLP alone.

**Fig. 3.** Immunoblot analysis of NF-κB p65 protein in tissues of mice with CLP-induced sepsis. A, The p65 protein levels in the nuclear fractions from lung, heart, liver, kidney, and spleen tissues are evidently increased in 24-h CLP mice as compared with sham controls. No apparent difference in p65 expression in the total fraction is found between sham control and CLP tissues. B, Effects of transfection of NF-κB decoy ODN or its scrambled form on nuclear translocation of NF-κB p65 protein in spleen tissues of 24-h CLP mice. In the top trace, typical Western blots are shown. Actin served as loading control, and lamin A as a nuclear membrane marker. In the bottom trace, the summary of quantification of densitometric measurement as ratio of nuclear p65 relative to total p65 is presented. Values are means ± S.E. ($n = 4$). *, $P < 0.05$ compared with sham control. #, $P < 0.05$ compared with CLP alone.

**Fig. 4.** Effect of systemic administration of $H_4$ receptor siRNA on NF-κB activation in spleen tissues of mice with CLP-induced sepsis. A, Representative RT-PCR data showing that systemic delivery of $H_4$ receptor siRNA successfully eliminated $H_4$ receptor mRNA in the lung, heart, liver, kidney, and spleen 24 h after CLP. B, Nuclear translocation of NF-κB p65 protein in spleen tissues. Mice underwent sham procedure, CLP, CLP and $H_4$ receptor siRNA administration, or CLP and scrambled siRNA administration, and tissues were harvested 24 h after surgery. In the top trace, typical Western blots are shown. Actin served as loading control, and lamin A as a nuclear membrane marker. In the bottom trace, the summary of quantification of densitometric
measurement as ratio of nuclear p65 relative to total p65 is presented. Values are means ± S.E. (n = 4). *, P<0.05 compared with sham control. #, P<0.05 compared with CLP alone.

**Fig. 5.** Immunoblot analysis of expression of pro- and anti-apoptotic molecules in spleen tissues of mice with CLP-induced sepsis. Mice underwent sham procedure, CLP, CLP and H₄ receptor siRNA administration, or CLP and H₄ receptor siRNA/NF-κB decoy ODN administration, and tissues were harvested 24 h after surgery. A, Typical Western blots are shown. Actin served as loading control. B, The summary of quantification of measurement as ratio of the target molecules relative to actin is presented. Values are means ± S.E. (n = 4). *, P<0.05 compared with sham control. #, P<0.05 compared with CLP alone. †, P<0.05 compared with CLP treated with H₄ receptor siRNA.

**Fig. 6.** Immunoblot analysis of caspase-3 expression in spleen tissues of mice with CLP-induced sepsis. Mice underwent sham procedure, CLP, CLP and H₄ receptor siRNA administration, or CLP and H₄ receptor siRNA/NF-κB decoy ODN administration, and tissues were harvested 24 h after surgery. A, Typical Western blots of caspase-3 which is depicted as the proenzyme of 32 kDa (top) and activation-associated cleavage product of 17 kDa (middle) by using the two antibodies that react with pro- and cleaved forms of enzyme, respectively. Actin (bottom) served as loading control. B, The summary of quantification of measurement as ratio of active caspase-3 of 17 kDa relative to actin is presented. Values are means ± S.E. (n = 4). *, P<0.05 compared with sham control. #, P<0.05 compared with CLP alone. †, P<0.05 compared with CLP treated with H₄ receptor siRNA.
Fig. 7. Spleen tissue sections analyzed by an in situ TUNEL assay. Mice underwent sham procedure (A), CLP (B), CLP and H4 receptor siRNA administration (C), or CLP and H4 receptor siRNA/NF-κB decoy ODN administration (D). Tissues were harvested 24 h after surgery. Shown are representative micrographs from four independent experiments in which the same results were obtained. Lower panels show high-magnification images.
Fig 1

A

- CLP alone
- CLP + NF-κB Decoy
- CLP + Scrambled Decoy

Plasma Histamine Concentration (μM)

Time after CLP (h):
- 0
- 1
- 12
- 18
- 24

B

HDC

74 kDa
53 kDa

Actin

Relative Densities of HDC/Actin

24-h Sham
24-h CLP

NF-κB Decoy
Scrambled Decoy
Fig 2

A

24-h Sham
- H4-R
- β-Actin

24-h CLP
- H4-R
- β-Actin

24-h CLP + NF-κB Decoy
- H4-R
- β-Actin

24-h CLP + Scrambled Decoy
- H4-R
- β-Actin

Lung, Heart, Liver, Kidney, Spleen

B

H4-R

β-Actin

Relative Densities of H4-R mRNA/β-Actin mRNA

24-h Sham

24-h CLP

NF-κB Decoy

NF-κB Decoy

* #
**Fig 3**

A

24-h Sham

Nuclear p65

Total p65

24-h CLP

Nuclear p65

Total p65

Lung  Heart  Liver  Kidney  Spleen

B

Spleen

Nuclear p65

Lamin A

Total p65

Actin

Relative Densities of Nuclear p65/Total p65

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<tr>
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<th>24-h Sham</th>
<th>24-h CLP</th>
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<tr>
<td>NF-κB Decoy</td>
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<tr>
<td>Scrambled Decoy</td>
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* P < 0.05 compared with 24-h Sham
Fig 6

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<tr>
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<th>H4-R siRNA</th>
<th>NF-κB Decoy</th>
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<td>24-h CLP</td>
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A

Caspase-3

32 kDa

32 kDa

17 kDa

12 kDa

Actin

B

Relative Densities of 17-kDa Caspase-3/Actin

24-h Sham       | 24-h CLP       |